表　　題　MRSA におけるダプトマイシンとバンコマイシン交差耐性メカニズムの解明
(Elucidation of cross-resistance mechanism to daptomycin and vancomycin in MRSA)

論 文 の 区 分　博士課程

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ABSTRACT

We first reported a phenomenon of cross-resistance to vancomycin (VCM) and daptomycin (DAP) in methicillin-resistant *Staphylococcus aureus* (MRSA) in 2006, but mechanisms underlying the cross-resistance remain incompletely understood. Here, we present a follow-up study aimed at clarifying the genetic mechanism of cross-resistance. Using 12 sets of paired DAP-susceptible (DS) and DAP-nonsusceptible (DNS) MRSA isolates from 12 patients who had DAP monotherapy, we (i) assessed susceptibility to VCM and DAP, (ii) compared whole-genome sequences, (iii) investigated the identified mutations of cross-resistance, and (iv) determined the impact of altered gene expression and metabolic pathway on the cross-resistance. We found that all 12 DNS strains exhibiting cross-resistance carried mutations in *mprF*, while one DNS strain with resistance to only DAP carried a *lacF* mutation. On the other hand, among the 32 vancomycin-intermediate *S. aureus* (VISA) strains isolated from patients treated with VCM, 5 out of the 18 strains showing cross-resistance to VCM and DAP carried a *mprF* mutation, while 14 strains resistant to only VCM had no *mprF* mutation. Moreover, substitution of *mprF* in a DS strain with mutated *mprF* resulted in cross-resistance and vice versa. The *mprF* mutation elevated lysyl-phosphatidylglycerol (L-PG) production, positive membrane surface charges, and cell wall (CW) synthetic pathways. These results demonstrated that the *mprF* mutation contributed to cross-resistance to VCM and DAP in MRSA.

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Abbreviation	Definition
ABC transporter	ATP-binding cassette transporter
ACC	acetyl-CoA carboxylase
acetyl-CoA	acetyl-coenzyme A
ACP	Acyl carrier protein
acyl-P	acyl-phosphate
agr	accessory gene regulator
Ala	alanine
AMPs	antimicrobial peptides
BHI	brain-heart infusion
Ca^{2+}	calcium ion
CAMPs	cationic antimicrobial peptides
ccr	cassette chromosome recombinase
cDNA	complementary deoxyribonucleic acid
CDP	cytidine diphosphate
CFU	colony forming unit
CL	cardiolipin
cls	cardiolipin synthease
CLSI	clinical and laboratory standards institute
CM	cell membrane
CO_2	carbon dioxide
COGs	clusters of orthologous groups
CTP	cystidine triphosphate
CW	cell wall
DAG	diacylglycerol
DAP	daptomycin
dgkB	diacylglycerol kinase
DNS strain	DAP-nonsusceptible strain
DS strain	DAP-susceptible strain
EDTA	ethylenediaminetetraacetic acid
EUCAST	European committee on antimicrobial susceptibility testing
F-6P	fructose-6-phosphate
fabG	β-ketoacyl-ACP reductase
fabI	Enoyl-A ACP reductase
fabZ	β-hydroxyacyl-ACP
FASII	type II fatty acid synthesis
FDA	US Food and Drug Administration
fem	factor essential for methicillin resistance
G3P	glycerol-3-phosphate
Glc1P	glucose-1-phosphate
Glc6P	glucosamine-6-phosphate

Abbreviation	Definition
glmM	phosphoglucosamine-mutase
glmS	glucosamine-F-6P aminotransferase
glmU	UTP-Glc1P uridyltransferase
Glu	glutamic acid
Gly5	pentaglycine
graSR	glycopeptide resistance-associated
GSH	glutathione
gtaB	UTP: α-Glc1P uridyltransferase
GUVs	giant unilamellar vesicles
H_2O_2	hydrogen peroxide
HCl	hydrochloric acid
JMUB	Jichi medical university bacterialbank
KCl	potassium chloride
L-PG	lysyl-phosphatidylglycerol
LTA	lipoteichoic acid
ltaA	glycolipid permease
ltaS	LTA synthase enzyme
Lys	lysine
MAN	*N*-acetylmannosamine
Mg^{2+}	magnesium ion
MH	Muller-Hinton
MIC	minimum inhibitory concentration
MLST	multi-locus sequence typing
MOPS	3-(N-morpholino) propanesulfonic acid
mprF	multiple peptide resistance factor
mraY	phospho-NAM-pentapeptide translocase
MRSA	methicillin-resistant *Staphylococcus aureus*
murA	UDP-N-acetylglucosamine transferase
murB	UDP-N-acetylmuramate dehydrogenase
murG	undecaprenyl-PP-NAM-pentapeptide-UDP-NAM transferase
N/A	not available
NAG	*N*-acetylglucosamine
NAM	*N*-acetylmuramic acid
NaOAc	sodium acetate
NARSA	network on antimicrobial resistance in *Staphylococcus aureus*
OD	optical density
PAP-AUC	population analysis profiling-area under the curve
PBP	penicillin-binding protein
PEP-PTS	lactose phosphoenolpyruvate phosphotransferase system
PG	phosphatidylglycerol
PG-P	PG-phosphate
pgcA	α-phosphoglucomutase

Abbreviation	**Definition**
PL	phospholipid
Poly-GroP	polyglycerolphosphate
PtdOH	phosphatidic acid
rot	repressor of toxins
SCC*mec*	staphylococcal cassette chromosome *mec*
SD	standard deviation
SNP	single nucleotide polymorphism
TAs	teichoic acids
tar	teichoic acid ribitol
TCRS	two-component regulatory system
TEM	transmission electron microscopy
TLC	thin-layer chromatography
TMSs	*transmembrane* segments
TSB	tryptic soy broth
UDP	uridine diphosphate
UDP-Glc	UDP glucose
UDP-GlcNAc	UDP-N-acetyl-glucosamine
UPRT	uracil phosphoribosyltransferase
UTP	uridine triphosphate
VCM	vancomycin
VISA	VCM-intermediate *Staphylococcus aureus*
vraSR	VCM-resistance-associated sensor/regulator
VRSA	VCM-resistant *Staphylococcus aureus*
VSSA	VCM-susceptible *Staphylococcus aureus*
WT	wild type
WTA	wall teichoic acid

CHAPTER I

INTRODUCTION

Infections with methicillin-resistant *Staphylococcus aureus* (MRSA) are serious clinical problems in all parts of the world causing high morbidity and mortality. MRSA is resistant not only to the β-lactam antibiotics, but also the other classes of antibiotics such as aminoglycosides, tetracyclines, or fluoroquinolones, restricting the available antibacterial agents for MRSA treatment [1-4]. Vancomycin (VCM), a glycopeptide antibiotic exerting bactericidal activity by binding to D-Ala-D-Ala residues of peptidoglycan to inhibit bacterial cell wall (CW) synthesis, is the first-line antibiotic against MRSA infections [5]. Emergence of MRSA with reduced susceptibility to VCM has therefore further limits the scarcely available treatment options [3, 4, 6-8].

Daptomycin (DAP), a cyclic lipopeptide antibiotic with potent bactericidal activity, is frequently used as salvage therapy after failure of VCM treatment [9]. In the presence of calcium, anionic DAP molecule attained its active cationic peptide form which will then insert its lipophilic tail into the negative-charged cell membrane (CM) [10, 11]. The interaction between DAP and CM causes potassium leakage and membrane depolarization that ultimately contribute to cell death [12]. These mean that DAP and VCM differ in not just chemical structure, but also in their bactericidal mechanisms [7, 13]. Nevertheless, MRSA strains with cross-resistance to DAP and VCM have been frequently isolated from patients treated with either DAP or VCM, with the first isolation reported by our group in 2006 [8, 14-16].

Multiple peptide resistance factor (MprF) is known to mediate DAP-nonsusceptibility in *S. aureus* by alteration of net surface charges on CM. Mutation of *mprF* gene causes a gain-in-function, in which lysinylation of phosphatidylglycerol (PG) will be enhanced and thus increasing membrane lysyl-PG (L-PG) production [17, 18]. These positively-charged L-PG

will then be translocated from the inner membrane to the outer leaflet of CM by flippase domain of MprF protein, causing an increased net positive charge on CM [19]. Eventually, the more positively-charged CM surface will serve as a protective barrier against DAP binding [20, 21]. Besides changes in CM properties, increased thickness of CW is also proposed to cause ineffective binding of DAP to CM [14, 22]. DAP-nonsusceptibility is accompanied by an increased expression of genes involved in CW metabolism, such as *murAB* or *pbp2*, a response similar to those induced by VCM and the other CW-targeting agents [23, 24]. As a salient feature of VCM-intermediate *S. aureus* (VISA), CW thickening could be a potential factor of VCM resistance in DAP-nonsusceptible strain. In fact, mutations in either *walK*, encoded for the sensor protein kinase of a two-component regulatory system, or *vraSR*, involved in cell envelop homeostasis, both of which resulted in CW thickening, is sufficient to cause DAP/VCM cross-resistance [25, 26]. However, phenotypic change in CW thickness was not consistently observable in all DAP-nonsusceptible strains [22, 27]. Consequently, the mechanism(s) conferring resistance of *S. aureus* to the two different classes of antibacterial agents remains largely unknown.

This study clarifies the mechanism of cross-resistance between DAP and VCM in clinically isolated MRSA. A total of 12 sets of DAP-susceptible (DS) and DNS MRSA isolates collected from different hospitals in Japan were compared for their genotypic and phenotypic characteristics. Our results suggested that DAP and VCM cross-resistance was regulated by *mprF* mutation via increased L-PG production, subsequent alteration of membrane surface charge, and CW biosynthetic pathways. This proposed mechanism was supported by transcriptional analysis that revealed an enhanced CW/CM metabolism in cross-resistant strain and was found to contribute more substantially to DAP and VCM cross-resistance than changes in CW thickness.

CHAPTER II

LITERATURE REVIEW

1.1. *Staphylococcus aureus* (*S. aureus*)

S. aureus are Gram-positive spherical bacteria (round and grape-like shape) that grow under both aerobic and anaerobic conditions, called facultative anaerobic organism. The bacteria can grow on rich agar medium forming yellowish-orange colonies, which is caused by production of staphyloxanthin pigment following carbohydrate fermentation [28]. A positive catalase test (active bubbling as a result of conversion of hydrogen peroxide (H_2O_2) to water and CO_2) is used to distinguish staphylococci from the other members of Gram-positive cocci. While positive-coagulation, activating the transition of fibrinogen to fibrin, differentiated *S. aureus* from the other *Staphylococcus* species [29, 30]. *S. aureus* normally harmlessly inhabit mucous membrane and skin (ranged from 20 to 50%) and were found on clothing or medical equipment [31]. However, *S. aureus* is also one of the most common pathogenic bacteria frequently isolated from community- and healthcare-associated infections, causing various diseases such as pneumonia, osteomyelitis, skin infection or sepsis, as well as enterotoxin-mediated food poisoning [30, 32]. High prevalence of *S. aureus* and difficulties in treating staphylococcal infections due to its rapid adaptation against many types of antibiotics lead to the increased rate of morbidity and mortality [33]. Thus, understanding the mechanism of drug resistance are crucial for development of new strategies against resistant bacteria.

1.2. *S. aureus* cell envelop

1.2.1) Cell wall (CW)

The prokaryotic cells have a simpler structure than eukaryotic cells because they lack many membrane-bound organelles such as ribosome, mitochondria or nucleus [34]. However,

unlike eukaryotic cells, a complex multilayer structure known as CW peptidoglycan, locates outside bacterial CM to support cell structure and protects bacteria from environmental stresses [35]. Different properties of CW between Gram-positive and Gram-negative bacteria have also been used for bacterial characterization, with a thicker peptidoglycan observed in Gram-positive bacteria [36]. The peptidoglycan (murein) is consists of three important parts; 1) disaccharide units composed of alternating *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) connected with β-1,4 linkage, which serves as backbone for peptidoglycan [37], 2) pentapeptide side chains (L-Ala-D-Glu-L-Lys-D-Ala-D-Ala) cross-linked to L-Lys of another peptide chain at the 4th amino acid (D-Ala) with 3) pentaglycine cross-link, resulting in the cleavage of the terminal D-Ala residue [38].

There are three stages of peptidoglycan biosynthesis (Figure 1). In the first stage, nucleotide sugar-linked precursors (uridine diphosphate-NAM (UDP-NAM) and UDP-NAG) are synthesized in the cytoplasm. *glmS* (glucosamine-F-6P aminotransferase) catalyzed the conversion of fructose-6-phosphate (F-6P), the substrate from glycolysis pathway, to glucosamine-6-phosphate (Glc6P), which are then used for the generation of uridine UDP-NAG. The latter reaction of which is catalyzed by *glmM* (phosphoglucosamine-mutase) and *glmU* (UTP-glucose-1-phosphate uridyltransferase) [39]. The UDP-NAM molecules are generated from UDP-NAG molecules through reactions with two transferase enzymes *murA* (UDP-NAG transferase) and *murB* (UDP-*N*-acetylenolpyruvoylglucosamine reductase) [40]. Finally, the ligase enzymes (MurC - F) catalyzed the construction of pentapeptide by adding L-Ala, D-Glu, L-Lys and dipeptide D-Ala (D-Ala-D-Ala) sequentially to UDP-NAM, generating the Park's nucleotide. The second stage of peptidoglycan biosynthesis which occurs in inner membrane, involved *mraY* (phospho-NAM-pentapeptide translocase)-catalyzed transferring of Park's nucleotide to lipid carrier undecaprenyl-diphosphate located at CM, forming "lipid I" (NAM-(pentapeptide)-pyrophosphoryl-undecaprenol). The UDP-NAG

synthesized in the first stage is then linked to lipid I by *murG* (undecaprenyl-PP-NAM-pentapeptide-UDP-NAM transferase), generating "lipid II" (NAG-β-(1,4)-NAM-(pentapeptide)-pyrophosphoryl-undecaprenol). These lipid II-molecules are the building block for CW synthesis. Five glycyl-tRNA (glycine residues) were added at the D-Lys position of pentapeptide side chain by *femXAB* (factor essential for methicillin resistance) and the D-Glu position were deaminated by couple enzymes *murT*/*gatD* before lipid II molecules can be cross-linked to form peptidoglycan polymers [41, 42]. The final stage involved translocation of complete lipid II molecules (peptidoglycan) across cytoplasmic membrane for cross-bridge formation with the other lipid II, as catalyzed by penicillin binding proteins (PBPs), a family of proteins which serve as either mono-functional or bi-functional transglycosylase and/or transpeptidase [43]. Transglycosylase catalyzed the cross-linking between NAM from one peptidoglycan with NAG of other peptidoglycan, with release of lipid carrier; while transpeptidase cross-linked the L-Lys of pentapeptide side chain to D-Ala (4^{th} position) of another pentapeptide chain with pentaglycine (Gly5) bridge following cleavage of the terminal D-Ala residue (5^{th} position) [44-46].

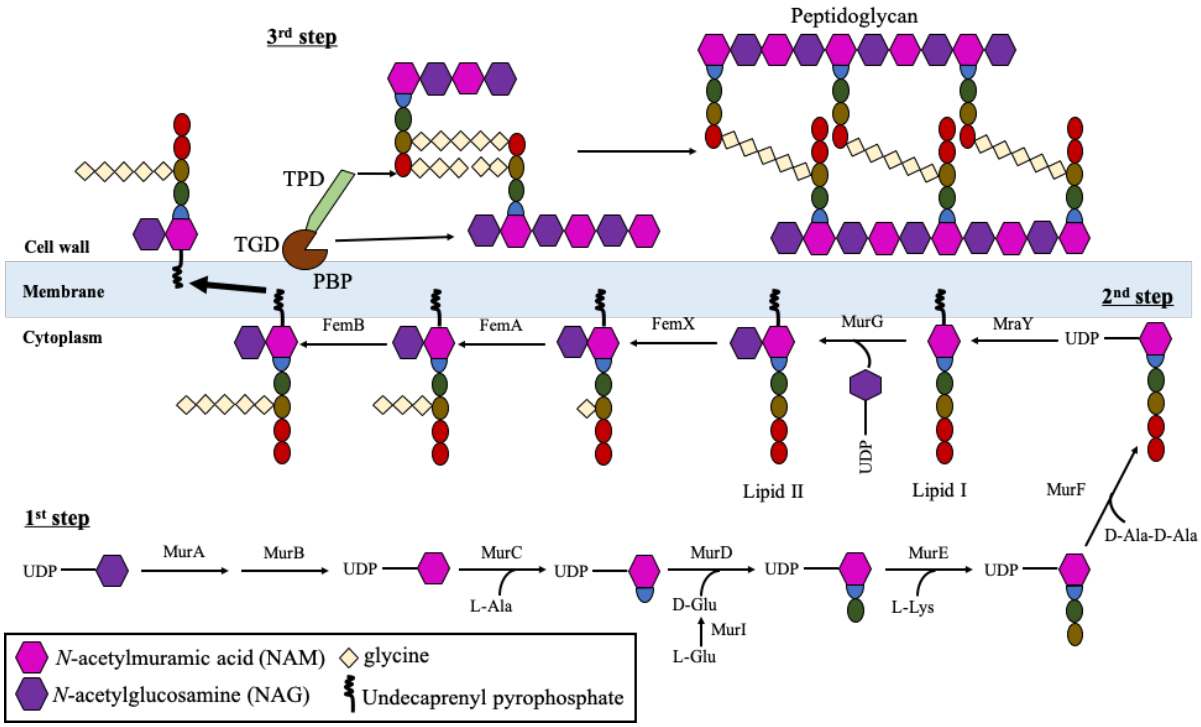

Figure 1: Schematic model of peptidoglycan synthesis.
NAG and NAM are the backbone of peptidoglycan which is the building block for generation of CW. NAG is synthesized from the substrate of glycolysis pathway and converted to NAM as catalyzed by MurAB. Then, Mur ligases (MurC to F) catalyzed the formation of pentapeptide by sequentially added amino acids L-Ala, D-Glu, L-Lys and dipeptide D-Ala (D-Ala-D-Ala) to NAG, generating the Park's nucleotide. MraY-mediated membrane translocation subsequently attached Park's nucleotide to lipid carrier undecaprenyl-diphosphate at CM, yielding lipid I. The following MurG-catalyzed NAG linkage to Park's nucleotide generated lipid II. The five glycine residues are attached to lysine residue of lipid II in a process catalyzed by FemX/A/B proteins. After translocation of pentaglycine-lipid II to outer membrane, penicillin binding proteins (PBPs) generated CW from peptidoglycan units through transglycosylation and transpeptidation. This figure is modified from previous study [47].

Table 1: Genes involved in peptidoglycan synthesis

Genes	Description
glmS	glucosamine-6-phosphate synthase fructose-6-phosphate → glucosamine-6-phosphate
glmM	phosphoglucosamine mutase glucosamine-6-phosphate → glucosamine-1-phosphate
glmU	glucosamine-1-phosphate acetyltransferase glucosamine-1-phosphate → UDP-*N*-acetylglucosamine (UDP-NAG)
murA	UDP-*N*-acetylglucosamine 1-carboxyvinyltransferase UDP-NAG + phosphoenolpyruvate → UDP-NAG enolpyruvate
murB	UDP-*N*-acetylenolpyruvoylglucosamine reductase UDP-NAG enolpyruvate + NADPH → UDP-*N*-acetylmuramic acid (UDP-NAM)
murC	UDP-*N*-acetylmuramate-L-alanine ligase UDP-NAM + L-Alanine → UDP-NAM-L-Alanine
murI	glutamate racemase L-Glutamine → D-Glutamine
murD	UDP-*N*-acetylmuramoylalanine-D-glutamate ligase UDP-NAM-L-Alanine + D-Glutamine → UDP-NAM dipeptide
murE	UDP-*N*-acetylmuramoyl-L-alanyl-D-glutamate-L-lysine ligase UDP-NAM dipeptide + L-lysine → UDP-NAM tripeptide
murF	UDP-*N*-acetylmuramoyl-tripeptide-D-alanyl-D-alanine ligase UDP-NAM-tripeptide + D-Ala-D-Ala → UDP-NAM pentapeptide
mraY	phospho-*N*-acetylmuramoyl-pentapeptide transferase UDP-NAM pentapeptide + *cis*-undecaprenyl phosphate → lipid I
murG	UDP-NAG-NAM-pentapeptide pyrophosphoryl-undecaprenol NAG transferase lipid I + UDP-NAG → lipid II
femX	lipid II:glycine glycyltransferase lipid II + glycyl-$tRNA^{Gly}$ → lipid II-Gly1
femA	aminoacyltransferase lipid II-Gly1 + 2 (glycyl-$tRNA^{Gly}$) → lipid II-Gly3
femB	aminoacyltransferase lipid II-Gly3 + 2 (glycyl-$tRNA^{Gly}$) → lipid II-Gly5
murT/*gatD*	lipid II isoglutaminyl synthase lipid II-Gly5 → lipid II(D-isoglutamine-NH_2)-Gly5-
pbp	transpeptidase and transglycosylase

Teichoic acids (TAs), anionic glycosylated poly(alditolphosphates), also located on CW. TAs served as a surface antigen and are involved in cell division, maintenance of cell shape, cation homeostasis and protection of cells against extreme conditions (salt or temperature) as well as antimicrobial peptides (AMPs) [48, 49]. There are two types of TAs: wall teichoic acids (WTAs) and lipoteichoic acids (LTAs) [48, 50]. The anionic WTAs consist of glycopolymers of ribitol-phosphate repeats that covalently linked to NAM residues in every ninth peptidoglycan (Figure 2) by WTA linkage unit [51]. The WTA synthesis occurs in cytoplasm and is regulated by many *tar* (teichoic acid ribitol) genes [52]. This process is initiated by the synthesis of WTA linkage unit. *tagO* gene first transferred the NAG residue to undecaprenyl phosphate carrier anchored at inner CM. NAG transferase *tagA* then catalyzed the formation of β-1,4-linked *N*-acetylmannosamine (MAN) and NAG disaccharide complex by transferring MAN to C4 hydroxyl residue of NAG. Following that, phosphoglycerol is attached to C4 hydroxyl residue of MAN in MAN-NAG complex by glycerophosphate transferase *tarBF* genes. Finally, ribitol-repeating units are attached to the glycerol phosphate of the disaccharide linkage unit by polymerase enzyme *tarL* gene [53, 54] and the complete WTAs polymers are translocated to outer cellular membrane by ABC-dependent transporter complex (TarGH) before attached to CW peptidoglycan. The enzymes involved in WTAs linkage to peptidoglycan in *S. aureus* have not been elucidated.

The ribitol repeat units in WTAs are important for cation binding or phage attachment, as well as protection from antibiotic actions [55-61]. In addition, the negatively-charged phosphate group in the ribitol-repeating units of WTAs allow metal cations binding, which is important for CW rigidity [62]. Previous reports have shown that bacteria lacking WTA have increased mortality rate due to imbalanced ion homeostasis. On the other hand, increased concentration of bound Mg^{2+} in bacteria with enhanced WTA synthesis improved bacterial

survival [63, 64]. Moreover, metal cations binding at WTA increased surface positive charges which then contributed to bacterial resistance against many antibiotics [58-61].

