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Title:
Characterisation of a membrane protein, MspA, that is critical for Staphylococcus aureus pathogenesis

Characterisation of a membrane protein, MspA, that is critical for *Staphylococcus aureus* pathogenesis

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Abstract

Staphylococcus aureus is one of the most common human pathogenic bacteria. It can cause various infections, ranging from superficial skin infections to sepsis and other life-threatening diseases. Due to the rise of antibiotic resistance, this microorganism has become a challenge to treat. To prevent disease and develop effective treatments, we need to understand the mechanisms used by this organism to cause disease. One approach undertaken to address this in Prof. Massey's lab is to apply a functional genomics approach, which has identified a novel membrane-bound protein gene called *mspA* (membrane stabilizing protein A). When the encoded protein is absent, the bacteria were found to lose their ability to produce cytolytic toxins, produce staphyloxanthin, and they became unable to control their iron homeostasis or adapt to iron-rich media, and they increased their resistance to β-lactam antibiotics and increased the amount of penicillin binding proteins PBPs in the cell membrane. Using a site-directed mutagenesis approach domains and amino acids within MspA that are critical to its activity have been identified. While work is still underway to understand how this protein exerts its pleiotropic effects on *S. aureus,* this work has characterised these effects and identified potential targets within this protein that could be used to develop a novel therapeutic approach to treat these bacteria in the future.

Dedication

To my late father,

  As I was finishing off my thesis, all I could think about was how much I wanted to show you my work and how I would give anything to listen to your feedback and hear you say "I'm proud of you Alaa" like you have always done. I felt that it was only right to keep this page for you since you are the main reason I am here today. I would like to dedicate my thesis to you, so I can always be reminded that the support of the people most important to you could give you the boost you need to make your dreams come true. From the day I told you I wanted to study microbiology and the moment you realized my passion for it, you made it your mission to provide me with everything I need to make my dreams come true. I can still hear you say "my dream is to watch my children's dreams come true and to see them happy" which I think is the greatest representation of the unconditional love that you have provided my siblings and I with throughout the years. I truly could not have wished for a more incredible person to have my back. To say that I am grateful to have had your support would be an understatement, you have always been ready to give up anything for the sake of your children and for that, I will be eternally grateful. I cannot put into words just how much I miss you, I cannot count just many times I had a nice result and reached for my phone only to realize that you are no longer there, and how many times I became frustrated and needed your comforting words and not being able to hear them. These difficult and frustrating moments have set me back slightly but I kept reminding myself of what you would have said if you were still here, and how you would have kept me motivated when I questioned my choices. As difficult as it has been working through the grief of losing you, I hope that you are proud of me and I hope that I can set an example for my children based on all of what you have taught me.

With love,
Alaa