Unlike WTAs, the anionic LTAs is consisted of polyglycerolphosphate (poly-GroP) chain, which are attached to diglucosyl-diacylglycerol (Glu_2-DAG) at CM and extended into the peptidoglycan layer [65]. The process of LTA synthesis takes place in cytoplasm (Figure 2). Firstly, glucose-6-phosphate, a substrate in glycolysis, is converted to glucose-1-phosphate (Glc1P) by PgcA (α-phosphoglucomutase) enzyme. After that, GtaB (UTP: α-glucose-1-phosphate uridyltransferase) enzyme produces UDP-Glc (uridine diphosphate glucose) from Glc1P. Two UDP-Glc moieties will be transferred to diacylglycerol (DAG) at CM by YpfP (glycosyltransferase) enzyme, generating Glc_2-DAG [66], which are then translocated to the outer leaflet of membrane by LtaA (glycolipid permease) [67]. Finally, LTA synthase (LtaS) cleaves GroP subunits from head group of CM phospholipids and adds onto Glc_2-DAG to generate the poly-GroP chains (Figure 2) [68]. LTAs is required for cell division, bacterial invasion, as well as protection from cationic antimicrobial peptides [69-71].

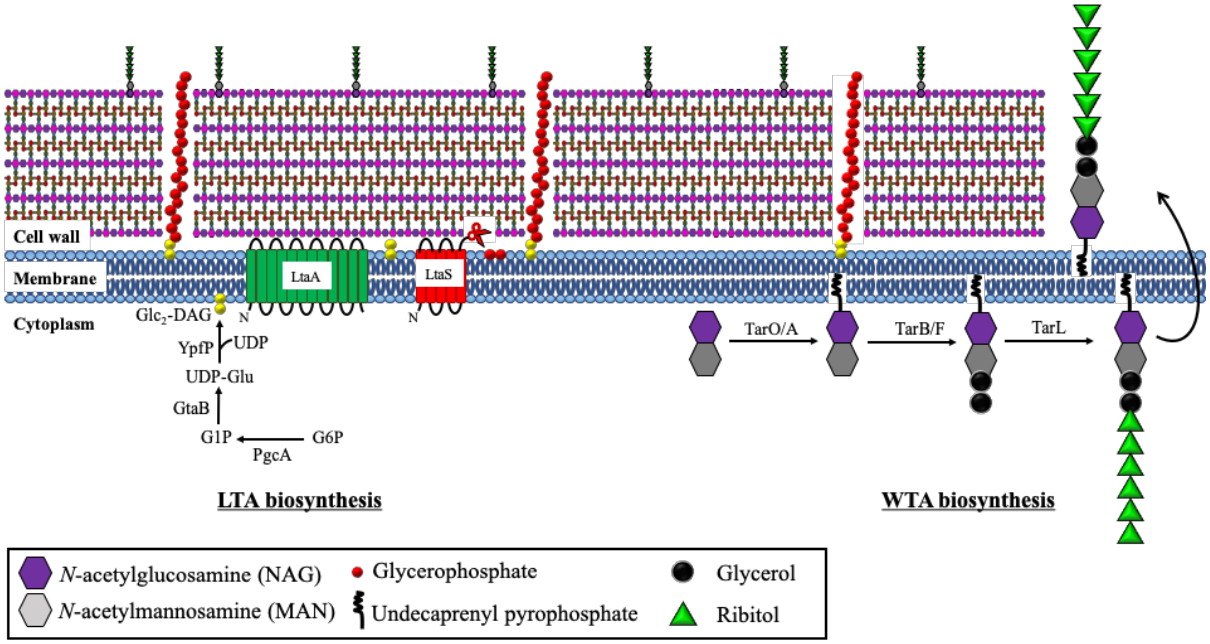

Figure 2*:* Schematic model of teichoic acid production.
Wall teichoic acids (WTAs) and lipoteichoic acids (LTAs) are located between peptidoglycan contributing to CW rigidity. WTAs is comprised of *N*-acetylmannosamine (MAN)-*N*-acetylglucosamine (NAG) disaccharides attached to undecaprenyl pyrophosphate and anchored with glycerol and polyribitol by a series of enzymes: *tarO*/*A*, *tarB/F* and *tarL*. Meanwhile, glucose-6-phosphate (G6P) is the precursor for LTA production, from which Glu_2-DAG will be generated and attached with polyglycerophosphate cleaved from CM by LtaS membrane protein.

Table 2*:* Genes involved in teichoic acid synthesis

Gene	Description
Wall teichoic acid (WTA)	
tarO	undecaprenyl-phosphate *N*-acetylglucosaminyl 1-phosphate transferase
tarA	*N*-acetylglucosaminyldiphosphoundecaprenol *N*-acetyl-β-D-mannosaminyltransferase
tarB	teichoic acid glycerol-phosphate primase
tarF	teichoic acid poly (glycerol phosphate) polymerase
tarL	teichoic acid ribitol-phosphate polymerase
Lipoteichoic acid (LTA)	
pgcA	phosphoglucomutase α-D-glucose 1-phosphate → α-D-glucose 6-phosphate
gtaB	UTP-glucose-1-phosphate uridylyltransferase α-D-glucose 6-phosphate +H^+ + UTP → UDP- α-D-glucose + diphosphate
ypfP	diacylglycerol β-glucosyltransferase 2(UDP- α-D-glucose) + 1,2-diacyl-*sn*-glycerol → 1,2-diacyl-3-O-(β-D-glucopyranosyl)-*sn*-glycerol (Glc_2-DAG)
ltaA	glycolipid permease intracellular Glc_2-DAG → extracellular Glc_2-DAG
ltaS	lipoteichoic acid synthase phosphatidylglycerol → glycerolphosphate

1.2.2) Plasma membrane

The bacterial membrane, which consists of monounsaturated fatty acids and lacks sterols, is different from eukaryotic cells. It is composed of 40% phospholipids (PLs) and 60% proteins. Most proteins are embedded in the membrane for selective transport, secretion of molecule or protection from harmful substrate. The amphipathic PLs consist of a polar head (hydrophilic) that is attached with two non-polar tails (hydrophobic) by ester bond [72]. These PLs bilayer completely surround a bacterial cell to avoid the leakage of intracellular molecules such as DNA and ribosome, and protect the bacterial cell from environmental stresses such as high osmolarity and extreme pH. Besides, PLs bilayer contained many membrane-bound

proteins which are involved in energy production through selective permeation of protons. In addition, many antibiotics as well as human immunity targeting CM are associated with PLs properties [73].

There are three types of PLs; PG (phosphatidylglycerol), L-PG (lysyl-PG) and cardiolipin (CL) [74]. The first step of PL synthesis involved acyl-acyl carrier protein (acyl-ACP) elongation via type II fatty acid synthesis (FASII) cycle (Figure 3). Initially, acetyl-CoA is converted into malonyl-CoA by acetyl-CoA carboxylase (ACC). The malonyl-CoA is then covalently linked with ACP by malonyl-CoA:ACP transacylase enzyme *fabD* forming malonyl-ACP. After that, β-ketoacyl-ACP is generated from malonyl-ACP by 3-oxoacyl-ACP synthase. Both malonyl-ACP and β-ketoacyl-ACP will be used for the production of acyl-ACP, a multi-step process catalyzed by *fabG* (β -ketoacyl-ACP reductase), *fabZ* (β -hydroxyacyl-ACP) and *fabI* (enoyl reductase) [75]. Acyl-ACP is subsequently transformed into acyl-phosphate (acyl-P) by membrane associated protein PlsX. Other than FASII cycle, acyl-P can be generated from extracellular fatty acids by either of the two fatty-acid binding proteins (FakB1 and FakB2) depending on the properties of the fatty acids, with the former specific for saturated fatty acids while the latter is specific for unsaturated fatty acids [76]. Following that, membrane-bound acyl transferase PlsY will catalyzed the acylation of glycerol-3-phosphate (G3P) to 1-acyl-G3P using acyl-P as the substrate [77], and PlsC, another acyltransferase, will transfer a second acyl-ACP to the carbon-2 position of 1-acyl-G3P to form phosphatidic acid (PtdOH) [78]. The synthesis of PtdOH is completed when a long chain acyl-ACP generated from FASII cycle is added to the 2^{nd} position of 1-acyl-G3P [79]. Then, phosphatidate cytidylyltransferase CdsA converts PtdOH and cystidine triphosphate (CTP) to cytidine diphosphate-DAG (CDP-DAG), which are then changed into phosphatidylglycerolphosphate (PG-P) when CDP-diacylglycerol-G3P 3-phosphatidyltransferase PgsA catalyzes the substitution of cytidine monophosphate in CDP-DAG with G3P [80]. The

phosphatidylglycerophosphatase enzyme PgpP dephosphorylates PG-P into PG, that is the key intermediate for phospholipid production [81]. The generated PG can then be converted to L-PG and CL by MprF and CL synthases (c*ls1* & *cls2*), respectively (as explained below) [82, 83]. The turnover of PLs turnover is regulated by phosphorylation of DAG to PtdOH by diacylglycerol kinase DgkB, in the process of LTA production [84].

PLs is a selective barrier for ions, proteins and some other molecules, whereby it regulates the bacterial membrane fluidity and hence the penetration of molecules into intracellular compartment. The properties of PLs are dependent on three factors: 1) temperature, 2) carotenoid (in replacement of cholesterol in eukaryotic cell membrane) and 3) saturated or unsaturated fatty acids [85-87]. The structure of PL is more rigid (crystallization) at low temperature and make it difficult for the membrane molecules to move. This reduced cell permeability, causing the cells to be easily broken. In contrast, high temperature increased the distance between PL and ultimately leads to nonselective permeability and loss of membrane structure. On the other hand, the carotenoid which are randomly inserted between PLs rendered CM not too tight during low temperature, while preventing the uptake of unwanted substrates during high temperature [88, 89]. Lastly, fatty acids comprising the tailed part of PLs are made up of saturated/unsaturated fatty acids depending on the presence of double bonds between carbon atoms. Increased amount of unsaturated fatty acids in CM enhances fluidity due to increased distance between PLs. Overall, these factors indicated that the balance of PLs distance is crucial for maintenance of bacterial structure and membrane permeability [90].

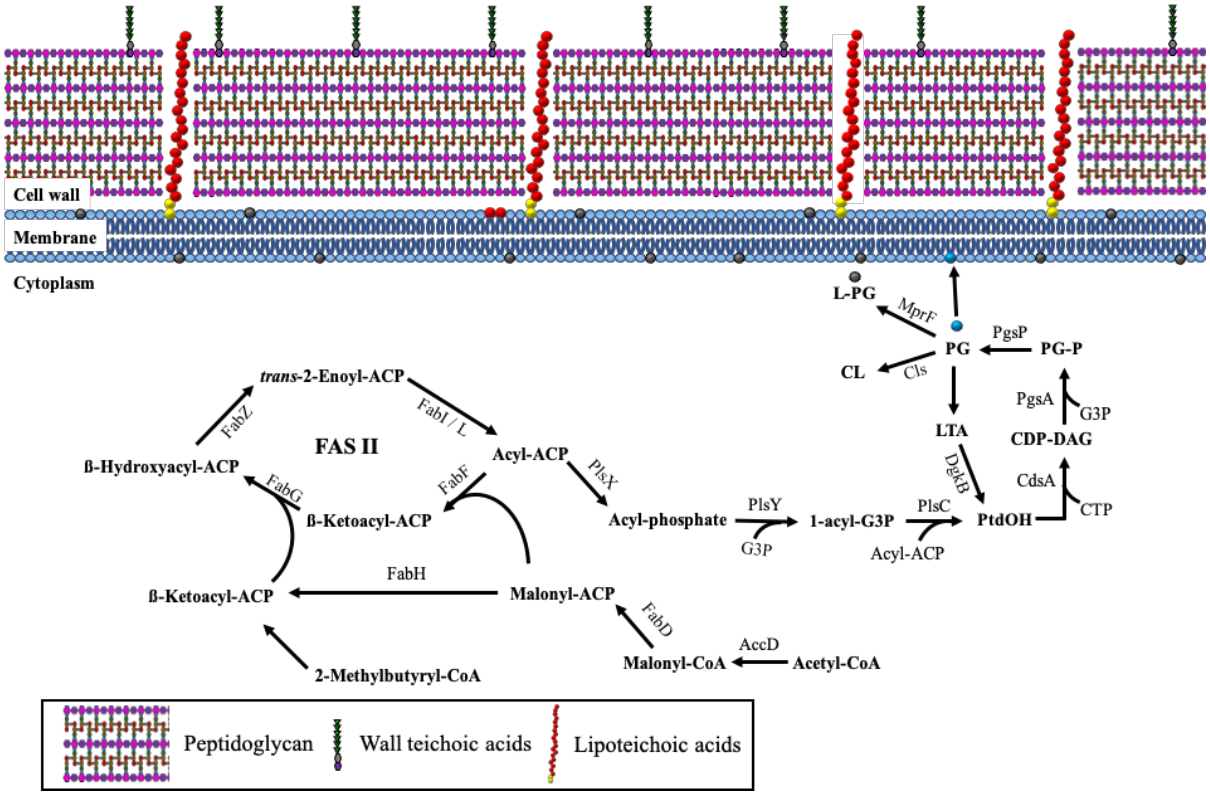

Figure 3*:* Schematic model of phospholipid production.
Acyl-ACPs (acyl-acyl carrier proteins) generating through FASII pathway by using acetyl CoA as substrate is an intermediate substrate for phospholipid synthesis. The synthesis of phosphatidic acid (PtdOH) from Acyl-ACPs or LTA recycle produces phospholipids (PGs) that generate the other kind of phospholipids such as positive-charge PG by *mprF* or cardiolipin (CL) by *cls* or lipoteichoic acid (LTA) by *ltaS*.

Table 3: Genes involved in phospholipid synthesis

Genes	Description
accD	acetyl-coenzyme A carboxylase carboxyl transferase acetyl-CoA → malonyl-CoA
fabD	malonyl CoA-acyl carrier protein transacylase malonyl-CoA + holo-[ACP] → malonyl-[ACP]
fabH	3-oxoacyl-[acyl-carrier-protein] synthase 3 malonyl-[ACP] + acetyl-CoA → 3-oxobutanoyl-[ACP] +CO_2 + CoA
fabF	3-oxoacyl-[acyl-carrier-protein] synthase 2 malonyl-[ACP] + acyl-ACP → β-ketoacyl-ACP
fabG	3-oxoacyl-[acyl-carrier-protein] reductase β-ketoacyl-[ACP] + NADPH → hydroxyacyl-[ACP] + $NADP^+$
fabZ	3-hydroxyacyl-[acyl-carrier-protein] dehydratase hydroxyacyl-[ACP] → *trans*-2-enoyl-[ACP] + H_2O
fabI/L	enoyl-[acyl-carrier-protein] reductase *trans*-2-enoyl-[ACP] + NADPH → acyl-[ACP] + $NADP^+$
fakB	fatty acid kinase fatty acid (FA) + FakB-acyl-PO_4 → acyl-PO_4 + FakB-FA
fakA	fatty acid kinase FakB-FA → FakB-acyl-PO_4
plsX	phosphate acyltransferase acyl-[ACP] + phosphate → acyl phosphate
plsY	glycerol-3-phosphate acyltransferase acyl phosphate + glycerol 3-phosphate → 1-acyl-*sn*-glycero-3-phosphate + phosphate
plsC	1-acyl-*sn*-glycerol-3-phosphate acyltransferase 1-acyl-*sn*-glycero-3-phosphate + acyl-[ACP] → 1,2-diacyl-*sn*-glycero-3-phosphate (PtdOH)
cdsA	phosphatidate cytidyltransferase 1,2-diacyl-*sn*-glycero-3-phosphate + CTP → CDP-1,2-diacyl-*sn*-glycerol + diphosphate
pgsA	CDP-diacylglycerol-glycerol-3-phosphate 3-phosphatidyltransferase CDP-1,2-diacyl-*sn*-glycerol + glycerol 3-phosphate → phosphatidylglycerolphosphate (PG-P) + CMP
pgsP	phosphatidylglycerophosphatase PG-P → phosphatidylglycerol (PG)
mprF	phosphatidylglycerol lysyltransferase PG + Lys-tRNA → lysyl-phosphatidyl-glycerol (L-PG)
cls	cardiolipin synthase 2(PG) → cardiolipin + glycerol

Genes	Description
dgkB	diacylglycerol kinase 1,2-diacyl-*sn*-glycerol + ATP → PtdOH + ADP

1.3. The emergence of methicillin-resistant *S. aureus* (MRSA)

β-lactam is the first antibiotic used for the treatment of *S. aureus* infection [91, 92]. Penicillin, cephalosporins and carbapenems are the antibiotics characterized as β-lactams. This group of antibiotics contain β-lactam ring that is nitrogen atom in β-carbon (three carbons). All β-lactam antibiotics are selective inhibitors of bacterial CW biosynthesis which covalently bind to nucleophilic serine in different PBPs involved in peptidoglycan cross-link to form acyl-enzyme complex (PBP-β-lactam complex), thereby causing cytoplasm leakage and eventually cell death [93-95]. Staphylococcal PBPs can be divided two groups depending on their molecular mass: low molecular mass PBPs, monofunctional enzyme involved in transpeptidation; high molecular mass PBPs, bifunctional enzyme catalyzed transglycosylation and transpeptidation [96]. *S. aureus* carries 4 types of PBPs (PBP1-4) [97]. Different β-lactams may bind either specific or multiple PBPs based on the structure of the antibiotics. For example, the first β-lactam antibiotic (penicillin G or benzylpenicillin) having a similar structure as D-Ala-D-Ala residues in nascent peptidoglycan will bind to the active site serine of PBP1 and PBP3 causing cell lysis and inhibition of cell division, respectively [91, 92]. Methicillin, β-lactamase-resistant β-lactam, is a semisynthetic penicillin capable of avoiding bacterial β-lactamase activity owing to the replacement of phenol group of penicillin G with methoxy group [97]. Methicillin was first used in the early 1960s and shown to be more stable and effective in killing bacteria compared with penicillin [98].

However, the first methicillin-resistant *S. aureus* (MRSA) was isolated from England soon after the clinical application of methicillin and start to spread to many other countries [99, 100]. MRSA are resistant towards all β-lactams and many other classes of antibiotics [101].

Comparison between methicillin-sensitive *S. aureus* (MSSA) and MRSA by Hartman and Tomasz group identified a new PBP2a that exhibited low affinity for β-lactam binding, while attaining its function as nascent PBP [102, 103]. Multidrug resistance in MRSA is not mediated by plasmid as β-lactamase-mediated destruction of β-lactam ring that rendered β-lactam antibiotics ineffective, but it occurs via horizontal transfer of a foreign methicillin resistance gene *mecA* onto the bacterial chromosome [104, 105]. There are two regulatory genes controlling the expression of *mecA*, namely *mecI* (a repressor) and *mecR1* (a sensor protein) [106]. The *mecA* gene is located on staphylococcal cassette chromosome *mec* (SCC*mec*) that is a group of a distinct mobile genetic element [107]. Moreover, a cassette chromosome recombinase (*ccr*) gene complex and three J regions encoding for antibiotic and heavy metal resistance were co-localized on SCC*mec* and are used for MRSA typing [108]. Nowadays, MRSA have been characterized as one of the ESKAPE pathogens which are difficult-to-treat due to its resistance to many antibiotics, such as cephalosporin (CW inhibitor), tetracycline (protein synthesis inhibitor) or rifampin (inhibition of nucleic acid synthesis) [101]. The timeline summary of antibiotic resistance in *S. aureus* is shown in Figure 4.

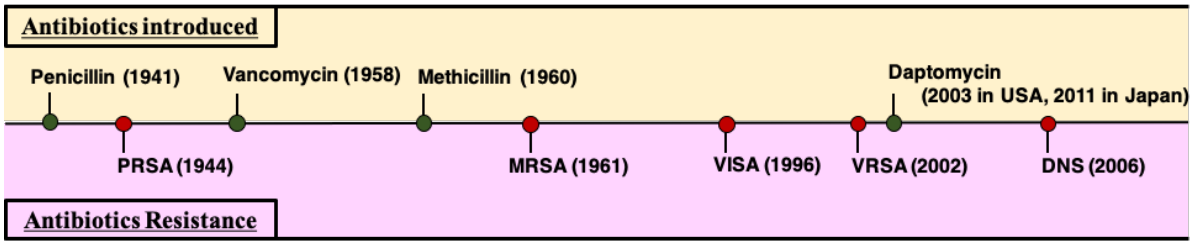

Figure 4*:* Brief history of antibiotic therapies and resistance in *S. aureus*.
PRSA: Penicillin-resistant *S. aureus* / MRSA: Methicillin-resistant *S. aureus* / VISA: VCM intermediate *S. aureus* / VRSA: VCM-resistant *S. aureus* / DNS: DAP-nonsusceptible *S. aureus*

1.4. Antibiotics for treatment of MRSA infection

Daptomycin (DAP) and vancomycin (VCM) have been approved by the US Food and Drug Administration (FDA) to treat MRSA infections [9].

1.4.1) Vancomycin (VCM)

VCM is categorized as the Group I glycopeptide antibiotic that consists of glycans attached with peptides [109, 110]. VCM, derived from *Amycolatopsis orientalis* in 1956 by the Lilly Research Laboratories, was first reported on killing MRSA in 1958 and has continuously been used to treat multidrug-resistant Gram-positive organisms ever since, both aerobic and anaerobic bacteria [5, 111, 112].

VCM is a high molecular mass heptapeptide (1450 Da) made up of five proteinogenic residues (Alanine, Asparagine, Glutamic acid, Leucine and Tyrosine) and two nonproteinogenic amino acid residues (β-hydroxytyrosine and 4-hydroxyphenylglycine,3,5-dihydroxyphenylglycine) [113]. The N-terminal leucine residue plays a crucial role in inhibition of CW synthesis by binding to C-terminal dipeptides D-Ala-D-Ala of peptidoglycan precursor with 5 hydrogen bonding, causing weakened CW and enhanced swelling under osmotic pressure (Figure 5) [114, 115]. Moreover, VCM can penetrate bacterial membrane and inhibit RNA synthesis [109, 116].

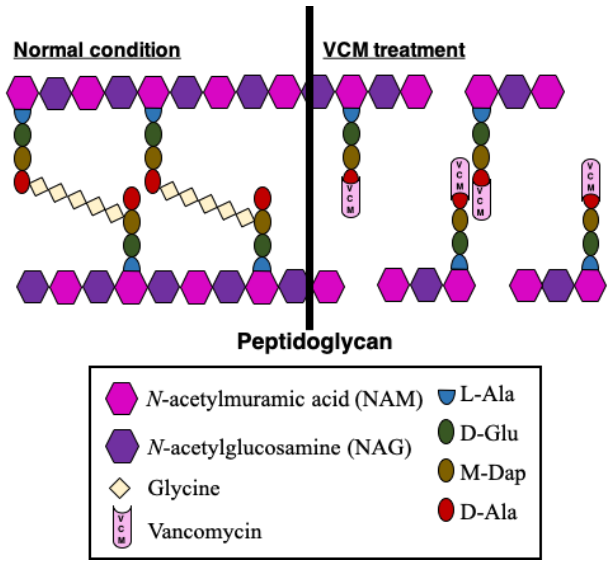

Figure 5: Schematic model of inhibition of peptidoglycan synthesis by VCM.
The peptidoglycan is comprised of NAM-NAG backbone, pentapeptide and five glycine residues. VCM binding at terminal D-Ala-D-Ala of pentapeptide chain inhibit the formation of glycine cross-bridge between peptidoglycan.

1.4.2) Daptomycin (DAP)

DAP ($C_{72}H_{101}N_{17}O_{26}$) was first derived from the soil bacterium *Streptomyces roseosporus* in 1980s [117]. It is a high molecular weight (1620.67 Da) lipopeptide antibiotic that consists of three parts; 1) lipophilic tail, 2) water-soluble hydrophilic core and 3) thirteen amino acids. DAP has a stronger bactericidal activity than glycopeptide antibiotics against aerobic and anaerobic Gram-positive bacteria, such as MRSA and VCM-resistant enterococci, but is not lethal to Gram-negative bacteria. DAP has been approved by FDA and the Ministry of Health, Labor and Welfare to be used for treatment of complicated infections since 2003 in USA and 2011 in Japan [12, 118, 119]. It is known that ionized calcium (Ca^{2+}) is required for DAP killing activity, the binding with which forms DAP micellar structure (octameric complex) that allows interaction of the antibiotic with CM (Figure 6) [11, 120], though the exact mechanism(s) succeeding DAP-CM interactions that finally causes bacterial cell death has not been elucidated [121]. Nonetheless, two theories are currently regarded to be the mechanisms of actions of DAP. Firstly, positively-charged DAP-Ca^{2+} complex directly binds

the negatively-charged PG located on the outer leaflet of CM through electrostatic interaction [10]. Translocation of DAP oligomers into the inner leaflet of CM sequentially formed multifunctional pore-like structure [122, 123] that causes ion leakage. Both PG and CL are essential for the formation of DAP-Ca^{2+} complex pore-like structures [10, 124]. Muraih *et al*. showed that even a single molecule of PG is sufficient for the formation of pore-like structures, whereas the lack of PG failed to induce DAP oligomerization [10, 122]. On the other hand, Zhang *et al*. reported the crucial role of CL in translocation of DAP oligomers into inner leaflet of CM [124]. A second theory suggested that interaction of DAP with PG removed lipid molecules from phospholipid bilayer (lipid extracting effect), thereby altering PLs compositions [125]. Both theories unanimously proposed that DAP disrupts CM properties and causes ion leakage. This in turn results in CM depolarization and finally leads to cell death [123, 125, 126].

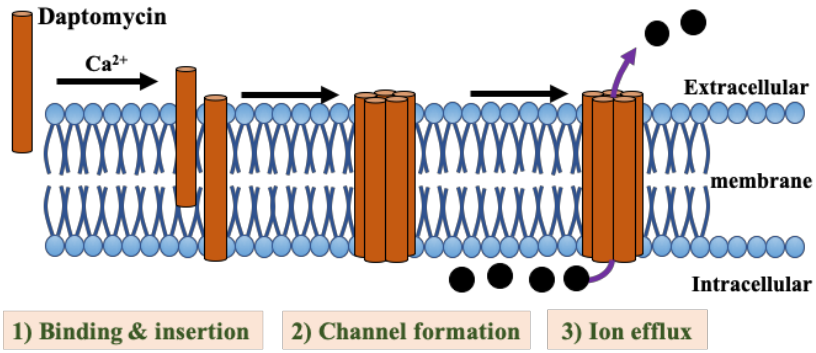

Figure 6: Schematic model of cell membrane disruption upon DAP exposure.
Pore-like structures formed by DAP in the presence of calcium ion induces efflux of intracellular ions, leading to cell death.

1.5. VCM and DAP resistance in MRSA

Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines define the *in vitro* susceptibility breakpoints for VCM and DAP to be ≤2 μg/mL ≤1 μg/mL, respectively [127]. MRSA strains resistant to DAP or VCM have been reported.

1.5.1) VCM resistance

Due to the increased incidence of MRSA infections, VCM has been widely used for the treatment of complicated staphylococcal infections since 1980s [128]. VCM resistance appeared in many forms according to the VCM minimum inhibitory concentration (MIC) of *S. aureus*, including VCM-intermediate *S. aureus* (VISA; 4 - 8 μg/mL) and VCM-resistant *S. aureus* (VRSA; ≥ 16 μg/mL) [127]. The first VRSA strain was isolated in the United States in 2002 [129]. VRSA exhibits vancomycin resistance following the acquisition of *vanA* operon carried on a plasmid-borne transposon Tn*1546* from VCM-resistant enterococci. The function of *vanA* operon is to catalyze the alteration of terminal D-Ala-D-Ala residues of peptidoglycan pentapeptide side chain (target site of VCM) to D-Ala-D-lactate (D-Ala-D-Lac) [130]. The *vanA* operon is consists of regulator-sensor system (*vanR* and *vanS*), and structural genes for D-Ala-D-Lac (*vanH*, *vanA* and *vanX*) and peptidase (*vanY* and *vanZ*) synthesis. During VCM exposure, the two-component regulator-sensor system activates transcription of genes responsible for D-Ala-D-Lac synthesis [131]. Structural genes (*vanH*, *vanA, vanX*) is essential for VCM resistance. Peptide-bond cleavage at D-Ala-D-Ala by *vanX*-encoded D,D-dipeptidase provide the substrate for formation of new D-Ala-D-Lac dipeptide. D-Lac amino acid is synthesized by D-lactate hydrogenase, a process regulated by *vanH*, and ligated by *vanA* [130]. D,D-carbozylpeptidase encoded by *vanY* also plays a role in cleaving dipeptide bonds of D-Ala-D-Ala at pentapeptide stem providing precursors for D-Ala-D-Lac synthesis [132].

However, the function of *vanZ* expression in contribution to VCM resistance has not been elucidated (Figure 7).

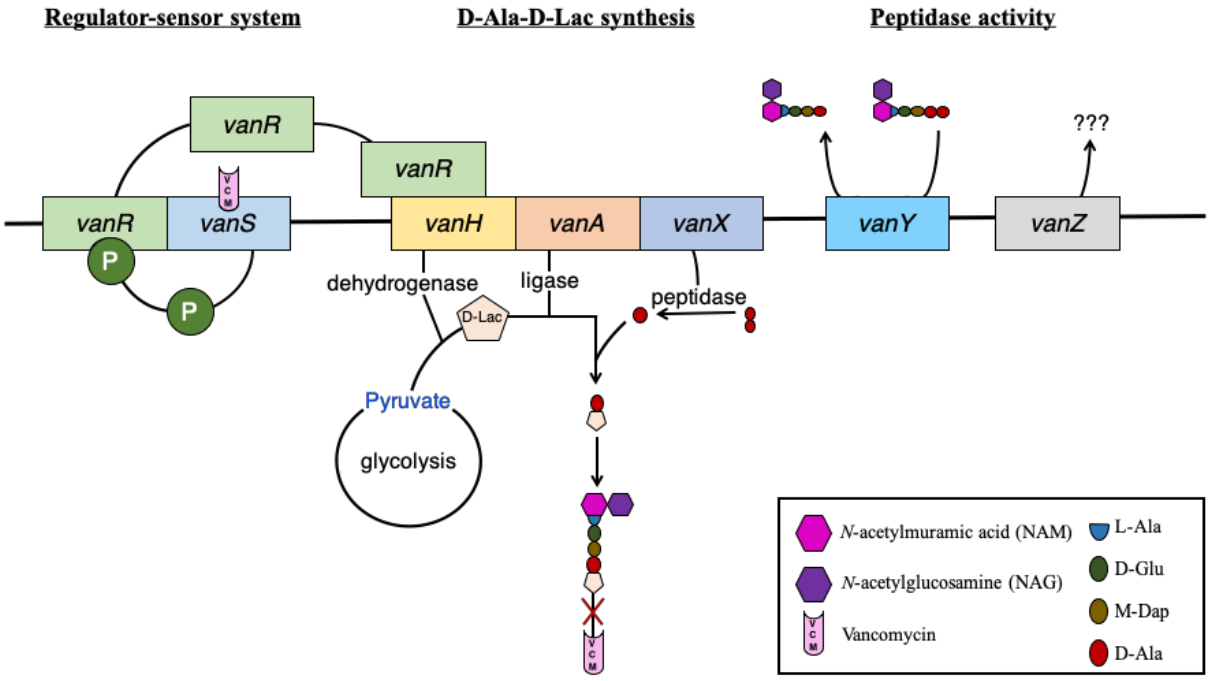

Figure 7: Schematic model of *vanA* operon-mediated VCM resistance in VRSA. Phosphorylation of *vanR* after sensing of VCM by *vanS* activates the genes responsible for D-Ala-D-Lac synthesis: *vanX* encodes for peptidase that cleaves dipeptide bond of D-Ala-D-Ala, *vanH* encodes for D-lactate dehydrogenase that regulates D-Lac production, and *vanA* which catalyzes the ligation of D-Ala and D-Lac. Cleavage of dipeptide bond in D-Ala-D-Ala of pentapeptide stem is regulated by *vanY*.

Table 4: Genes contributed to VCM-resistance in VCM-resistant *Staphylococcus aureus* (VRSA)

Genes	Description
vanR	VCM response regulator
vanS	sensor histidine kinase
vanH	VCM resistance protein
vanA	D-alanine-D-lactate ligase
vanX	D-alanyl-D-alanine dipeptidase

vanY	D-alanyl-D-alanine carboxypeptidase
vanZ	Predicted integral membrane protein

The emergence of VISA strain was first reported in 1996 in Japan [133] and has subsequently appeared in USA [134], Europe [4] and Asia [135]. In VISA strains, alteration of CW metabolism increased the synthesis of 'false target' (D-Ala-D-Ala) which served to trap the VCM molecules from binding to its lethal target site (division septum), resulting in protection from inhibition of CW synthesis [136]. Reduced VCM susceptibility in VISA strains is attributable to cumulative effects of mutations that contributed to altered CW metabolism, such as increased CW thickness, reduced peptidoglycan cross-linking or reduced rate of autolysis [137-139]. A few two-component regulatory systems (TCRSs), including GraSR, WalKR and VraSR, have been associated with VCM resistance in VISA strains [140, 141]. The interplay between TCRSs and VCM resistance was first reported based on upregulated expression of VCM-resistance-associated sensor/regulator (VraSR) observed in VISA and VRSA strains [142]. Histidine kinase VraS is a sensor for CW inhibitor agents, which upon activation will phosphorylate response regulator VraR to promote the transcription of CW biosynthesis genes such as *pbp2*, *fmtA* or *murZ* [143]. Previous reports showed that point mutation in *vraSR* reduced VCM susceptibility by upregulation of CW biosynthesis genes and increased CW thickness [24, 142, 144].

The glycopeptide-resistance-associated sensor/regulator (GraSR) is involved in capsule biosynthesis, CW signal transduction pathways and resistance against cationic antimicrobial peptides (CAMPs) [140, 145]. Studies have reported that activation of response regulator GraR by sensor kinase GraS upon exposure to CAMPs increased expression of *mprF* or *dlt* operon, both of which are involved increasing the positive charges on cell envelope [146]. Moreover, VISA isolates were found to exhibit upregulation of *graS* gene and VCM-sensitive strain

became resistant to VCM when *graS* was overexpressed [147]. Cui *et al*. also showed that transformation of only *vraS* or combination of *vraS-graR* into VSSA Mu50Ω supported its transition into VISA strain [148].

WalKR is another TCRS crucial for bacterial growth and CW metabolism [80, 149]. Similar to all TCRS, response regulator WalR is phosphorylated by histidine kinase WalK that ultimately activate CW modification [150]. Mutation in either *walK* or *walR* causes an increased CW thickness due to the decreased rate of CW turnover and have hence been associated with the development of VCM resistance [26, 151, 152]. In addition, downregulated expression of genes involved in autolysis (*lytM* and *ssaA*), proteins degradation (*fnbA* and *fnbB*), and cytolysis (*hla* and *hlb*) are observed in *walR*-mutated bacteria compared with those carrying wild-type allele [150, 151, 153]. Despite mutations in TCRSs, many other genes contributing to reduced VCM sensitivity have been reported (Table 5).

Table 5: Gene mutations contributed to the development of VISA

Mutated Genes	Impact on VCM resistance	Description	Reference
graRS	- increased VCM sensitivity in strain Δ*graRS*	respond to CW damage	[140, 154, 155]
	- *graS* substitution (T136I) reduced VCM susceptibility in VSSA		[155]
vraSR	- *vraS* mutation (I5N) caused reduced VCM susceptibility in Mu3 (hVISA) or Mu50 (VISA)	Respond to CW damage	[156]
	- introduction of mutated *vraS* in Mu50Ω reduced its VCM susceptibility		[148]
walKR	- *walKR* overexpression reduced VCM susceptibility	CW metabolism, autolysis and cell death	[141, 157]
	- reduced autolysis rate and increased CW thickness in hVISA carrying *walKR* mutation		[158, 159]
vraFG	- hypersensitivity to VCM in Mu50 lacking *vraG*	ABC transporter permease	[140]
yycH	- reduced VCM susceptibility in VISA strain (JH6; 8 μg/ml) with loss of function in *yycH* compared with VISA strain (JH5; 6 μg/ml)	CW synthesis and autolysis	[139]
pbp4	- increased MIC of VCM in VSSA with *pbp4*Δ and reduced MIC of VCM in VISA with PBP4 overexpression	CW remodeling with increased transpeptidase activity	[160]
agr	- the association between loss of *agr*II or *agrC* and induction of hVISA upon VCM exposure	attainment of virulence factors	[161]
	- reduced VCM susceptibility in VISA strain (JH6; 8 μg/ml) with loss of function in *agrC* compared with VISA strain (JH5; 6 μg/ml)		[139]

Mutated Genes	Impact on VCM resistance	Description	Reference
rpoB	- reduced VCM susceptibility and increased CW thickness in	DNA transcription	[162, 163]
clpP	- increased VCM resistance and increased CW thickness in N315LR5P1 with *clpP* mutation	proteolytic regulatory protein	[26]
isdE	- reduced VCM susceptibility in VISA strain (JH6; 8 µg/ml) with *isdE* mutation (A84V) compared with VISA strain (JH5; 6 µg/ml)	heme-iron transport	[139]
prsA	- reduced VCM susceptibility in VISA strain (JH6; 8 µg/ml) with frameshift mutation of *isdE* compared with VISA strain (JH5; 6 µg/ml)	chaperone involved in post-translational folding	[139]
mgrA / sarA	increased VCM sensitivity in double deletion in *mgrA* and *sarA* with reduced production of autolysins	negative regulators of autolysis	[164]
stp1	- increased VCM MIC in VCM sensitive strain (ISP794) with *stp1* mutation (Q12stop)	serine/threonine phosphatase	[165]
rsbU	- increased VCM MIC in strain carrying *rsbU* mutation	stress response	[166]
spoVG	- increased VCM resistance in strain Δ*spoVG*	capsule production	[167]
trfA/trfB	- increased VCM resistance in VSSA RN4220 lacking *trfA*/*trfB*	unknown function	[168]

1.5.2) DAP nonsusceptibility

In case of DAP, only the susceptibility breakpoint (<1 µg/mL) is established since there is no clear distinction between sensitive and resistant MIC values. In 2001, reduced DAP

susceptibility was first reported in *S. aureus* isolated from patients who had received ≥ 5 days of DAP treatment in phase 2 and 3 clinical trials (MIC of 4.0 μg/mL). Increased cases of DAP treatment failure are also demonstrated in clinics since 2006 following its approval for clinical uses by FDA in 2003 [169-173]. Multiple mechanisms involving CM and CW alterations have been proposed to be associated with DAP nonsusceptibility in *S. aureus*. These include mutations in genes responsible for phospholipid metabolism, such as *mprF*, *pgsA* and *cls2* [174].

Multiple peptide resistance factor (MprF) is a large membrane protein that consists of two domains: lysinylation and flippase domains (Figure 8). Lysinylation domain regulates the modification of anionic PG in inner leaflet of CM to lysyl-PG (L-PG; positively-charged membrane) via L-lysine addition, while the flippase domain translocates L-PG to outer leaflet of CM [175]. The expression of *mprF* is regulated by GraSR [176]. Previous reports showed that deletion or inactivation of *mprF* gene increased DAP sensitivity in DNS strains [13, 177]. *mprF* mutation that resulted in MprF gain-in-functions, either increased L-PG pools by lysinylation domain or enhanced L-PG translocation to outer leaflet of CM by flippase domain, can cause DAP nonsusceptibility [13, 178, 179]. Although the exact underlying mechanisms is still unclear, DAP nonsusceptibility mediated by *mprF* mutation seems to be related more closely with alteration of PG content than changes in surface charge of CM because not all DAP-nonsusceptible isolates carrying *mprF* mutation showed alteration of membrane surface charges compared with DAP-sensitive strains [177, 180]. In addition, reduced PG content in *B. subtilis* and *Enterococcus* or mutation in PG synthesis gene *pgsA* in *S. aureus* were shown to contribute to DAP nonsusceptibility [174, 181, 182]. Other than *mprF* gene, PG can also be modified via production of CL by CL synthetase *cls* gene [183]. CL is involved in respiratory chain, proton uptake and structural integrity [184-186]. There are two *cls* genes in *S. aureus*; *cls1* is expressed during membrane stress response such as low pH or high salt, *cls2* takes role

in cardiolipin synthesis [83, 187]. *cls2* mutation induces a less anionic surface charge in *S. aureus* which then causes reduced DAP susceptibility in those strains [83]. The other gene mutation that affect membrane surface charge is found on *dlt* which catalyzes the D-alanylation of WTAs. Previous reports showed that overexpression of *dlt* operon or *dlt* mutation can cause DAP nonsusceptibility [58, 61].

Membrane fluidity is the movement of PLs in CM that can regulate the passage of molecules across membrane[188]. As aforementioned, viscosity of CM can be affected by many factors, including temperature, phospholipid content or carotenoid expression [189, 190]. Membrane fluidity is also a possible pathway associated with DAP nonsusceptibility. It is hypothesized that alteration of membrane fluidity leads to reduced DAP binding at CM (target site of DAP) [181, 194]. However, contrasting features of membrane fluidity was observed between clinical and laboratory-derived DNS isolates. Clinical DNS isolates showed increased fluidity causing increased distance between PLs. On the other hand, DNS isolates generated *in vitro* showed decreased fluidity and have a more rigid membrane [181, 194, 195]. Moreover, only some DNS isolates showed alteration of membrane fluidity and the exact mechanism as to how membrane fluidity affects DAP susceptibility is still unclear [192].

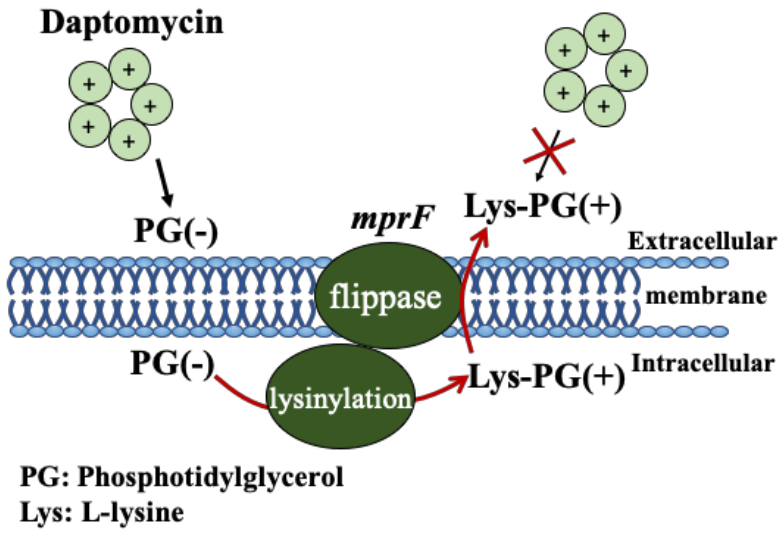

Figure 8: Schematic model of reduced DAP susceptibility mediated by *mprF* mutation. Cationic DAP binds to negatively charged CM through charge-charge interaction. Lysinylation domain of MprF changes negatively-charged PG into positively-charged Lys-PG by addition of lysine. Then, intracellular Lys-PG is translocated to outer leaflet by flippase domain to protect cells from DAP binding.

1.5.3) Cross-resistance between DAP and VCM

Cui *et al*. first reported the emergence of cross-resistance between DAP and VCM in VISA strain Mu50 and associated this phenomenon with increased CW thickness [14]. Since then, cross-resistance has been continuously reported by the other groups [27, 191, 192]. Increased CW thickness seems to be the contributing factor to reduced DAP binding at the CM [14, 192]. Upregulated CW metabolism genes, such as TCRS (*vraSR*), CW metabolism (*pbp2* or *mecA*), peptidoglycan biosynthesis (*lrgAB* or *murAB*) and autolytic enzyme (*lytH*), a similar observation as in VISA strains, are demonstrated in DNS strains compared with DS strains [60, 177, 193]. Besides, many reports showed that DNS strains carrying *walKR* mutation exhibited VCM resistance [17, 60, 194, 195], which leads to the hypothesis that reduced DAP susceptibility mediated by *walKR* mutation is associated with alteration of membrane fluidity [195]. On the other hand, inactivation of CW sensor regulator *vraSR* reverted cross-resistant strains back to DAP and VCM sensitive, coupled with a thinner CW thinner [177, 191]. However, increased CW thickness has been observed in some *S. aureus* which displayed DAP

nonsusceptibility but are sensitive to VCM [27, 59, 174]. Similarly, not all DNS strains showed increased CW thickness [27, 59].

In addition to increased CW thickness, alteration in membrane surface charge (a well-established contributor of DAP nonsusceptibility) is somewhat related to DAP and VCM cross-resistance. The *tag* gene involved in generation of anionic peptidoglycan is upregulated in DNS strains [59, 60]. Previous reports also showed increased DAP and VCM sensitivity in *S. aureus* following disruption of their negatively-charged WTAs [196, 197]. Not only does surface charges on CW, but also CM charges, are involved in DAP/VCM cross-resistance. The introduction of *rpoB* mutation (RNA polymerase β subunit) into DS strain rendered them DAP/VCM cross-resistance and exhibited phenotypic alteration of membrane surface charges [162]. Nonetheless, some DNS strains did not show alteration of membrane surface charges compared with DS strains [198]. Therefore, the mechanism of cross-resistance between DAP and VCM need to be further investigated to facilitate the generation of new ways against DAP/VCM cross-resistant bacteria.

CHAPTER III

OBJECTIVES

Emergence of MRSA causes a serious problem in the treatment of *S. aureus* infections due to its ability to develop resistance to a wide array of antibiotics. DAP, acting via disruption of bacterial CM, and VCM, functioning as inhibitor of bacterial CW synthesis, are the first choice of antibiotics indicated for treatment of complicated MRSA infections. Unfortunately, these antibiotics have each elicited different resistance mechanisms in bacteria. Alteration in surface charge of bacterial CM associated with DAP nonsusceptibility and increased bacterial CW thickness contributing to VCM resistance have been reported in *S. aureus*. Despite differences in bactericidal modes of action and resistance mechanisms triggered in bacterial cells, cross-resistance to both DAP and VCM in MRSA are increasingly identified and this phenomenon further limits the already scarcely available treatment options. The mechanisms leading to this phenotype remains unknown, therefore, our study aims to determine the possible pathways of DAP/VCM cross-resistance in MRSA. This will facilitate the identification of novel target sites and development of new therapeutic agents, contributing to the management of difficult-to-treat bacterial infections.

Aim 1: To characterize the pattern of drug resistance between single-drug resistance and DAP/VCM cross-resistance

Studies on single resistance of MRSA to DAP and VCM have been extensively documented. However, majority of these studies were limited by the number of isolates included (both clinical or laboratory-derived isolates) and did not focus on DAP/VCM cross-resistance. Our study will be the first attempt to reveal the mechanism of cross-resistance by conducting a systematic genetic study on a reasonable number of paired clinical isolates. Our studied strains can be separated into two groups; DAP treatment group and VCM treatment

group. 12 sets of DS and DNS isolates (each set collected from the same patient in Japan before and after daptomycin treatment), were included in this study. In addition, we also studied on 32 VISA strains collected worldwide from 9 countries, all of which are clinical isolates from patients receiving vancomycin therapy. The DAP and VCM MICs of all isolates will be determined according to the guideline of CLSI and each strain will then be categorized into different groups as follows:

1) DAP/VCM cross-resistance (MICs: DAP > 1 mg/L, VCM > 2 mg/L)

2) DAP nonsusceptiblity (MICs: DAP > 1 mg/L, VCM ≤ 2 mg/L)

3) VCM resistance (MICs: DAP ≤ 1 mg/L, VCM > 2 mg/L)

Aim 2: To determine the genetic mutation associated with DAP/VCM cross-resistance

The genetic determinants of DAP/VCM cross-resistance has not been clarified. Whole genome sequences of paired DS and DNS MRSA strains in each of the 12 sets of patients' isolates were compared to identify point mutation(s) in DNS strains. Then, mutation in the gene(s) of interest presumably involved in DAP/VCM cross-resistance will be transformed into DAP-sensitive strains. Furthermore, mutated gene(s) carried by resistant strains will be reverted to wild-type gene(s). Drug resistance pattern of all generated mutants will be determined to confirm the role of each selected mutations on DAP/VCM cross-resistance. If the transformed mutated gene(s) does play an important role in drug resistance, the sensitive strains introduced with mutated gene(s) will be rendered DAP/VCM cross-resistance.

Aim 3: To elucidate the possible pathway(s) in contribution of DAP/VCM cross-resistance

To consolidate our findings, genetic determinant(s) that contribute to bacterial resistance will be verified phenotypically. In case of phenotypic experiments, the thickness of

CW and the property of CM are investigated. Increased CW thickness was previously reported to reduce bacterial susceptibility towards VCM. Hence, we will measure the size of CW of each isolate. On the other hand, alteration of membrane surface charge is involved in DAP nonsusceptibility and this property will also be measured. Not only the phenotypic changes, but differential gene expression of paired DS and DNS strains from single-drug resistant or cross-resistance group will be determined to clarify the pathway(s) associated with single-drug resistance and DAP/VCM cross-resistance.

CHAPTER IV

MATERIALS AND METHODS

4.1 Materials

4.1.1) Chemicals and reagents

Acidic-phenol saturated	FUJIFILM Wako Pure Chemicals, Japan
Anhydrotetracycline	Sigma-Aldrich, USA
Brain-heart infusion (BHI)	Becton Dickinson Co., Ltd., USA
Chloroform	FUJIFILM Wako Pure Chemicals, Japan
Cytochrome *c*	Sigma-Aldrich, USA
Daptomycin	Pfizer Inc., USA
DNase I Recombinant, RNase-free	Roche, Germany
DNeasy blood and tissue kit	Qiagen GmhH, German
EDTA	Invitrogen, CA
Etest strip (DAP and VCM)	BioMérieux, USA
Ethanol	Kanto Chemical Co. Inc., Japan
Gateway BP clonase II Enzyme mix	Thermo Scientific, Wilmington, DE
HCl	Kanto Chemical Co. Inc., Japan
KCl	FUJIFILM Wako Pure Chemicals, Japan
Lysostaphin	Sigma-Aldrich, USA
Methanol	Kanto Chemical Co. Inc., Japan
Miseq reagent kit version 3	Illumina, Inc., USA
Molybdenum blue	Merck, USA
MOPS	DOJINDO, Japan
Muller-Hinton (MH)	Becton Dickinson Co., Ltd., USA
NaOAc	Kanto Chemical Co. Inc., Japan
Nextera Mate Pair Library Prep kit	Illumina, Inc., USA
Nextera XT DNA Library Prep Kit	Illumina, USA
Ninhydrin	FUJIFILM Wako Pure Chemicals, Japan
PicoGreen dsDNA detection kit	Invitrogen, CA
PrimeScript™ Double Strand cDNA Synthesis Kit	Takara, Japan
Ribo-Zero rRNA Removal Kit	Illumina, USA
RNeasy Mini Kit, Part 2	Qiagen, German
Tris-base	Sigma-Aldrich, USA
Tryptic soy (TS)	Becton Dickinson Co., Ltd., USA
Vancomycin	Sigma-Aldrich, USA

4.1.2) Bacterial strain

The list of bacterial MRSA isolates used in this study are displayed in **Table 6** for DAP treatment group and **Table 7** for VCM treatment group. In addition, the bacteria used for experiment of transformation are listed in **Table 8**.

Table 6: Clinical isolates from the same patient with DAP treatment

Patient	Strain	Collected Source	DAP Exposure	DAP MIC from original source		JMUB[a]	Reference
				DAP (μg/mL)	Methods		
A	A-1	Wound	-	0.25	VITEK2	JMUB480	[199]
	A-2	Wound	7 days	4	VITEK2	JMUB481	[199]
B	B-1	Purulent	-	0.5	VITEK2	JMUB483	[199]
	B-2	Purulent	14 days	4	VITEK2	JMUB484	[199]
C	C-1	Purulent	-	0.25	VITEK2	JMUB486	[199]
	C-2	Purulent	15 days	0.25	VITEK2	JMUB487	[199]
	C-3	Purulent	26 days	4	VITEK2	JMUB488	[199]
	C-4	Purulent	33 days	4	VITEK2	JMUB489	[199]
D	D-1	Purulent	-	0.25	VITEK2	JMUB491	[199]
	D-2	Purulent	24 days	4	VITEK2	JMUB492	[199]
E	E-1	Blood	-	0.5	WalkAway40 puls	JMUB473	This study
	E-2	Wound	21 days	>1	WalkAway40 puls	JMUB474	This study
F	F-1	Sputum	-	0.5	WalkAway40 puls	JMUB475	This study
	F-2	Brain abscess	45 days	>1	WalkAway40 puls	JMUB476	This study
G	G-1	Wound	-	0.5	WalkAway40 puls	JMUB477	This study
	G-2	Wound	7 months	>1	WalkAway40 puls	JMUB478	This study
H	H-1	N/A[b]	-	ND	N/A[b]	JMUB611	This study
	H-2	N/A[b]	5 days	ND	N/A[b]	JMUB612	This study
	H-3	N/A[b]	27 days	ND	N/A[b]	JMUB613	This study

Patient	Strain	Collected Source	DAP Exposure	DAP MIC from original source		JMUB[a]	Reference
				DAP (µg/mL)	Methods		
	H-4	N/A[b]	2 months	ND	N/A[b]	JMUB614	This study
	H-5	N/A[b]	3 months	ND	N/A[b]	JMUB615	This study
I	I-1	Blood	-	0.5	BMD[c]	JMUB1250	This study
	I-2	Blood	-	1	BMD[c]	JMUB1251	This study
	I-3	Blood	2 month	>2	BMD[c]	JMUB1252	This study
J	J-1	Blood	-	ND	N/A[b]	JMUB1276	This study
	J-2	Blood	51 days	>1	BMD[c]	JMUB1277	This study
	J-3	Blood	N/A[b]	2	BMD[c]	JMUB1278	This study
	J-4	Blood	N/A[b]	2	BMD[c]	JMUB1279	This study
K	K-1	Blood	-	0.5	WalkAway40SI	JMUB1358	This study
	K-2	Blood	6 days	>1	WalkAway40SI	JMUB1359	This study
L	L-1	Blood	-	1	WalkAway40SI	JMUB1360	This study
	L-2	Blood	-	>1	WalkAway40SI	JMUB1361	This study
	L-3	Blood	13 days	<0.5	WalkAway40SI	JMUB1363	This study

[a] Jichi Medical University bacterialbank

[b] not available

[c] broth microdilution method

-: Before DAP treatment

Table 7: Worldwide VISA collection upon VCM treatment

Strain Name	Year	Country	Code	MLST[a] (ST)	Reference or Source
NJ (HIP5836)	1997	USA	NRS4	5	[7]
HIP07256	1999	USA	NRS19	5	[7]
LIM2	1995	France	NRS36	247	[7]
HIP09740	2000	USA	NRS51	5	[200]
BR15	1998	Brazil	NRS54	239	[7]
HIP10540	2000	USA	NRS73	8	[200]
P1V44	1999	Belgium	NRS272	247	[200]
99/3759-V	1999	UK	NRS39	247	[7]
AMC11094	1997	Korea	NRS49	5	[7]
LY-1999-03	1998	Oman	NRS65	372	[200]
C2000001227	2000	USA	NRS76	5	[200]
NRS118	2002	USA	NRS118	247	[200]
NRS126	2000	USA	NRS126	5	[200]
98141	1998	France	N/A[b]	247	[7]
MI (HIP5827)	1997	USA	NRS3	5	[7]
SA MER	1998	France	NARSA	5	[200]
SA MER-S6	1998	France	NRS12	5	[200]
SA MER-S20	1998	France	NRS14	5	[200]
HIP06297 (PC)	1998	USA	NRS17	5	[7]
HIP08926	2000	USA	NRS23	5	[200]
HIP09143	2000	USA	NRS24	5	[200]
HIP12864	2003	USA	NRS402	5	[200]
HIP13057	2004	USA	NRS403	5	[200]
HIP13036	2004	USA	NRS404	5	[200]

Strain Name	Year	Country	Code	MLST[a] (ST)	Reference or Source
Mu50 (ATCC700699)	1996	Japan	NRS1	5	[7]
HIP06854	1998	USA	NRS18	5	[200]
HIP09313	2000	USA	NRS26	8	[200]
HIP09662	2000	USA	NRS28	8	[200]
LY-1999-01	1998	Oman	NRS63	372	[200]
99/3700-W	1999	UK	N/A[b]	239	[200]
28160	1998	South Africa	N/A[b]	247	[7]
BR5	1999	Brazil	NRS56	239	[7]

[a] Multi-locus sequence typing

[b] not available

"NARSA Catlog Number" refers to strain identification according to the Network on Antimicrobial Resistance in *Staphyloccocus aureus* (NARSA) (http://www.narsa.net)

Table 8: Bacterial transformation

Strain	Description
***S. aureus*-transformed strains**	
H-3_*mprF* (L291I)	allelic replacement with MprF (L291I) from H5 into H3-DS strain
H-5_*mprF* (WT)	allelic replacement with wild type MprF from H1 into H5-DNS strain
***E. coli* strain**	
DH5 α	Gene amplification, Amp^S

WT: wild type

4.1.3) Primers and plasmid

The primers for sequence confirmation and gene substitution, including plasmids used in this study are listed on **Table 9** and **Table 10**.

Table 9*:* Primers used in this study

Target gene and region	Primer	Oligonucleotide sequence (5'-3')
Primer for sequence confirmation		
mprF	mprF-F1	CCA TAT TGT TCT GTT TGA GAT AG
	mprF-F2	GTT TTG TAG GAT TGT ACT GCA
	mprF-F3	CAA TTT TAT TAA TCA CAG TGG C
	mprF-F4	GAT CAA CAC ATG CCT TTA TAT C
	mprF-R1	CAA TAA AGG ACA TGA ATG ATA C
	mprF-R2	ACA GAT GTA TGC ATT TCA ATC
	mprF-R3	GCG CTT TAG ATA AGT ATT CTT C
	mprF-R4	CGA CTT AAC TTA AGC TCA TTT C
agrA	agrA-F	GCA GAA TTA AGA ACT CGA ATT
	agrA-R	TGC ATT TGC TAG TTA TCT TGT
pbp2	pbp2-F	ACA CAA CAA GTT GTT AAA GAT G
	pbp2-R	AAC TTG TTG GTC TTT ATT CTT G
clfB	clfB-F	GGT CAA ATG TAG TCT TTT CTA AC
	clfB-R	CTA ATC TAG AAA TTG AAA TGG AG
arlS	arlS-F	GTG AAG TAG AAA CAA ATG TCG
	arlS-R	ATA AAC TGA CAA GCG GTT TA
lacF	lacF-F	TAC ATT TAC TTA AGT GCT GGT G
	lacF-R	CAC GTA TGC AAT TAA TAG AAA G
hisF	hisF-F	AGC TTA ATG CTA GAA GTG ATT G
	hisF-R	GAT TAT CTT GTA AAA TTG CTG G

Target gene and region	Primer	Oligonucleotide sequence (5'-3')
F1_0571	602762_F1-F	GGT GTT AAA GTT ACT TTG CAA
	602762_F1-R	TCT AAA CAG AAA AAA CCA GG
F1_0943	985260_F1-F	CAA AAA TTG ATG GAT GGT AC
	985260_F1-R	ACC TAG TCG TTC TAA AAC ATC T
F1_0110	2497349_F1-F1	CTT GTC TAA GGT TCA TAC CTC TA
	2497349_F1-F2	GAT TAT TTT CAT GTA GGA TTC
	2497349_F1-F3	TGG TAT GTA TAT CGT CGT ATT C
	2497349_F1-R1	CAT TAA TGG CAT CTT TAG GA
	2497349_F1-R2	TTT GTG TTA AGA CGT GTA ACC
	2497349_F1-R3	TTA GTA CAG CAT TAA CCA CTG T
626772_G1	626772_G1-F	GTA AAA CAT AAA TCT CAA AGG C
	626772_G1-R	CCA TCT TCT GGA TAA CAA AT
1286339_C1	1286339_C1-F	CTT TAG TAC GCT GTA ATC CAT T
	1286339_C1-R	GTT CCT ACA AAA ACT TAA TGG T
347486^347487_C1	347486^347487_C1-F	ACT TTG CAA ATA GCT TGT GTA
	347486^347487_C1-R	CGA TTA ATT GAC CAG ACT ATT AT
611436_K3	611436_K3-F	CTT GTA TCA ACA TGG TAC ATT AG
	611436_K3-R	ACC TGT TCC TAA TAT CGC TAA
356035_B1	356035_B1-F	GCC TCT TTT AAT TAT GCG TAT A
	356035_B1-R	CTT CAC GAA TAA CCA TGT AAC
B1_0504	553881_B1-F	AGC TGC AAG AAC TTA TAA TTT G
	553881_B1-R	ACT TTA AAA TGA ACT AAG CTC G
H1_0704	699807_H1-F	GCG TAC GAT TAT GTA TTT TTA AC
	699807_H1-R	TTA AAT GAC TTT TTG CAC CT
H1_2608	2641320_H1-F	AGA AAA GAT AAT CGC TGA TTA C
	2641320_H1-R	CGC ATG ATA ATT TAA TGC TAC

Target gene and region	Primer	Oligonucleotide sequence (5'-3')
306457^306458_C1	306457^306458_C1-F	GTG AGA ATC ATT GTC AAT TAG A
	306457^306458_C1-R	TTT ATC TAA ACC GTC TTG TCC
2284224_C1	2284224_C1-F	CAC TAA AAA ATC CAT TAA CTG G
	2284224_C1-R	CTT TTT GTC CTT CTC TTG TTA C
B1_1709	1840742^1840743_B1-F	TGT CTG AAT AAA TCG ATA AGG T
	1840742^1840743_B1-R	CGT TGA TCT ACG AAT CAT AAA
1394139_C1	1394139_C1-F	GAA TCA TAA TAG GTA CTT CTT GG
	1394139_C1-R	TAA GCA ATG TTT AGT TGC CT
255186_D1	255186_D1-F	GAT GAT TGT CCT TCA GCT TA
	255186_D1-R	AAA CAT CAT TTC TGT CTT ACC T
472436_D1	472436_D1-F	TCC ATA GCC TTT GAA GAT AA
	472436_D1-R	AAT TCG GTA AAG GAT TTG T
Primer for gene substitution		
M13M3	pKOR1 plasmid	GTA AAA CGA CGG CCA GT
M13RV		CAG GAA ACA GCT ATG AC
Forward	pKOR1-*lacF*	GGGGACCACTTTGTACAAGAAAGCTGGGTTCTTAAATATATGTTACGTG
Reverse		GGGGACAAGTTTGTACAAAAAAGCAGGCTCAAGTATACATTGAACAAGG
lacF-extra	pKOR1-*lacF*-Chromosome	ACT TGA GAA TAA GAT GAC AG
Forward	pKOR1-*mprF*	GGGGACCACTTTGTACAAGAAAGCTGGGTAAATATTCTTATCTGTACCG
Reverse		GGGGACAAGTTTGTACAAAAAAGCAGGCTAACACTTAAGATTCATCCAC
mprF-extra	pKOR1-*mprF*-Chromosome	GTT AGG CGA CTT AAC TTA A

Table 10: Plasmid used in this study

Plasmid	Description	Reference
pKOR1	*E. coli*-*S. aureus* shuttle vector for allele replacement	[201]

4.2 Methods

4.2.1) Bacterial strains and drug susceptibility test

The bacterial isolates used in this study included 12 pairs of DS and DNS strains, each collected from the same patient before and after DAP treatment (Table 6), and 32 VISA strains isolated from patients receiving VCM therapy (Table 7). All bacteria were kept in a final concentration of 40% glycerol at -80°C. Unless otherwise stated, the bacterial glycerol stocks were revived through cultivation in MH broth (Becton Dickinson, USA) at 37°C with constant agitation.

Two methods of drug susceptibility tests were employed in this study: Etest for determining DAP and VCM MIC for all studied strains, and broth microdilution for determining sensitivity toward DAP and VCM. Etests were performed following the guidelines of the Clinical and Laboratory Standard Institute. Briefly, each bacterial culture with 0.5 McFarland turbidity was streaked onto an MH agar plate, and DAP and VCM Etest strips (bioMerieux, France) were placed on the bacterial lawn. The inhibition zone break points for each isolate were read after 48 h. For the broth microdilution method, the ranges of DAP (0.5, 1, 1.5, 1.75, 2, 2.25, 2.5, and 3 mg/L) and VCM (0.5, 1, 1.5, 2, 2.25, 2.5, 3 and 4 mg/L) were tested against each bacterial culture. According to their susceptibility profile, each strain was classified as single DAP- or VCM-resistance or DAP/VCM cross-resistance.

4.2.2) Growth curve and doubling time

Growth kinetics of bacterial strains in DAP treatment group were determined. Overnight bacterial culture was adjusted to an optical density (OD) at 600 nm (OD_{600}) of 0.3. Following that, the OD-adjusted culture was diluted 1: 1000 with fresh brain-heart infusion broth (BHI; Becton Dickinson, USA) yielding a final concentration of 10^5 CFU/mL. Bacterial suspensions were then incubated at 37^oC with continuous agitation at 25 rpm in a temperature

gradient rocking incubator (TVS126MB; ADVANTEC, Japan). The bacterial density at OD_{600} was recorded every 5 min over a period of 24 h. Growth curve was then generated by plotting OD measurements against time gave a growth curve and doubling time of bacteria were then determined with equation described previously [7, 202].

4.2.3) Population analysis profiling-area under the curve (PAP-AUC) analysis

PAP-AUC is adapted from previous studies to confirm the DAP and VCM MICs of clinical MRSA isolates [7, 203]. Briefly, an overnight culture of MRSA strain was adjusted to OD_{600} of 0.3 and serially diluted 10-fold over a range of 10^{-3} to 10^{-10}. Then, 100 µL of each dilution was spread on drug-free MH agar, MH agar with DAP (0.5-3.0 µg/mL) and MH agar with VCM (0.5-4.0 µg/mL). After 48 h, the number of bacterial colonies were calculated and plotted semi-logarithmically.

4.2.4) DNA extraction and purification

20 mL of *S. aureus* overnight culture grown in tryptic soy broth (TSB; Becton Dickinson, USA) at 37^{o}C was harvested by centrifugation at 7,000 rpm for 10 min at 4^{o}C. The pellet was resuspended with digestion buffer (989 µL of TE buffer (pH 8.0) and 20 µg/mL of lysostaphin (Sigma-Aldrich, USA) and 5 unit of achromopeptidase (FUJIFILM Wako Pure Chemicals, Japan) and 100 µg of RNase A (Sigma-Aldrich, USA). After incubation at 37^{o}C for 1 h. 88 µL of 10% SDS and 7 µL of protease K solution (Qiagen, German) were added and the mixture was incubated at 56^{o}C for 2 h. Equal volume of tris-saturated phenol (pH 8.0) was then added and the solution was mixed well by inverting the tube for 5 min. Subsequently, the mixture was centrifuged at 8,000 rpm for 10 min. The aqueous phase (top layer) was transferred into new tube and an equal volume of a 24: 1 chloroform: isoamyl alcohol (vol/vol) was added. After inverting the tube for 5 min, the sample was centrifuged at 8,000 rpm for 10 min and the upper aqueous phase was transferred into new tube. The DNA was precipitated by adding 1/25

volumes of 5 M NaCl and 2.5 volumes of cold absolute ethanol, and pelleted by centrifugation at 8,000 rpm for 10 min at 4^{o}C. The supernatant was removed and the pellet was washed by 500 μL of 80% ethanol. Finally, the DNA pellet was air-dried briefly and resuspended in an appropriate volume of TE buffer. The extracted DNA was purified by DNeasy Blood and Tissue Kit (Qiagen GmhH, German) according to the manufacturer's instructions and the DNA concentration was measured by Nanodrop Lite spectrophotometer (Thermo Scientific, DE) and PicoGreen dsDNA detection Kit (Invitrogen, CA).

4.2.5) MLST and single nucleotide polymorphisms (SNPs) determination by whole genome sequencing

Each DS strain was used as reference sequences for comparison. The integrative analysis of genomic DNA sequence was performed without size selection using Nextera Mate Pair Library Prep Kit. Standard protocols and MiSeq instrument (2 x 301 bp) with the MiSeq reagent kit version 3 (Illumina, USA), indicating in coverage of an average raw genome, were investigated. The quality score of 30 (QC30) was used as a benchmark for quality trimming in FASTQ toolkit version 2.0.0. Quality-trimmed sequences were then assembled by using the Velvet de novo assembly version 1.2.10 algorithm.

For DNS strains, library preparation was performed with the Nextera XT DNA sample preparation and index kits. The prepared DNA-libraries were sequenced using the MiSeq platform (Illumina, USA) with 300-bp paired end reads. The genetic background of clinical MRSA isolates is characterized by multilocus sequence typing (MLST), containing the sequences of ~450-bp internal fragment of housekeeping genes (*arcC*, *aroE*, *glp*, *gmk*, *pta*, *tpi*, and *yqiL*) and checked on the "Center for Genomic Epidemiology" (CGE) website (https://cge.cbs.dtu.dk/services/MLST/). On the other hand, other gene mutations were identified by mapping the sequenced genomes against corresponding reference sequences by using CLC Genomics Workbench software (CLC bio, Denmark). The single nucleotide

polymorphisms (SNPs) and deletion or insertion mutations were identified and verified by PCR and Sanger sequencing with ABI3130x1 genetic analyzer (Applied Biosystems, USA).

4.2.6) Gene replacement into the chromosome

To investigate the effect of *mprF* (L291I) mutation, identified in DNS isolates of H patient, on drug susceptibility, gene replacement was performed using pKOR1 plasmid [26, 148]. In brief, *mprF* genes were amplified from each H-1 (DS) and H-5 (DNS) strain with primer sets listed in Table 9. The PCR fragments were individually cloned into pKOR1 plasmid using Gateway BP clonase II Enzyme mix (Thermo Scientific, DE) and recombinant plasmids were selected through CcdB-based positive selection system in *Escherichia coli* DH5α. The plasmid carrying wild-type *mprF* gene was then introduced into DNS strain H-5, while the mutated *mprF* gene was transformed into DS strain H-3. This was achieved by electroporation using NEPA21 electroporator (NEPA Gene, Japan) following the parameters reported previously [204]. Chromosomal gene replacement involved single-crossover plasmid integration at 43^{o}C followed by overnight incubation in drug-free medium at 37^{o}C to eliminate plasmid. Anhydrotetracycline was used to select for non-plasmid-carrying mutants. The presence of gene mutations was confirmed by PCR and targeted gene sequencing with ABI3130x1 genetic analyzer (Applied Biosystems, USA).

4.2.7) *In vitro* induction by stepwise DAP exposure

Overnight bacterial cultures (C-1 and K-1 strains) were streaked onto MH agar supplemented with 50 mg/L calcium and a range of concentrations of DAP (0.5-4 mg/L). After incubation at 37^{o}C for 2 days, the colonies that grown on MH agar containing the highest concentration of DAP were picked and then streaked again onto fresh MH agar containing DAP at different concentrations. The colonies that can grow on 4 mg/L DAP were further

investigated for their DAP and VCM MICs, along with presence of *mprF* or *lacF* mutation by Sanger sequencing.

4.2.8) Transmission electron microscopy (TEM)

CW thickness of all bacterial isolates form DAP treatment group was determined using TEM [138, 205]. *S. aureus* was grown overnight in 4 mL of BHI medium with shaking at 37^{o}C. 4 mL of bacterial suspension was then inoculated into new 4 mL of BHI and further cultured at 37^{o}C to an OD_{620} of 1.0. 1.5 mL of bacterial culture was centrifuged at 10,000 rpm for 1 min at 4^{o}C to harvest the cells. The bacterial pellet was washed 2 times with 0.1 M phosphate buffer (pH 7.4) by centrifugation at 10,000 rpm for 1 min at 4^{o}C. Bacterial precipitation was finally fixed overnight in cold 2.5% glutaraldehyde (vol/vol) in cold 0.1 M phosphate buffer (pH 7.4) and washed 2 times in 0.1 M phosphate buffer (pH 7.4). After fixation with cold 2% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) for 2 h and dehydration through an ascending series of ethanol, the fixed bacteria was embedded in EPON epoxy resin mixture and then cut with an ultra-thin sectioning. The sections were then stained with 2% uranyl acetate and Reynolds lead citrate, and were ready for visualization with a Hitachi H-7600 TEM. Thirty cells of each bacterial strain were examined for CW thickness measurement at nearly equatorial cut surfaces. The results were presented as mean $\pm$ standard deviations (SD).

4.2.9) Evaluation of membrane surface charge

Cytochrome *c* binding assay was performed as described previously [196] to measure membrane surface charges of bacterial isolates from DAP treatment group and transformed mutants carrying wild-type or mutated *mprF* gene. In brief, overnight bacterial cultures in MH broth were harvested and washed twice with 20 mM MOPS buffer (3-(N-morpholino) propanesulfonic acid; pH 7.0). Thereafter, the cells resuspended in the same buffer were adjusted to an OD_{600} of 7.0. The bacterial suspension was incubated at room temperature in 0.5

mg/mL cytochrome *c* (95% purity; Sigma-Aldrich Chemie GmbH, Germany) for 10 min before centrifuged at 6,000 x g for 10 min. The supernatant was collected for determination of the amount of unbound cytochrome *c* with spectrophotometry at an OD_{410}. Cytochrome *c* binding values in each DNS strain were determined from three-independent studies with normalization to DS strains of the same set.

4.2.10) Determination of L-PG production

PLs extraction from clinical MRSA isolates was adapted from Bligh-Dyer procedure [206]. Briefly, cells were harvested from overnight cultures of bacterial isolation from DAP treatment group grown in 20 ml of TSB broth for 12 h by centrifugation at 3,000 *g* for 15 min at 4^{o}C. The pellet was then washed with normal saline and bacterial density was adjusted to an OD_{600} of 20. After centrifugation at 3,000 *g* for 10 min at 4^{o}C, the bacterial pellet was directly digested with a mixture of chloroform/methanol/water (1.75: 3.5: 1.4; v/v/v) by vortexing. After that, 1.75 ml of chloroform and 1.6 ml 0.85% KCl were sequentially added to the mixture and vortexed well. The lower phase after centrifugation (1,000 rpm for 10 min) was then collected and washed with authentic upper phase that was prepared using dH_2O in place of sample, together with the same ratio of chloroform/methanol/water. The extracted organic layers were concentrated by evaporation before separation with thin layer chromatography (TLC) using Silica 60 $WF_{254}S$ thin layer chromatography (TLC; Silica-Gel 60-W-F254s, Merck, USA). The plates were developed with chloroform/methanol/water (65: 25: 4; v/v/v). L-PG was visualized by ninhydrin spray (FUJIFILM Wako Pure Chemicals, Japan), while total PLs were detected with Molybdenum Blue (Merck, USA). The L-PG production in ninhydrin and total PLs in each sample was quantified by ImageJ software (Rasband, 1997 - 2014). The L-PG levels in relative to total PLs of DNS strains were calculated from three independent studies by comparing with DS strains of the same set.

4.2.11) RNA extraction and RNA expression analysis

Overnight bacterial cultures (H-3/H-5 and K-1/K-2) diluted 1: 100 in 10 mL of MH broth was incubated at 37^{o}C until an OD_{600} of 0.8. The bacterial pellet was harvested and resuspended with 6 mL of pre-cooled $T_{10}E_{10}$ buffer (10 mM Tris-HCl, 10 mM EDTA; pH 8.0), followed by addition of 10 μg/mL lysostaphin (Sigma-Aldrich, USA) for complete bacterial lysis. Following that, 7 mL of acidic-phenol saturated with 20 mM NaOAc (pH 4.8) (FUJIFILM Wako Pure Chemicals, Japan) and 600 μL of 3 M NaOAc (pH 4.8) were added. The mixture was subjected to 3 cycles of 20 min freezing at -80^{o}C and 5 min thawing at 65^{o}C. Bacterial RNA was then extracted by phenol-chloroform method and ethanol precipitation. The RNA pellet was dissolved with DNase I Recombinant, RNase-free (Roche, Germany) and purified by RNeasy Mini Kit, Part 2 (Qiagen, German) before re-extraction with phenol-chloroform and ethanol precipitation. Finally, ribosomal RNAs in total RNA preparations were depleted by Ribo-Zero rRNA Removal Kit (Illumina, USA). The extracted RNAs were first converted into complementary DNA (cDNA) and subsequently be made double-stranded DNA (dsDNA) by PrimeScript^{TM} Double Strand cDNA Synthesis Kit (Takara, Japan). The generated dsDNAs were the used as template for cDNA library preparation using Nextera XT DNA Library Prep Kit (Illumina, USA) as previously described. The fold change of RNA expression between DS and DNS strain was determined by CLC bio (Denmark).

4.2.12) Statistical analysis

Student's *t* test was employed for all statistical analyses.

CHAPTER V

RESULTS

5.1 Reassessment of VCM and DAP susceptibility

This study began with analysis and validation of VCM and DAP susceptibilities for all paired isolates collected from 12 patients from whom DNS MRSA strains were generated during DAP monotherapy (Table 6). DAP and VCM susceptibility tests on the 13 DNS strains could classify the DNS strains into two different resistance groups, judged by minimum inhibitory concentrations (MICs) and population analysis profiles, namely, resistance to both DAP and VCM (termed as cross-resistance) and resistance to only DAP (termed as single resistance) (Table 11). Among the 13 DNS strains, 12 strains that belonged to the cross-resistance group showed MICs of 1.5 to 3.0 mg/L for DAP and 1.5 to 3.0 mg/L for VCM, which increased by 3.0 to 8.0 and 1.33 to 2.0 times, respectively, when compared to the corresponding DS parent strains. One strain, K-2, showed resistance to only DAP with a MIC of 2.0 mg/L but had no change in VCM susceptibility compared to its parent strain K-1 (Table 11). These resistant patterns were also confirmed by analysis of a resistant subpopulation against DAP and VCM (Figure 9) and determination of MICs with a broth microdilution method (Table 12). In addition, almost all DNS strains had increased doubling time compared to their DS counterpart, but there were two exceptions (D-2 and G-2) (Table 11).

5.2 Comprehensive mutation identification

To determine genomic alterations regarding reduced susceptibilities to DAP and VCM between DS and DNS isolates, whole-genome sequences of 12 pairs of DS and DNS MRSA strains from 12 patients were determined. Comparative genome analysis found that all DNS strains with reduced susceptibility to either DAP and VCM (cross-resistance) or DAP only (single resistance) carried at least one non-synonymous mutation. All mutations identified in DNS strains were validated using PCR-based sequencing and are listed in Table 11. Interestingly, all DNS strains with cross-resistance to DAP and VCM carried mutations on the *mprF* gene encoding an L-PG synthetase, which is known to synthesize positively charged lipid L-PG and subsequently translocate it from the inner membrane leaflet to the outer membrane leaflet. On the other hand, the DNS strain K-2 that is only resistant to DAP had an insertion mutation in *lacF*, which encodes a conserved ATP-binding domain homologous to ABC transporters known in bacteriocin immunity systems [207]. The *lacF* of K-1 differed from that of K-2 for the presence of one thymine deletion at position 125 that generated a premature stop codon (Figure 10A) and resulted in LacF truncation at position 42 (Figure 10B), indicating that the restoration of LacF function is responsible to the reduced susceptibility of K-2 to DAP.

For the *mprF* mutation, 10 types of point mutations were identified in this study, most of which were located on the lysinylation domain of MprF. Among those, three mutations, *mprF*(L291I), *mprF*(W424R), and *mprF*(L776S), were identified for the first time in this study (Figure 11). In addition, as shown in Table 11, DNS strains D-2 and G-2 showed intergenic region mutations besides the *mprF* mutation. Three out of eleven cross-resistant DNS strains had additional mutations that resulted in amino acid substitutions of B1_1709(N31_fs) in DNS strain B-2, *agrA*(T210I) and F1_0943(A363T) in DNS strain F-2, L1_0548(T134I) in DNS strain L-2, and *hisF*(G207_del) and H1_0704(C241Y) in DNS strains H-5. The mutations of *hisF*(G207_del) and H1_0704(C241Y) could also be found in DS strain H-3, indicating that

these mutations are not involved in the mechanism of the cross-resistance. In summary, *mprF* mutations were commonly found in the MRSA isolates with cross-resistance to VCM and DAP that were isolated from patients who had DAP monotherapy.

Table 11: Summary of MIC, gene mutation, MLST and doubling time

Patient	Strain	DAP MIC[a]		VCM MIC		Doubling Time (min)	MLST	Mutation in:	
		mg/L	Ratio	mg/L	Ratio			*mprF*	Others
Cross-resistance group (to DAP and VCM)									
A	A-1	0.38	1.00	1.5	1.00	26.6	764	-[b]	-
	A-2	1.5	3.95	2	1.33	27.0	764	T345I	-
B	B-1	0.19	1.00	1.5	1.00	30.0	764	-	-
	B-2	1.5	7.89	2	1.33	30.7	764	L776S	B1_1709 (N31_fs)[c]
C	C-1	0.5	1.00	2	1.00	29.5	764	-	-
	C-3	1.5	3.00	3	1.50	30.2	764	A475P	-
	C-4	3	6.00	3	1.50	30.9	764	L459_H466 del	-
D	D-1	0.25	1.00	1.5	1.00	32.8	1	-	-
	D-2	2	8.00	2	1.33	27.6	1	L826F	ir-1, ir-2[d]
E	E-1	0.25	1.00	1	1.00	32.6	764	-	-
	E-2	1.5	6.00	1.5	1.50	36.2	764	L826F	-
F	F-1	0.25	1.00	0.75	1.00	33.3	764	-	-
	F-2	2	8.00	1.5	2.00	36.5	764	L826F	*agrA*(T210I), F1_0943(A363T)
G	G-1	0.5	1.00	1	1.00	32.2	2809	-	-
	G-2	1.5	3.00	1.5	1.50	31.4	2809	T345A	ir
H	H-1	0.75	1.00	2	1.00	27.1	5	-	-
	H-3	0.5	0.67	2	1.00	25.9	5	-	*hisF*(G207_del), H1_0704(C241Y)
	H-5	3	4.00	3	1.50	26.6	5	L291I	*hisF*(G207_del), H1_0704(C241Y)
I	I-2	0.75	1.00	2	1.00	27.0	NT	-	-
	I-3	3	4.00	3	1.50	33.8	NT	W424R	-
J	J-1	0.38	1.00	1.5	1.00	27.1	764	-	-
	J-3	1.5	3.95	2	1.33	30.1	764	L341S	-

Patient	Strain	DAP MIC[a]		VCM MIC		Doubling Time (min)	MLST	Mutation in:	
		mg/L	Ratio	mg/L	Ratio			*mprF*	Others
L	L-1	0.25	1.00	1.5	1.00	26.5	380	-	-
	L-2	2	8.00	2	1.33	29.9	380	S337L	L1_0548(T134I)
DAP single-resistant strains (to DAP)									
K	K-1	0.38	1	1.5	1	24.7	764	-	*lacF*(trunc)[f]
	K-2	2	5.26	1.5	1.00	27.1	764	-	*lacF*(H41)

a) MIC ratio of DNS strain to its parent DS strain; b) no mutation; c) fs: frameshift; d) ir: intergenic region; e) not determined; f) truncated at position 42

Table 12: Daptomycin and vancomycin MICs determined by using broth microdilution method

Patient	Strain	MICs (mg/L)	
		DAP	VCM
Cross-resistance group (to DAP & VCM)			
A	A-1	1	1
	A-2	>3	2
B	B-1	1	1
	B-2	>3	1.5
C	C-1	1	1.5
	C-3	>3	2.25
	C-4	>3	2.25
D	D-1	<0.5	1
	D-2	3	1.5
E	E-1	1	1
	E-2	>3	1.5
F	F-1	1	1
	F-2	>3	2
G	G-1	0.5	1
	G-2	2.5	1.5
H	H-1	1	2
	H-3	1	2
	H-5	>3	2.25
I	I-2	1	2
	I-3	>3	>4
J	J-1	1	1.5
	J-3	>3	2
L	L-1	1	1.5
	L-2	>3	4
Single-resistance group (to DAP)			
K	K-1	1	1.5
	K-2	2	1.5

DAP: Daptomycin; VCM: Vancomycin

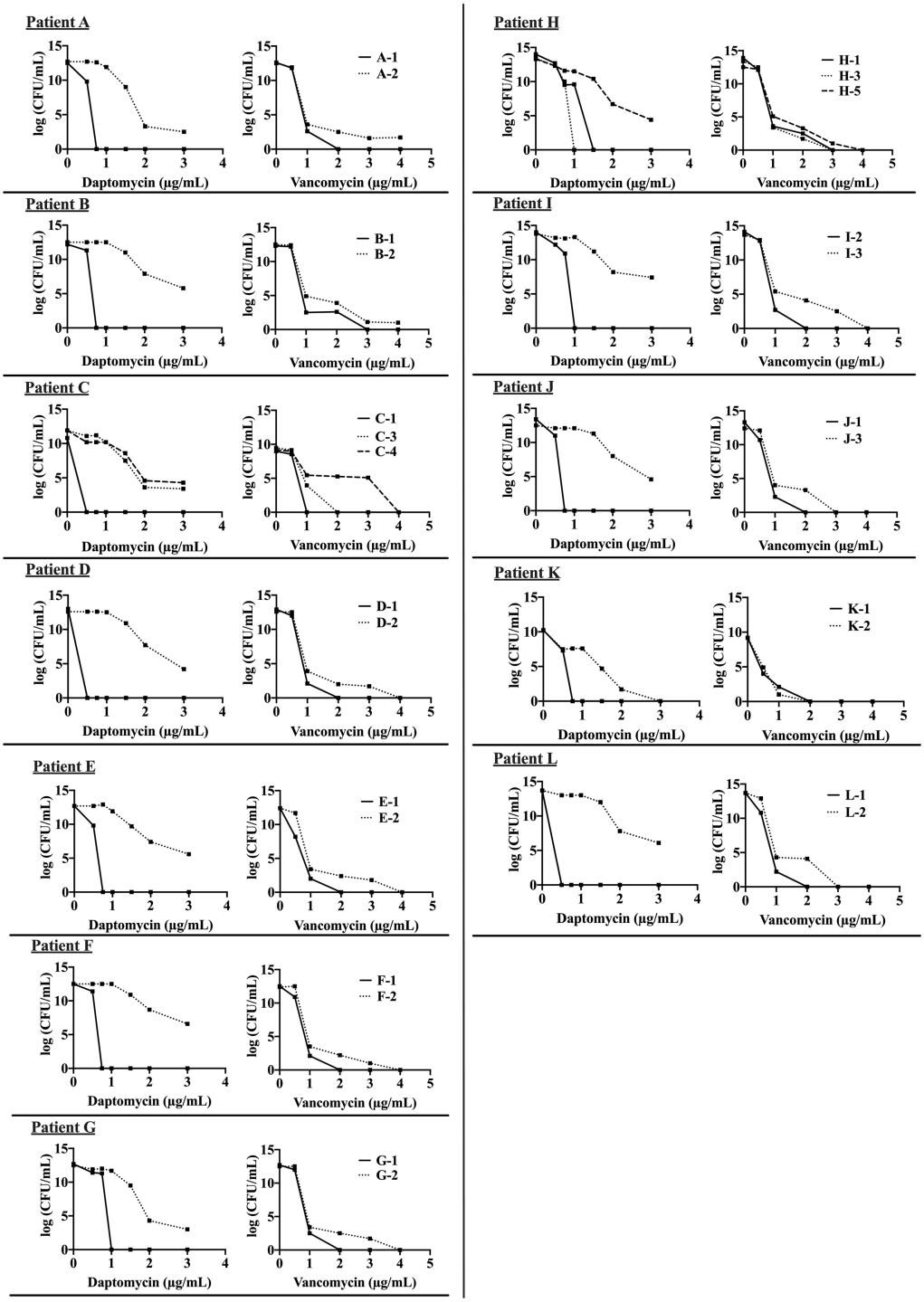

Figure 9: Population analysis profiles of clinical MRSA isolates in DAP treatment group. The bacteria were spread on MH agar in the presence of DAP (0.5 to 3 mg/L) or VCM (0.5 to 4.0 mg/L) to observe the distribution of resistant subpopulations in DAP and VCM. The number of CFU per mL was determined after 48-h incubation and plotted on the graph. All DNS strains displayed alterations in DAP susceptibility when compared with their DS strain (left graph). However, there is the same pattern of VCM susceptibility in DNS strain K-2, but not in the other sets.

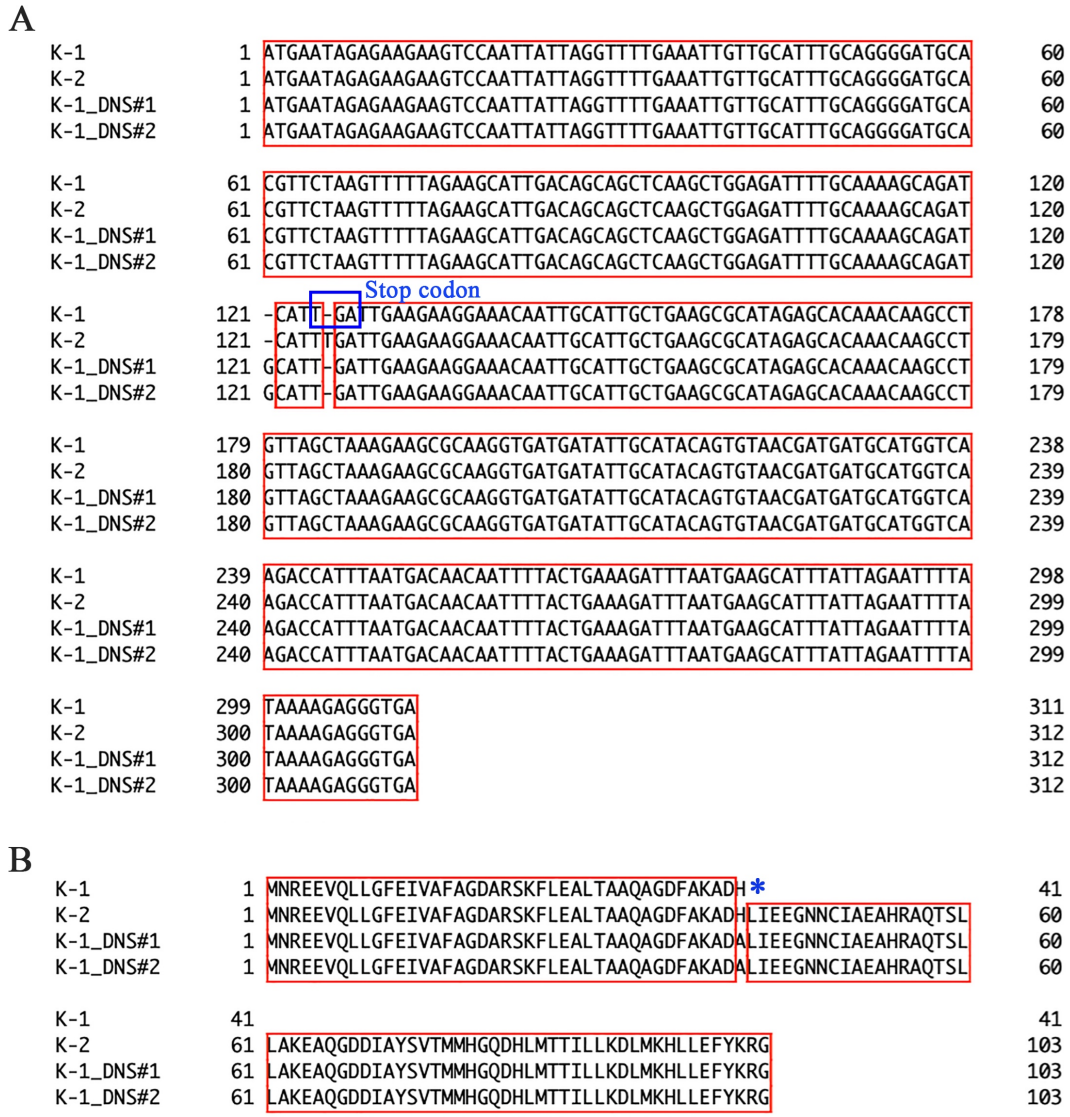

Figure 10: Sequence alignment for *lacF* of K-1 and its DAP-resistant mutants.
The *lacF* of K-1 had a premature stop codon at position 121 (A) results in the LacF truncation at position at 42 (B) when compared to K-2, K-1_DNS#1, and K-1_DNS#2.

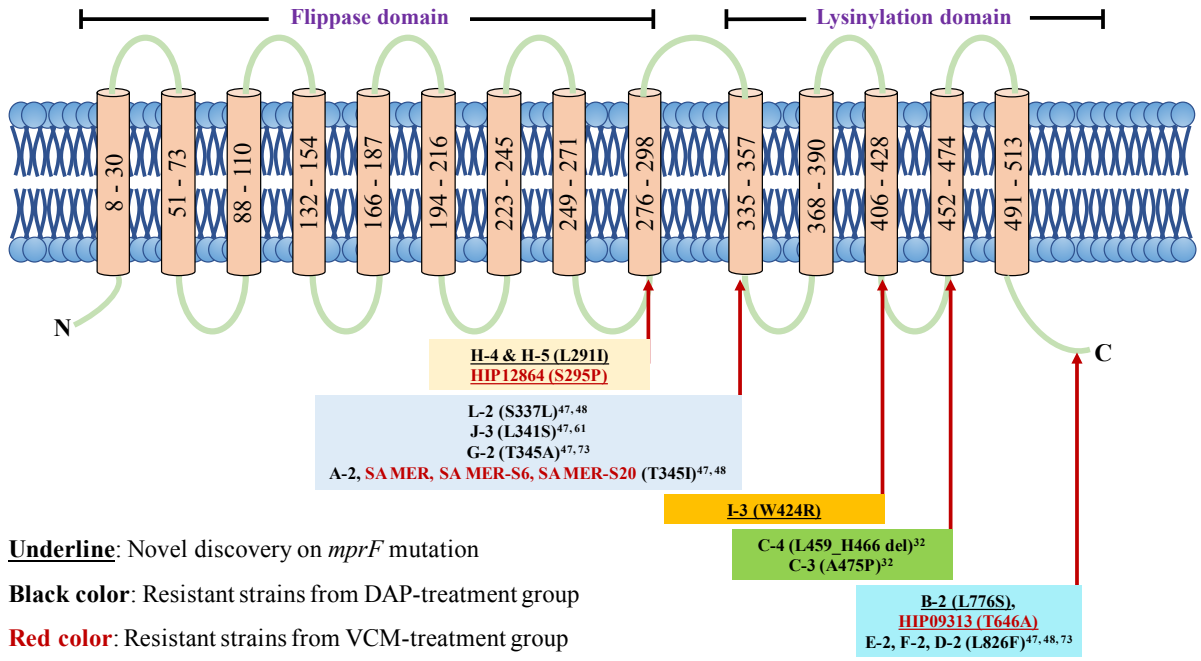

Figure 11: The location of *mprF* mutations in DNS strains and VISA strains. Most DNS (black text) and VISA (red text) isolates in this experiment carried *mprF* mutations on the lysinylation domain. The underlined mutations refer to newly discovered *mprF* mutations. The MprF structure is modified from previous studies [13].

5.3 Detection of genes reported to be associated with decreased susceptibility to VCM or DAP in *S. aureus*

Many genes have been reported to be associated with conversion of vancomycin-susceptible *S. aureus* to VISA, including *walK*, *clpP*, *graSR*, *vraSR*, *msrR*, and *rpoB* [26, 158, 200]. Some genes were also reported to reduce DAP susceptibility in MRSA [158, 162]. Therefore, we examined the sequences of these genes for all strains, but no differences were found between any pair of DS and DNS strains. The phenomenon of DAP and VCM cross-resistance was first reported in VISA strains in 2016 [14], and afterward, it became recognized in clinical practice during the VCM therapy. We first investigated and characterized the pattern of DAP and VCM susceptibilities for 32 VISA strains isolated from patients worldwide who received VCM therapy without exposing to DAP. We found that 14 strains were resistant to only VCM and 18 strains were resistant to both VCM and DAP (cross-resistance) (Table 13). All 32 strains were examined for single-nucleotide polymorphisms (SNPs) associated with VCM resistance as shown on Table 13. In the current study, we determined whole sequences of *mprF* for all strains using PCR and Sanger sequencing methods, and the results were combined with our previous results on the SNPs of *walK*, *clpP*, *graSR*, *vraSR*, *msrR*, and *rpoB* and are listed in Table 13. We found point mutations in *mprF* in 5 out of 18 DNS VISA strains with cross-resistance, but not in any of the 14 strains with resistance to only VCM. No other mutations found in genes or intergenic region of DNS strains from DAP monotherapy patients were found in the VISA isolates. These results suggested that there is a high prevalence of cross-resistance among the VISA strains and that *mprF* mutations play a role in conferring cross-resistance to VCM and DAP in some VISA strains that were generated during the prolonged VCM chemotherapy.

Table 13: Summary of MIC and gene mutation of VISA strains

Strain Name	Etest MIC (mg/L)		Gene Mutations[a]								
	DAP	VCM	*mprF*	*walK*	*clpP*	*graS*	*graR*	*vraS*	*vraR*	*msrR*	*rpoB*
Cross-resistance group (to VCM and DAP)											
MI (HIP5827)	1.5	6	-	V494L[26]	-	-	-	-	-	-	R140S[200]
SA MER	2	3	T345I	-	ND	ND	ND	ND	ND	ND	ND
SA MER-S6	4	3	T345I	-	ND	ND	-	-	ND	-	ND
SA MER-S20	4	6	T345I	-	ND	ND	-	-	ND	-	-
HIP06297 (PC)	2	4	-	A567D[26]	ND	ND	-	-	ND	-	Q468L[200]
HIP08926	2	3	-	R222I, T492K[26]	ND	L26F, I59L, T224I[26]	D148Q[26]	-	ND	-	ND
HIP09143	1.5	3	-	-	ND	ND	-	-	ND	-	ND
HIP12864	2	4	S295P	-	ND	ND	-	-	ND	-	P519L[200]
HIP13057	1.5	4	-	R282C[26]	ND	ND	E15K[26]	-	ND	-	H481Y[200]
HIP13036	1.5	6	-	-	ND	ND	-	T104A[26]	ND	-	-
Mu50	1.5	6	-	-	-	-	N197S[26]	I5N[26]	-	E146K[200]	H481Y[200]
HIP06854	1.5	6	-	T492K[26]	ND	ND	ND	ND	ND	ND	ND
HIP09313	2	4	T646A	L10F, S437T[26]	R152H[26]	ND	ND	P327S[26]	E59D[26]	ND	ND
HIP09662	1.5	3	-	Ins 433N, Ins 434D[26]	ND	L26F, I59L, T224I[26]	D148Q[26]	-	E59D[26]	-	D471N, S486L[200]
LY-1999-01	1.5	4	-	N48K, R222K, A468T[26]	ND	L26F, I59L, T224I[26]	D148Q[26]	ND	E59D[26]	K321R[200]	R406S[200]
99/3700-W	1.5	3	-	R222K, V366M, A468T[26]	ND	L26F, I59L, T224I[26]	D148Q[26]	-	-	-	-

Strain Name	Etest MIC (mg/L)		Gene Mutations[a]								
	DAP	VCM	*mprF*	*walK*	*clpP*	*graS*	*graR*	*vraS*	*vraR*	*msrR*	*rpoB*
28160	1.5	3	-	ND	ND	L26F, I59L, T224I[26]	D148Q[26]	ND	E59D[26]	ND	S529L[200]
BR5	1.5	3	-	R222K, V366M, A468T[26]	ND	L26F, I59L, T224I[26]	D148Q[26]	-	E59D[26]	ND	I527M[26]
Single-resistance group (to VCM)											
NJ (HIP5836)	0.75	4	-	I28T, 1341V[26]	-	-	S79F[26]	A260V[26]	-	-	H481Y[200]
HIP07256	1	3	-	ND	ND	ND	ND	ND	ND	ND	ND
LIM2	1	3	-	ND	ND	L26F, I59L, T224I[26]	D148Q[26]	-	E59D[26]	ND	H481N, S529L[200]
HIP09740	0.75	3	-	V380I[26]	ND	ND	-	-	ND	-	H481D[200]
BR15	1	3	-	ND	ND	ND	ND	ND	ND	ND	ND
HIP10540	1	4	-	-	ND	L26F, I59L, T224I[26]	D148Q[26]	-	ND	-	A477V[200]
P1V44	0.75	3	-	-	ND	L26F, I59L, T224I[26]	D148Q[26]	-	ND	-	H481N, S529L[200]
99/3759-V	0.75	3	-	V156Q[26]	M1V[26]	L26F, I59L, T224I[26]	D148Q[26]	ND	E59D, H481N, S539L[26]	ND	H481N, S529L[200]
AMC11094	0.75	3	-	ND	ND	ND	-	-	A113V[26]	-	-
LY-1999-03	1	4	-	N48K, R222K, A468T[26]	ND	L26F, I59L, T224I[26]	D148Q[26]	-	E59D[26]	K312R[26]	-

Strain Name	Etest MIC (mg/L)		Gene Mutations[a]								
	DAP	VCM	*mprF*	*walK*	*clpP*	*graS*	*graR*	*vraS*	*vraR*	*msrR*	*rpoB*
C2000001227	1	4	-	A243T[26]	ND	ND	-	A314V[26]	ND	-	-
NRS118	1	4	-	F330S[26]	ND	ND	-	-	ND	-	H481N, S529L[200]
NRS126	0.5	3	-	-	ND	ND	-	-	ND	-	H481N[200]
98141	0.75	3	-	-	ND	L26F, I59L, T224I[26]	D148Q[26]	ND	E59D[26]	ND	H481N, S529L[200]

a) *mprF* mutation was determined in this study, and the other gene mutations were detected in the previous studies (references were indicated); -: no mutation; ND: Not determined.

5.4 Substitution of *mprF* with mutated *mprF* identified in the cross-resistant DNS strain caused cross-resistance of DS strain to VCM and DAP

To confirm the role of *mprF* mutations in DAP and VCM cross-resistance, a newly identified *mprF* mutation (*mprF*(L291I)) of DNS strain H-5 was cloned and introduced into its counterpart DS strain H-3 to substitute *mprF* for H-3. The H-3 strain (an isogenic strain of H-1 and H-5), isolated in between H-1 and H-5 during DAP therapy, was chosen for *mprF* substitution to eliminate the confounding effect of other gene mutations (*hisF* and H1_0704), which cannot be found in DS strain H-1 (Table 11). The MIC test showed that the H-3 strain carrying *mprF*(L291I) increased the MICs of both DAP and VCM from 0.5 and 2 to 3.0 mg/L, showing cross-resistance to the same levels of the H-5 strain; vice versa, replacement of *mprF*(L291I) of H-5 with *mprF* of H-3 resulted in decreased MICs of both DAP and VCM from 3 to 0.5 and 2 mg/L, respectively, for the H-5 strain (Table 14). These results demonstrated that *mprF* mutations are a direct cause of raising cross-resistance to DAP and VCM in the MRSA H-5 strain.

Table 14: MICs of DAP and VCM in the *mprF* substitution of H-set isolates

Strain	*mprF* gene	Drug susceptibility (mg/L)	
		DAP	VCM
Clinical isolates			
H-1	Parental strain	0.75	2
H-3[b]	*mprF*(WT[a])	0.5	2
H-5[b]	*mprF*(L291I)	3	3
Constructed mutant strain			
H-5 *mprF*(WT)	L291I > WT	0.5	2
H-3 *mprF*(L291I)	WT[a] > L291I	3	3

[a] wild type

[b] additional mutations of H1_0704 (C241Y) and *hisF* (618insTGG)

5.5 Cross-resistance resulting from *mprF* mutation was found in *in vitro* selected mutants

The clinical DNS strains from patients who had DAP monotherapy exhibited cross-resistance to DAP and VCM due to *mprF* mutations. The mutation position in *mprF* differed among patients, as shown in the above results (Table 11, Figure 11) and the findings of Kanesaka *et al* [199]. To understand whether this is also the case for *in vitro* selected DNS mutants with cross-resistance, we generated DNS with cross-resistance to DAP and VCM *in vitro* by exposing DS MRSA to DAP with gradually increasing concentrations and examined mutations for *mprF* and *lacF*. The two DNS mutants were obtained from DS strain C-1 (DAP MIC, 0.5 mg/L) by stepwise selection on Mueller Hinton (MH) agar containing increasing DAP concentrations from 0.5 to 4 mg/L. These mutants could grow in the presence of 4 mg/L DAP. We found that these two mutants reduced susceptibility to both DAP and VCM (cross-resistance), increasing the MICs of DAP from 0.5 to 3 and 6 mg/L and VCM from 2 to 3 and 4 mg/L, and are accompanied by *mprF* mutations, *mprF*(T472K) for the mutant C-1_DNS #1 and *mprF*(R50L) for mutant C-1_DNS #2, respectively (Table 15).

The DNS strain K-2 with resistance to only DAP associated with a *lacF* mutation that has not been previously reported (Table 11). To valuate this mutation, a similar stepwise DAP selection was performed on its DS counterpart strain K-1, and two DAP-resistant mutants (K-1_DNS#1 and K-1_DNS#2) were generated. Interestingly, these two *in vitro* selected mutants showed decreased susceptibility to both DAP and VCM (cross-resistance), increasing the MICs of DAP from 0.38 to 6 mg/L and VCM from 1.5 to 3 mg/L; it was also accompanied by a *mprF* mutation in addition to the restoration of LacF as seen in K-2 strain (Table 15 and Figure 10B). These results, together with the above study on clinical DNS strains, demonstrated that the phenomenon of cross-resistance to DAP and VCM in MRSA is strongly associated with *mprF* mutations.

Table 15: Summary of MIC, doubling time and mutations in *mprF* and *lacF* on *in vitro* derivatives of the C-1 and K-1 strains

Strain	Etest MIC (mg/L)		Doubling Time (min)	Mutation in	
	DAP	VCM		*mprF*	*lacF*
Clinical isolates from Patient C					
C-1	0.5	2	29.5	-	-
C-3	1.5	3	30.2	A475P	-
C-4	3	3	30.9	L459_H466 del	-
***In vitro* derivatives of C-1 strain**					
C-1_DNS #1	3	3	30.8	T472K	-
C-1_DNS #2	6	4	28.0	R50L	-
Clinical isolates from Patient K					
K-1	0.38	1.5	24.7	-	trunc*
K-2	2	1.5	27.1	-	H41
***In vitro* derivatives of K-1 strain**					
K-1_DNS #1	6	3	54.6	T472K	H41A
K-1_DNS #2	6	3	35.4	T472K	H41A

- : no mutation; DNS: Daptomycin-nonsusceptible strain; *: truncated at position 42.

5.6 Cross-resistance and CW thickness

Thickened CW is known as a phenotypic determinant for VCM resistance in VISA [7, 208]. Although not consistently reported, alteration of CW structure and/or changes in expression of genes involved in CW metabolic pathways have also been associated with DAP-nonsusceptibility [22, 27]. Therefore, alteration of CW structure might be one of the mechanisms involved in DAP and VCM cross-resistance. In order to test this hypothesis, the CW thicknesses of 30 cells from each strain were measured by transmission electron microscopy (TEM) for all DNS isolates. The results showed that only one strain in the cross-resistance group (I-3) carrying a *mprF* mutation (*mprF*(W424R)) displayed significantly increased CW thickness (25.55 ± 2.92 nm) compared with I-2 sensitive strain (22.11 ± 1.83 nm) (Figure 12A). The other 11 DNS strains in the cross-resistance group did not exhibit CW thickening compared with their corresponding parental strains (Figure 12A).

There was also no difference in CW thickness between DS isolate K-1 (21.50 ± 1.82 nm) and DNS isolate K-2 (21.15 ± 1.75 nm) exhibiting resistance to only DAP due to a *lacF* mutation (Figure 12B). Our results indicated that CW thickening did not appear to be the main mechanism of cross-resistance associated with *mprF* mutations or single-drug resistance associated with *lacF* mutations.

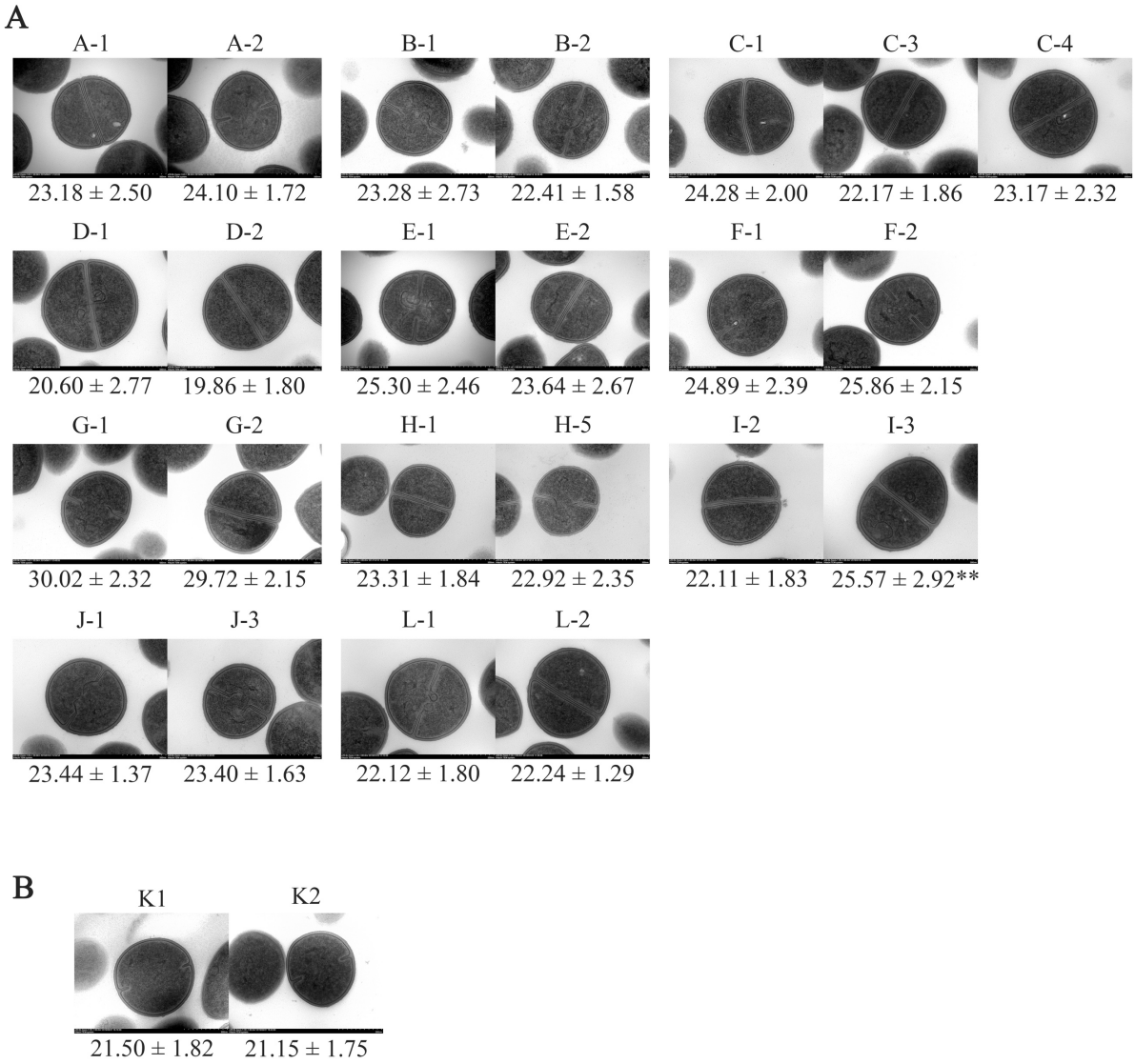

Figure 12: Comparison of cell-wall size in clinical MRSA isolates

The comparison of CW properties between DS and DNS strains in cross-resistance (A) and DAP-single resistance (B). An OD_{620} of 1.0 in each bacterium was washed 2 times with 0.1 M Phosphate buffer before analysis of the thickness of CW by TEM. There is no difference in CW thickness in most DNS strains. Data represent the means for 30 cells in each strain (nm) ± standard deviation (SD):

** P<0.01 indicate statistical significant relative to the parent strain (*t*-test; parametric tests)

5.7 *mprF* mutation and membrane surface charge

The *mprF* mutation had been previously reported in MRSA with reduced DAP susceptibility. MprF is a membrane-bound enzyme that adds lysine to phosphatidylglycerol in the cytoplasmic membrane. This modification changes the net charge of the bacterial CM to alter membrane permeability, which confers resistance to cationic antibiotics [13, 198]. To determine whether *mprF* mutations identified in this study resulted in such alterations, we carried out a cytochrome *c* binding assay on all DNS strains to examine the alteration of membrane surface charges. A cationic cytochrome *c* can bind a negatively charged membrane and, hence, has been widely employed to determine the relative surface charges of the cell envelope [178]. Our results showed that all strains carrying a *mprF* mutation had significantly reduced cytochrome *c* binding when compared to their parental strains, indicating that all *mprF* mutations identified in this study caused increased positive membrane surface charge (Figure 13A). Similarly, increased positive membrane surface charge was observed when the *mprF* of DS strain H-3 was replaced with mutated *mprF*, while replacement of mutated *mprF* in DNS strain H-5 with that of its wild-type counterpart reduced the positive membrane surface charge (Figure 13C). The DNS strain with resistance to only DAP carrying a *lacF* mutation did not exhibit reduced negative charges (Figure 13B). These results suggest that increased positive membrane surface charge is one mechanism causing *mprF* mutation-mediated DAP/VCM cross-resistance in MRSA.

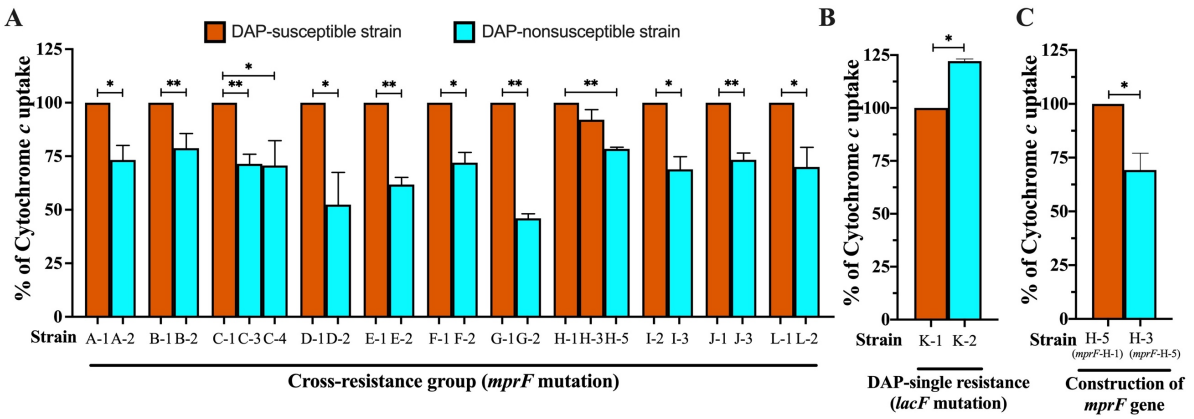

Figure 13: Measurement of membrane surface charge

Cell surface charge of DS (White bar) DNS (Grey bar) strains in cross-resistance group of clinical MRSA isolates (A) DAP-single resistance of clinical isolates (B) and construction of *mprF* gene (C). Overnight bacteria were incubated with 0.5 mg/ml cytochrome *c* solution for 10 min and calculated the percentage of cytochrome *c* bound compared with DS strains. Significant reduction of cytochrome *c* binding can be observed in cross-resistance group and bacterial construction containing *mprF* mutation but not in single-DAP resistance. Data represent the means of three independent experiments for each strain ± standard deviation (SD):

* $P<0.05$; ** $P<0.01$ indicate statistical significant relative to the parent strain (*t*-test; parametric tests)

5.8 *mprF* mutation and L-PG production

The increased cationic phospholipid L-PG production in cytoplasmic membranes has been reported to decrease DAP susceptibility in MRSA [209]. To understand whether the *mprF* mutations found in this study are implicated with L-PG production, we set out to determine membrane L-PG levels for all DNS and DS strains using TLC assay. Altered L-PG production of DNS strains over corresponding DS strains was calculated in relative values (percentages) and is summarized in Figure 14. As shown in Figure 14A and C, all DNS strains with cross-resistance (carrying a *mprF* mutation) showed increased L-PG production, which is correlated with increased positive membrane surface charges. Although a more than 50% increase in L-PG production can be found in most DNS strains, four strains (B-2, F-2, J-3, and L-2) harboring *mprF* mutations at different locations displayed only a marginal increase (10% or 20%) and showed a comparably lower level of change in membrane charge as determined by a cytochrome *c* assay (Figure 14A). These results suggested that increased L-PG production regulated by *mprF* mutation altered bacterial membrane surface charges to become more positive, thereby contributing to cross-resistance.

In addition, we found that the K-2 strain with resistance to only DAP that carries *lacF* mutation had decreased L-PG production compared to its DS counterpart, in agreement with the aforementioned increase in negative membrane charge (Figure 14B). This indicated that membrane charge alterations are not involved in *lacF* mutation-mediated DAP resistance.

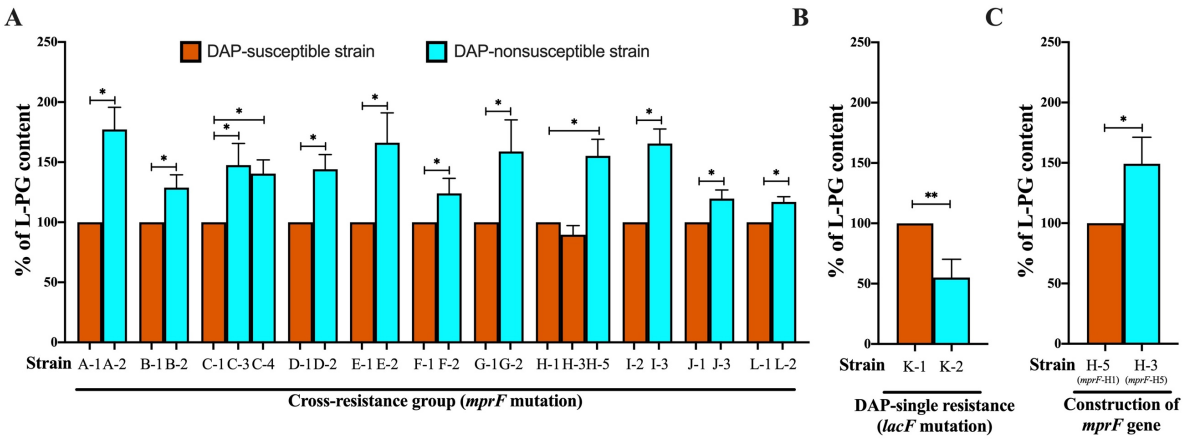

Figure 14: Evaluation of membrane L-PG production

The lipid extraction (10 µL) was spotted on Silica gel plates and the L-PG inner membrane was detected by ninhydrin spray. Cross-resistance group (A) and replacement with *mprF* mutation into H-3-DS strain (C) showed increased L-PG production compared with single-drug resistance (B). Data represent the means of three independent experiments for each strain ± standard deviation (SD):

* $P<0.05$; ** $P<0.01$ indicate statistical significant relative to the parent strain (*t*-test; parametric tests)

5.9 Transcriptional analysis on representative DNS strains with both single and cross-resistance and their DS counterparts

In the above results, the association of *mprF* mutation and altered membrane metabolic pathways with DAP and VCM cross-resistance was clearly demonstrated; however, the impact of the *mprF* mutations found in this study on metabolic regulations toward the resistance remains to be clarified. To this end, a representative pair of DS and cross-resistant DNS strains, H-3 and H-5, isolated from patient H were selected to analyze genome-scale gene expression profiles by RNA-sequencing. The *mprF*(L291I) mutation found in H-5 genome is the only difference between H-5 and H-3 and is considered to be responsible for cross-resistance to DAP and VCM. Its contribution to the cross-resistance was also verified by gene substitution experiments (see elsewhere above). A total of 103 genes differentially expressed more than fourfold between H-3 and H-5 were found (Table 16). Among them, 61 genes were upregulated (59.22%) and 42 genes were downregulated (40.78%). These genes could be roughly classified into four functional categories: metabolism (27.18%), information storage and processing (13.59%), cellular process and signaling (6.80%), and the others (52.43%). As shown in Table 16, a number of genes involved in fructose-6-phosphate (F-6P) synthesis are found to be upregulated in DNS strain H-5, such as *gatA* (5.2-fold), *tal* (8.4-fold), and *pmi* (4.8-fold). The genes mainly altered in expression were highlighted on the map of metabolic pathways of fatty acid and peptidoglycan synthesis (Figure 15). The F-6P biosynthesis pathway is associated with mevalonate pathway, which is essential for isoprenoid membrane lipid synthesis using acetyl-coenzyme A (acetyl-CoA) as a precursor [210-212]. In concordance with the above results, an exceptionally high upregulation of *hlb-2* (113.8-fold) was found. The *hlb-2* gene encodes phospholipase C, which is known to be deeply involved in the metabolism of glycerophospholipid, a main component of biological membranes. In addition, there were some other upregulated genes involved in fatty acid metabolism, such as *fadA* (4.3-fold) and *plsY*

(4.6-fold). On the other hand, the downregulation of the *narH* gene in nitrogen metabolism (-4.9-fold) was also found. This presumably caused the accumulation of nitrate inside cells and helped stabilize the membrane potential in DNS strain H-5, thereby preventing membrane depolarization during DAP treatment [193, 213, 214]. The accumulation of intracellular nitrate will in turn induce a nitrosative stress on cells, resulting in the upregulation of pyrimidine metabolism and increased uracil production that is a precursor for fatty acid metabolism [214]. Accordingly, the *pyrR* gene involved in pyrimidine metabolism was upregulated in DNS strain H-5 with a 4.1-fold. It was also noted that 7 out of 61 upregulated genes in DNS strain H-5 were involved in CW metabolism. Uridine diphosphate-*N*-acetylglucosamine (UDP-NAG) and UDP-*N*-aceylmuramic acid (UDP-NAM) serves as backbone for peptidoglycan [37]. Up-regulation of *nagD* (6.6-fold) will supply NAG production from conversion of glucosamine-6-phosphate, together with increased UDP pool by up-regulation of *pyrR* (4.1-fold) and *pmi* (4.8-fold) genes and down-regulation of *psuG* (-5.1-fold) gene. In addition, all 4 of these genes are associated with *staphylococcal* "cell wall stimulon" such as *spsA* (4.3-fold), *ssaA* (5.9-fold), *relP* (5.8-fold) and *sasA* (5.9-fold) [24, 215, 216]. Thus, cross-resistance mechanism by *mprF* mutation come from changes in CW/CM metabolism.

Table 16: Representatives of genes differentially expressed between cross-resistant strain H-5 and susceptible strain H-3

N315 locus tag	Gene	Function	Fold change (H-5/H-3)
Metabolism			
[C] Energy production and conversion			
SA0910	*qoxD*	Cytochrome aa3 quinol oxidase, subunit IV	5.6
SA1451		Luciferase family oxidoreductase, group 1	4.3
SA1988		Zinc-binding alcohol dehydrogenase family protein	4.0
SA2184	*narH*	Nitrate reductase, beta subunit	- 4.9
[E] Amino acid transport and metabolism			
SA1216		Oligoendopeptidase F (Peptidase family M3)	4.7
SA1980		Alanine racemase	4.3
SA0668	*pabA*	Glutamine amidotransferase of anthranilate synthase or aminodeoxychorismate synthase	- 9.3
SA2319	*sdaAB*	L-serine dehydratase, iron-sulfur-dependent, beta subunit	- 4.8
[F] Nucleotide transport and metabolism			
SA1041	*pyrR*	Hypoxanthine phosphoribosyltransferase	4.1
[G] Carbohydrate transport and metabolism			
SA0236	*gatA*	PTS system, fructose subfamily, IIA component	5.2
SA1599	*tal*	Transaldolase/Fructose-6-phosphate aldolase	8.4
SA1656	*hit*	Diadenosine tetraphosphate (Ap4A) hydrolase	7.5
SA2362		Ergot alkaloid biosynthesis protein, AFUA_2G17970 family	5.3
SA2435	*pmi*	Mannose-6-phosphate isomerase, class I	4.8
SA0903		Acyltransferase family	- 4.6
SA1758	*sak*	Staphylokinase/Streptokinase family	- 6.1
[H] Coenzyme transport and metabolism			
SA1412	*hemN*	Putative oxygen-independent coproporphyrinogen III oxidase	- 4.5
SA2390	*panD*	Aspartate 1-decarboxylase	- 4.5
[I] Lipid transport and metabolism			
SA0223	*fadA*	Acetyl-CoA C-acyltransferase	4.3
[P] Inorganic ion transport and metabolism			
SA0333	*fepC*	FTR1 family protein (Iron permease FTR1 family)	4.0
SA1977	*htsC*	Proposed F420-0 ABC transporter, permease protein	4.1

N315 locus tag	Gene	Function	Fold change (H-5/H-3)
SA0951	*potB*	Putative 2-aminoethylphosphonate ABC transporter	- 5.1
SA0110	*sirB*	Proposed F420-0 ABC transporter, permease protein	- 11.9
SA1212	*opp-2D*	Nickel import ATP-binding protein NikD	- 6.0
SA1219	*pstA*	Phosphate ABC transporter, permease protein PstA	- 4.0
[Q] Secondary metabolites biosynthesis, transport and catabolism			
SA1419		Malonyl-acyl carrier protein O-methyltransferase BioC (Methyltransferase domain)	6.1
SA0181		Pyrimidine utilization protein B	- 4.2
SA0301	*psuG*	CRISPR-associated endonuclease Cas1	- 5.1
Information storage and processing			
[J] Translation, ribosomal structure and biogenesis			
SA1067	*rpmB*	Ribosomal protein bL28	4.0
SA1116	*rpsO*	Ribosomal protein uS15	5.6
SA1459	*dtd*	D-tyrosyl-tRNA(Tyr) deacylase	5.8
SA1918		tRNA threonylcarbamoyl adenosine modification protein	5.6
[K] Transcription			
SA1191	*glcT*	GTP cyclohydrolase II (PRD domain)	11.1
SA1351	*argR*	arginine repressor	7.9
SA1851	*rex*	Transcriptional regulator	6.0
SA1998	*lacR*	CRISPR locus-related DNA-binding protein	4.0
SA2089	*sarR*	Staphylococcal accessory regulator family (MarR family)	4.0
SA0559		Ribosomal-protein-alanine acetyltransferase	- 6.0
[L] Replication, recombination and repair			
SA0987	*rnhC*	Ribonuclease HIII	5.0
SA1180	*sbcD*	Exonuclease SbcCD, D subunit (DNA repair)	4.7
SA1305	*hup*	Integration host factor, beta subunit	4.9
SA1489	*tag*	DNA-3-methyladenine glycosylase I	4.6
Cellular process and signaling			
[M] Cell wall / membrane / envelop biogenesis			
SA2291	*fnbA*	LPXTG cell wall anchor domain	- 6.0
[O] Post-translational modification, protein turnover and chaperones			
SA1179		Multicomponent Na+:H+ antiporter	- 6.0
SA1627	*splF*	Peptidase Do (Trypsin-like peptidase domain)	- 4.1

N315 locus tag	Gene	Function	Fold change (H-5/H-3)
SA1629	*splC*	Periplasmic serine peptidase DegS	- 4.0
SA1631	*splA*	Periplasmic serine peptidase DegS	- 6.1
[T] Signal transduction mechanisms			
SA2153		LytTr DNA-binding domain	4.2
[U] Intracellular trafficking, secretion and vesicular transport			
SA0825	*spsA*	Signal peptidase I	4.3
Poorly characterized			
[S] Function unknown			
SA0275	*essB*	type VII secretion protein EssB	6.0
SA0355		Abi-like protein (CAAX protease)	4.3
SA0513		HAD hydrolase, TIGR02253 family	4.6
SA0544	*hemQ*	Chlorite dismutase (heme-binding protein)	11.3
SA0649		Domain of unknown function (DUF296)	5.6
SA0664			4.8
SA0772		CsbD family protein	5.7
SA0914		Domain of unknown function (DUF5011)	7.9
SA1167		Cof-like hydrolase (haloacid dehalogenase-like hydrolase)	7.1
SA1187	*plsY*	acyl-phosphate glycerol 3-phosphate acyltransferase	4.6
SA1752	*hlb-1*	β-hemolysin	5.2
SA1811	*hlb-2*	a phospholipase C toward sphingomyelins	113.8
SA2093	*ssaA*	CHAP domain-containing protein	5.9
SA2101		Protein of unknown function (DUF1641)	4.2
SA2297	*relP*	RelA/SpoT family protein	5.8
SA2443	*asp3*	Accessory Sec system protein Asp3	4.3
SA2447	*sasA*	Signal peptidase I	4.3
SA0091	*plc*	Phosphatidylinositol-specific phospholipase C, X domain	- 6.0
SA0753		L-lysine exporter	- 4.5
SA0798		Disulfide oxidoreductase	- 6.1
SA0830		MSEP-CTERM protein	- 4.6
SA1209		Cof-like hydrolase (haloacid dehalogenase-like hydrolase)	- 4.1
SA1497	*engB*	Ribosome biogenesis GTP-binding protein YsxC	- 4.6
SA1613		Putative membrane protein insertion efficiency factor	- 4.5

N315 locus tag	Gene	Function	Fold change (H-5/H-3)
SA1756		Amidase	- 9.1
SA1784		dUTPase	- 6.0
SA2163		Protein of unknown function (DUF2871)	- 6.0
SA2225		Putative phosphoesterase (Calcineurin-like phosphoesterase)	- 9.1
SA2450		JNK-interacting protein leucine zipper II	- 6.1
Unknown			
SA0279	*esaE*	Domain of unknown function (DUF5081)	5.9
SA0743	*vwb*	Coagulase	4.6
SA0790	*nagD*	Predicted sugar phosphatases of the HAD superfamily	6.6
SA0806		U-34 5-methylaminomethyl-2-thiouridine biosynthesis protein	4.0
SA1161		Cell division protein FtsL	4.3
SA1304		Heptaprenyl diphosphate synthase (HEPPP synthase) subunit 1	4.4
SA2118		Protein of unknown function (DUF3397)	4.0
SA2139		Formate dehydrogenase, alpha subunit	4.3
SA2331		YozE SAM-like fold	5.3
SA2491		Short repeat of unknown function (DUF308)	4.5
SAS066	*agrD*	Cyclic lactone autoinducer peptide	6.6
SA0047		Phenylacetic acid degradation protein paaN	- 15.3
SA0203		Staphylococcus tandem lipoproteins (Csa1 family)	- 4.5
SA0382	*ssl1*	Exotoxin	- 4.6
SA1791		DnaD domain protein	- 4.6
SA2000		Conserved hypothetical protein	- 4.5
Mobile and extrachromosomal element functions			
		Terminase large subunit	8.1
		Conserved phage-associated protein	7.2
		Phage major capsid protein, HK97	11.8
	ear	Putative phage terminase, small subunit, P27 family	- 4.7
		Phi ETA orf 18-like protein	- 4.5
		Phage tail tape measure protein, TP901 family, core region	- 4.1
		Oxidoreductase (Phage antirepressor protein KilAC domain)	- 4.6
		Phage DNA-binding protein	- 6.6
		Phage protein	- 6.9

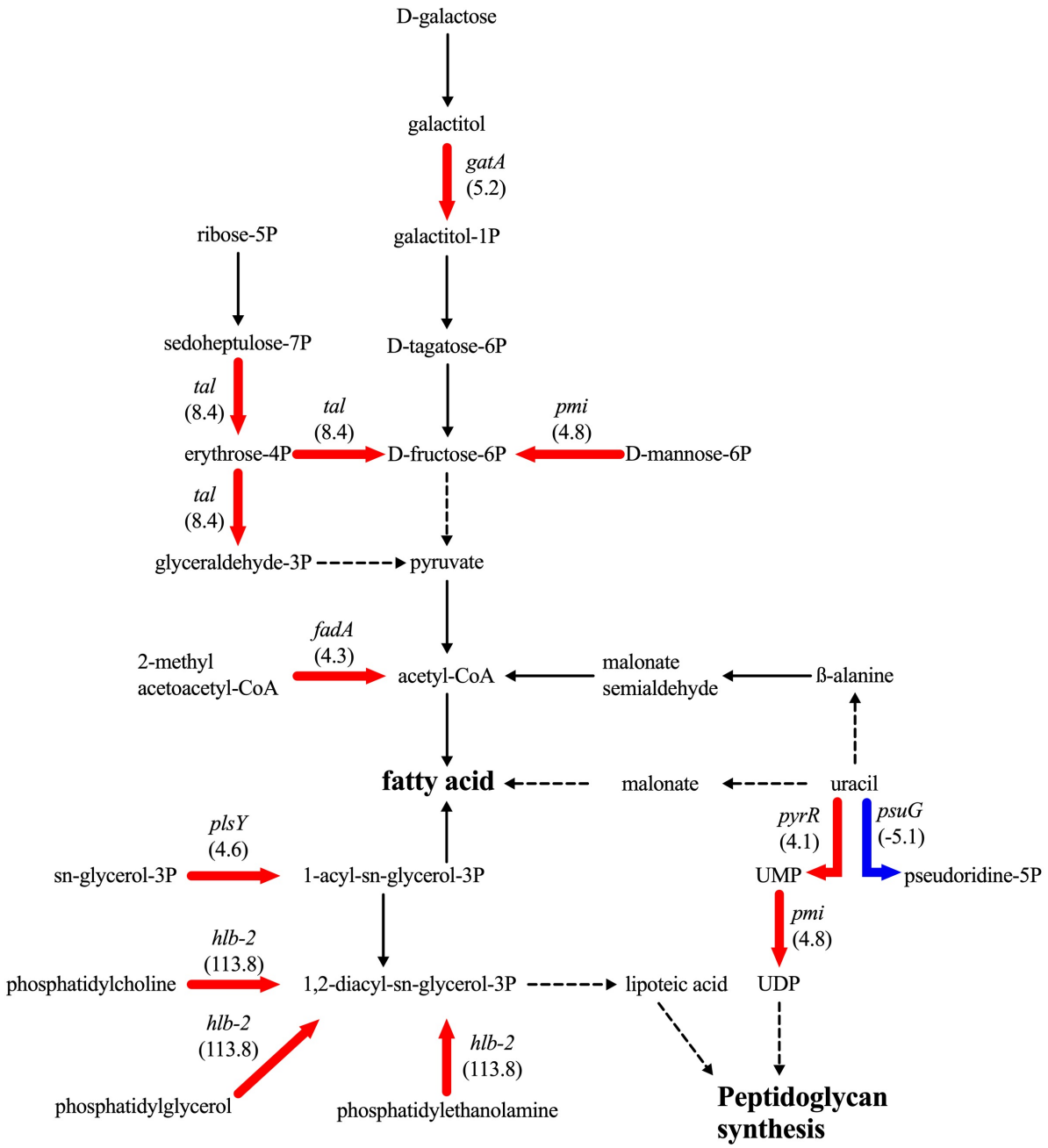

Figure 15: Gene expression in contribution to cross-resistance in DNS strain H-5. Increased gene expression in fatty acid and peptidoglycan via carbohydrate metabolism (galactitol, ribose, or mannose) was observed. The red arrows refer to gene upregulation. The blue arrows refer to gene downregulation.

Unlike the effects of *mprF* mutations on DAP and VCM cross-resistance, which are clearly identified in the current study, the regulatory function of a *lacF* mutation on DAP single resistance in the K-2 strain is still unclear, prompting us to perform RNA-Seq analysis. The gene expression profiles of K-1 and K-2 strains are shown in Table 17 and Figure 16. There are 37 (68.52%) upregulated and 17 (31.48%) downregulated genes with a difference of ±4-fold change between the DS (K-1) and DNS (K-2) strains. Fifty percent of the differentially expressed genes are involved in metabolic functions such as amino acid or carbohydrate transport and metabolism, energy production, and conversion or translation process. This is followed by 11.11% of genes associated with defense mechanism and 7.40% with CW, CM, and envelop metabolisms.

Most of the upregulated genes play a role in carbohydrate metabolism, especially lactose and galactose metabolism, with six genes in the *lac* operon (*lacABCDEG*) increasing from 15- to 75-fold. However, the significance of *lac* operon overexpression in DAP-nonsusceptibility is still unclear and requires further investigation. Nonetheless, expression changes in genes associated with the alteration of CM properties (established DAP-nonsusceptibility mechanism) were identified, including downregulated expression of lactate dehydrogenase (*lctE*; -14.81-fold) and increased expression of acylphosphatase (*acyP*; 4.85-fold) and D-methionine transport system permease (*metI*; 9.63-fold). These reported gene expression changes can increase pyruvate metabolism and subsequently synthesize fatty acid via acetyl-CoA. As shown in the results, the *fabZ* gene involved in fatty acid synthesis was upregulated (5.84-fold). Last, but not the least, alteration of cellular defense mechanisms was found in the K-2 isolate, as supported by expression changes in glutathione (GSH) metabolism gene *gpxA2* (6.78-fold) and oligopeptide ABC transporter gene *opp-4D* (-4.10-fold).

Table 17: Representatives of genes differentially expressed between daptomycin-nonsusceptible strain K-2 and -suscptible strain K-1

N315 locus tag	Gene	Function	Fold change (K-2/K-1)
Metabolism			
[C] Energy production and conversion			
SA1236	*acyP*	Carbamoyltransferase HypF	4.8
SA0232	*lctE*	L-lactate dehydrogenase	- 14.8
[E] Amino acid transport and metabolism			
SA0347	*metI*	Cystathionine beta-lyases/cystathionine gamma-synthases	9.6
SA1239	*brnQ3*	Component of the transport system	4.2
SA2081		Urea transporter	8.8
[G] Carbohydrate transport and metabolism			
SA0208	*malF*	Putative 2-aminoethylphosphonate ABC transporter	5.0
SA0209		Maltose ABC transporter, permease protein	10.9
SA0318	*ulaA*	PTS ascorbate transporter subunit IIC	4.3
SA1991	*lacG*	6-phospho-beta-galactosidase	74.2
SA1992	*lacE*	PTS system, lactose-specific IIC component	44.6
SA1994	*lacD*	tagatose 1,6-diphosphate aldolase	24.5
SA1995	*lacC*	tagatose-6-phosphate kinase	18.7
SA1996	*lacB*	D-galactose 6-phosphate degradation	24.3
SA1997	*lacA*	D-galactose 6-phosphate degradation	51.5
SA2167	*scrA*	PTS system lactose-specific IIBC component	6.9
SAS020		catalytic activity (Phosphoglycerate mutase family protein)	4.2
[H] Coenzyme transport and metabolism			
SA2215	*bioD*	Dethiobiotin synthase	- 5.1
[I] Lipid transport and metabolism			
SA1901	*fabZ*	beta-hydroxyacyl-(acyl-carrier-protein) dehydratase FabZ	5.8
[P] Inorganic ion transport and metabolism			
SA0417		Neurotransmitter:sodium symporter activity	5.8
Information storage and processing			
[J] Translation, ribosomal structure and biogenesis			
SA0009	*serS*	Serine-tRNA ligase	4.4

N315 locus tag	Gene	Function	Fold change (K-2/K-1)
SA0652		Cys-tRNA(Pro) deacylase	5.8
[K] Transcription			
SA1665		Cro/Cl family transcriptional regulator	- 5.2
[L] Replication, recombination and repair			
SA0538	*ung*	Uracil-DNA glycosylase	- 4.5
[M] Cell wall / membrane / envelop biogenesis			
SA0205		M23/M37 peptidase domain-containing protein	4.8
[O] Post-translational modification, protein turnover and chaperones			
SA1121		Predicted Zn-dependent peptidases	4.2
SA1543		Peroxiredoxin	5.8
SA2370		Putative flavoprotein monooxygenase	8.6
SA2414	*gpxA2*	Putative glutathione peroxidase Gpx7	6.8
		Protease subunit of ATP-dependent Clp proteases	7.7
[V] Defense mechanism			
SA0135	*phnE1*	Phosphonate ABC transporter	- 9.3
SA0851	*opp-4D*	Oligopeptide ABC transporter	- 4.1
SA1156		ABC transporter, ATP-binding protein	4.8
Poorly characterized			
[R] General function prediction only			
SA0683	*queF*	NADPH-dependent 7-cyano-7-deazaguanine reductase	5.8
[S] Function unknown			
SA1136		energy coupling factor transporter S component ThiW	4.4
SA2301		DedA family protein	4.9
SA1292		MazG nucleotide pyrophosphohydrolase	- 4.1
Unknown			
SA0211		Inositol 2-dehydrogenase	5.9
SA0400	*lpl4*	Staphylococcus tandem lipoproteins	4.8
SA0403	*lpl3*	Staphylococcus tandem lipoproteins	5.8
SA0489	*mrnC*	Correct processing of 23S rRNA precursor	7.8
SA0561		Putative cytosolic protein (YwhD)	6.8
SA0710		Probable autolysin LDP	- 7.3
SA1317		Uncharacterized Protein	- 4.6

N315 locus tag	Gene	Function	Fold change (K-2/K-1)
SA2372		Uncharacterized protein	- 4.1
SA2454		Acetyltransferase (GNAT) domain	- 4.1
Mobile and extrachromosomal element functions			
SA0195		Phage holin	6.3
		Phage-like protein	5.1
SA1793		beta-lactamase superfamily domain protein (Phage)	- 4.1
		Phage	- 7.3
		Phage packaging	- 6.2

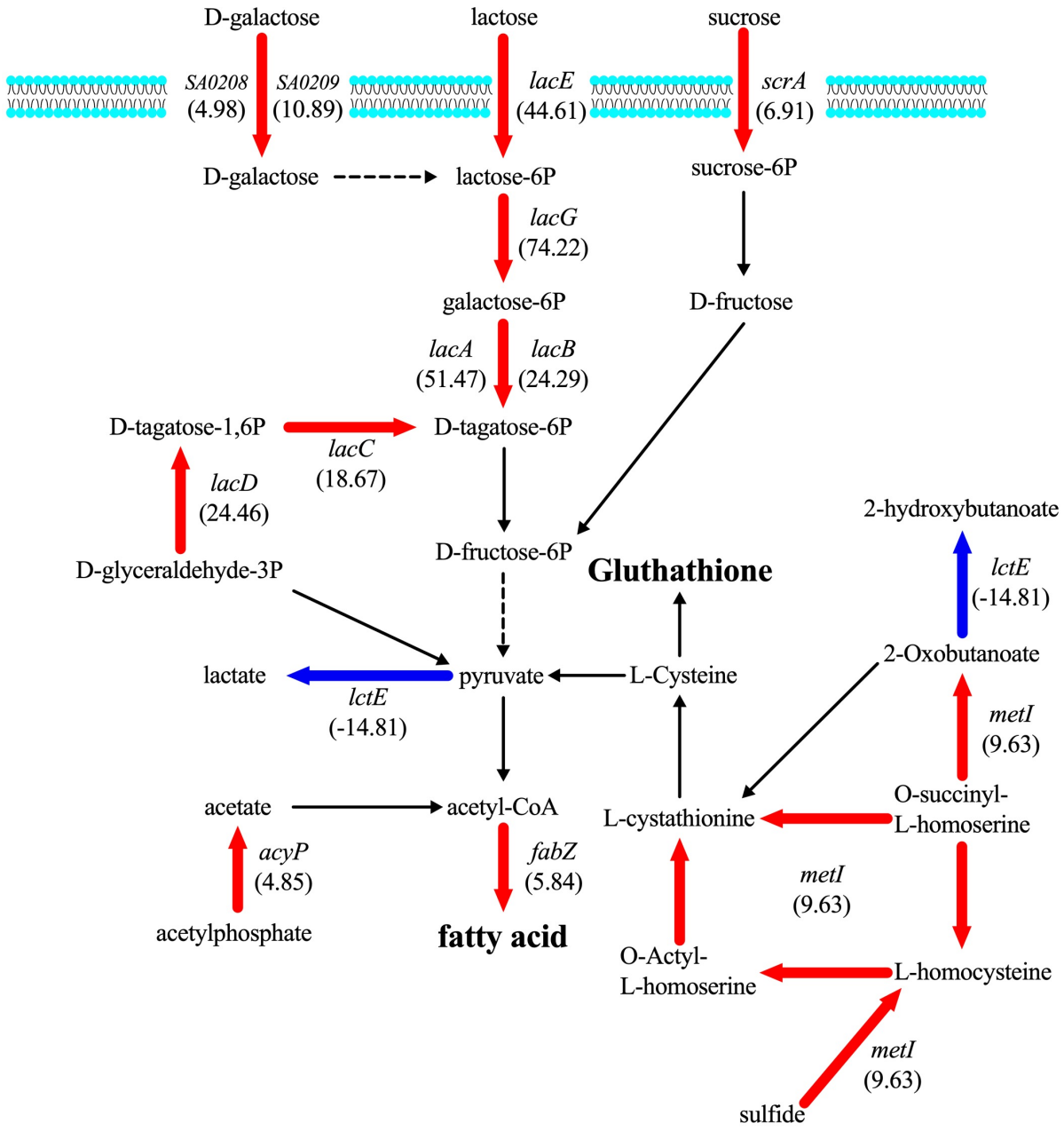

Figure 16: Gene expression in contribution to DAP-nonsusceptibility in DNS strain K-2. Acetyl-CoA, precursor for fatty acid metabolism, is produced via carbohydrate (lactose, tagatose, and sucrose) and cysteine methionine metabolism or acetate metabolism. The red arrows refer to gene upregulation. The blue arrows refer to gene downregulation.

CHAPTER VI

DISCUSSION

The current study was conducted with the aim of clarifying the mechanism(s) of DAP and VCM cross-resistance in MRSA. DAP and VCM are two different classes of antibiotics exhibiting distinct modes of bactericidal actions, thus triggering different resistance mechanisms in bacterial strains. Nevertheless, MRSA strains with reduced susceptibility to both DAP and VCM, a phenomenon known as DAP and VCM cross-resistance, have been reported [14-16]. Owing to the fact that DAP and VCM are primary treatment options for MRSA infections, understanding the regulatory pathways leading to cross-resistance is crucial to facilitate the identification of novel target sites and the development of new therapeutic agents, contributing to the management of difficult-to-treat bacterial infections.

Our results suggested that *mprF* mutations are major determinants of DAP and VCM cross-resistance in MRSA during DAP therapy but have only partial contribution during the course of VCM chemotherapy (Table 11 and 13). *mprF* mutation-mediated DAP and VCM cross-resistance was demonstrated in a gene replacement assay whereby introduction of *mprF* mutation from DNS strain H-5 to DS strain H-3 resulted in increased DAP and VCM MICs of DS strain H-3 (Table 14). MprF plays a role in protecting bacteria against cationic antimicrobial peptides (CAMPs), including DAP, by altering bacterial membrane surface charges. Principally, MprF regulates the transition of phospholipid PG to L-PG by the addition of a lysine residue, causing an increased positive charge in CM, which is repulsive toward cationic antibiotics [13, 16, 198]. Accordingly, cells lacking the *mprF* gene showed increased sensitivity toward many positively charged antibiotics, including CAMPs, DAP, or VCM [16, 217]. This evidence supported that cross-resistance to DAP and VCM in MRSA is associated

with *mprF* mutations. However, while mutation in the lysinylation domain of MprF alone was sufficient to cause DAP-nonsusceptibility [21, 218], DAP and VCM cross-resistance was not constantly linked to *mprF* mutation. Instead, other gene mutations were reported to be responsible for DAP and VCM cross-resistance in laboratory mutants (RNA polymerase *rpoB*) and clinical isolates (cardiolipin biosynthesis *cls* or PG production *pgsA*) [162, 174, 191, 219, 220]. In our study, we showed that *mprF* mutation is the sole contributor to DAP and VCM cross-resistance and that most mutations identified in the *mprF* gene of DAP and VCM cross-resistant DNS and VISA strains are located on the lysinylation domain (Figure 11). Moreover, the mutation in the lysinylation domain is not just limited to clinical isolates of DAP and VCM cross-resistant strains, since laboratory-derived DAP and VCM cross-resistant isolates obtained by stepwise DAP selection on either DS strains in cross-resistance group (strain C-1) or single-drug-resistance group (strain K-1) also carried *mprF* mutations in the lysinylation domain (Table 15).

Despite having a pronounced association with DAP-selected cross-resistant strains, involvement of *mprF* mutations in the DAP and VCM cross-resistance during VCM exposure is less evident. Regardless, glycopeptide-resistant bacterial isolates exhibiting DAP and VCM cross-resistance phenotype can be observed in previous [14, 221] and current studies (Table 13). This indicated that the *mprF* mutation is not a universal factor of DAP and VCM cross-resistance. VISA strains display thickened CW to allow increased binding of VCM to false targets in peptidoglycan (affinity trapping), thereby contributing to their reduced VCM susceptibility [8, 14]. Similar to VCM, the target site of DAP is located in the CM. DAP molecules need to penetrate the CW, the primary barrier of bacterial defense mechanism, before reaching their lethal targets. Therefore, one possible pathway leading to DAP-nonsusceptibility in VISA strains may be increased CW thickness [7, 208]. CW thickening could also explain the reduced DAP susceptibility in DAP-selected DAP/VCM cross-resistant

strains, as reduced DAP binding at CM was observed in DNS strains of *Enterococcus* [222, 223]. However, as shown by our TEM analysis, only one DNS strain (I-3; Figure 12) has thickened CW. Neither the remaining 11 sets of DNS strains from the DAP/VCM cross-resistance group carrying *mprF* mutation nor DNS strain with only DAP-nonsusceptibility carrying *lacF* mutation showed increased thickness of CW, albeit previous report on alteration of CW sizes between DS and DNS strains in patients A, B, and C [199].

Nonetheless, similar to previous observations [7, 22, 208], changes in the expression of CW-related genes have been identified in both DNS and VISA strains with cross-resistance to DAP and VCM. Many genes responsible for F-6P generation, such as those involved in the metabolism of galactose-tagatose, ribose and mannose, and the pentose phosphate pathway, were upregulated in DNS strain H-5 of DAP and VCM cross-resistance group (Table 16, Figure 15). In addition, DNS strain H-5 showed an increased expression of uracil phosphoribosyltransferase (UPRT). Both F-6P and UPRT play a role in bacterial CW biosynthesis. Fructose-6-phosphate is a substrate for the synthesis of CW precursor via the uracil diphosphate-*N*-acetyl-glucosamine (UDP-GlcNAc) pathway. The expression of this pathway was shown to be increased in DAP and VCM cross-resistant DNS and VISA strains in previous studies [59, 224]. On the other hand, the function of UPRT is to generate uracil monophosphate, which binds to the lipid carrier, undecaprenyl pyrophosphate, to facilitate the production of UDP-GlcNAc [225]. Our results also revealed upregulated expression of a few genes involved in glycerolipid production. The activated glycerophospholipid metabolism will probably increase the production of 1,2-diacyl-sn-glycerol, a lipoteichoic acid (CW component) precursor [226], and ultimately enhance the biosynthesis of CW. According to our RNA-Seq differential expression analysis, DAP and VCM cross-resistance seems to be associated with CW metabolism, although most DNS strains did not show significant differences in CW sizes compared with their parental strains. This contrasting phenomenon

might be due in part to the small range of VCM MIC changes observed between DS and DNS strains. DAP/VCM cross-resistance has been reported in both laboratory-derived and clinical isolates with no phenotypic characteristic of CW thickening [27]. It is therefore indicated that DAP/VCM cross-resistance is not the result of only one contributing factor; while increased CW thickness is associated with DAP and VCM cross-resistant VISA strains, alteration in membrane surface changes is more likely the causative factor of DAP and VCM cross-resistance in DNS strains. This hypothesis can be supported by several previous studies that reported that not only CW alteration but also changes in CM properties could be a substantial factor leading to DAP/VCM cross-resistance [11, 12, 178, 227, 228].

Daptomycin is a lipopeptide antibiotic acting on bacterial CM [10, 11]. Membrane depolarization and ion leakage can be observed when the positively charged Ca^{2+}-DAP complex binds to the negatively charged hydrophilic head group of PG in CM [11, 12]. Therefore, phenotypic alteration of membrane surface charges via *mprF* mutation is a commonly reported bacterial evolution to resist positively charged drugs, such as CAMPs and DAP [12, 178, 227, 228]. Interestingly, VCM molecules contain an ionizable amine and carboxylic group, which also display positive charge when administered [229, 230]. Moreover, disruption of negatively charged wall teichoic acids by deletion of the *dltABCD* operon involved in alanylation of teichoic acids was reported to increase the drug susceptibility of *S. aureus* Sa113 to both CAMPs, such as α-defensins or nisin, and glycopeptides, such as VCM or teicoplanin [196, 197]. Hence, we postulated that a change in net surface charge of CM as mediated by *mprF* mutation seems to be able to confer reduced DAP and VCM susceptibility in bacterial strains. However, Shrenik *et al.* have suggested otherwise. They claimed that the mechanism of DAP-nonsusceptibility in clinical isolates is not associated with alteration of membrane surface charge since no significant difference in cationic cytochrome *c* binding (referring to membrane charge) was observed between DS and DNS strains [198]. Thus, the

association between alteration of membrane charge and DAP-nonsusceptibility remains controversial.

Herein, every DNS isolate in the DAP/VCM cross-resistance group carrying *mprF* mutations in different positions, as well as DS strain H-3 transformed with *mprF* mutation, exhibited significant alteration of membrane surface charge as implicated by reduction of cytochrome *c* binding in these strains compared with the DS strains (Figure 13A and 13C). These observations attributed DAP/VCM cross-resistance to reduction of negative surface charges in the CM. According to our results, this mechanism seems to be regulated by *mprF*, although *rpoB* mutations have also been reported to alter membrane surface charges [162].

The alteration of bacterial membrane surface charges results from the modification of anionic PG to cationic L-PG by the lysinylation domain of MprF [231]. DNS isolates were indeed consistently reported to have increased L-PG production due to *mprF* mutations [178, 218, 232]. The *mprF* mutations identified in our studied strains were found to be located on four of the six *trans*membrane segments and C-terminal domain of MprF, which are responsible for lysinylation. Moreover, our results showed that L-PG production in DNS isolates of the DAP/VCM cross-resistance group increased in a mutation site-dependent pattern. Although different locations of *mprF* mutations have been proposed to affect L-PG production and consequently DAP susceptibility [218, 233], Mishra *et al.* have shown that the levels of L-PG production varied even when host cells do not carry *mprF* mutations or carry the same *mprF* mutation site, hence suggesting that there are other genes causing increased L-PG production [179]. The cause-effect relationship between increased L-PG production and *mprF* mutation is further confirmed in our study by transformation of mutated *mprF* in the DS strain (Figure 14A and 14C).

Furthermore, RNA-Seq analysis showed changes in gene expression that enhance fatty acid biosynthesis in DNS strain H-5. Among these gene expression changes, genes encoding

enzymes involved in F-6P production were upregulated. F-6P is the key substrate for pyruvate biosynthesis, a crucial metabolite of the citric acid cycle required for energy metabolism, other than CW biosynthesis. The upregulation of this process inevitably increases the intracellular pool of acetyl-CoA [234-236], which serves as a precursor for fatty acid biosynthesis catalyzed by acetyl-CoA carboxylase and many acyl-carrier protein [237]. DNS strain H-5 also had downregulated expression of serine dehydrogenase and upregulation of genes responsible for glycerolipid metabolism and fatty acid metabolism (Table 16, Figure 15), all of which ultimately increase generation of pyruvate and acetyl-CoA. We postulated that these differential gene expressions will favor alteration of membrane surface charge, since enhanced fatty acid synthesis will facilitate CM biosynthesis and subsequently increase the supply of building block for L-PG production. In support of our hypothesis, mutation in acetyl-CoA synthetase in combination with other mutations were reported to contribute to DAP-nonsusceptibility [17]. In short, our study suggested that changes in membrane surface charge via enhanced levels of L-PG production and alteration of CW metabolism, presumably regulated by *mprF*, can contribute to DAP and VCM cross-resistance.

Apart from the proposed DAP and VCM cross-resistance mechanism, the current study deduced another possible pathway conferring DAP-nonsusceptibility in single-drug-resistant strain, which is not related to *mprF*. Single-drug-resistant DNS strain K-2 carrying only a *lacF* mutation showed an increased binding of cytochrome *c* compared to DS strain, which was contradictory to DNS strains of DAP/VCM cross-resistance group (Figure 13B). This suggested a lack of a strong correlation between membrane surface charge and *lacF* mutation-associated DAP-nonsusceptibility, consistent with previous studies that reported that not all DNS isolates showed altered membrane surface charges [189, 194].

Mutations in *lacF*, the lactose phosphotransferase system (PTS), found in a DNS strain K-2 can cause only DAP-nonsusceptibility (Table 11). The association between mutations

affecting carbohydrate transportation and resistance to cationic peptide (including DAP) has been demonstrated in *S. aureus*, *E. faecalis*, and *Listeria monocytogenes* [238 - 240].

Listeria monocytogenes carrying mutation in the mannose PTS system showed resistance to bacteriocins, one of the cationic peptides capable of making pore-like structures in the membrane just as DAP, due to lower glucose consumption rate [240]. A previous study also reported that the bactericidal effect of DAP is enhanced by increased glucose concentration that eventually induces lysis protein activity [241]. Thus, reduced DAP susceptibility in the K-2 strain carrying a *lacF* mutation seems to be a result of decreased cell lysis caused by lowered glucose consumption. In addition, DNS strain K-2 demonstrated increased expression of genes involved in cysteine and methionine metabolism, which generates GSH (Table 17, Figure 16). This compound is a commonly known antioxidant protecting prokaryotic and eukaryotic cells from oxidative stresses [242]. However, the exact regulatory pathway(s) of GSH in conferring DAP-nonsusceptibility is not experimentally proven, although mutation in GSH has been reported to cause DAP-nonsusceptibility in *E. faecalis* [243]. Our results indicated that increased GSH metabolism coupled with reduced cell lysis are the cellular adaptations protecting bacterial isolates with *lacF* mutations from DAP toxicity.

CHAPTER VII

CONCLUSION

This study suggested that DAP/VCM cross-resistance in MRSA is associated with *mprF* mutations. The reduction of DAP and VCM susceptibility is mainly mediated by alteration of CM surface charge through increased L-PG production, while increased CW thickness is marginally involved. We also revealed a novel pathway leading to DAP-nonsusceptibility that is not related to the common genetic determinant *mprF*. In our study, single DAP-nonsusceptibility is believed to be caused by alterations in cellular metabolisms ensued from *lacF* mutations, but the exact mechanisms remain to be elucidated.

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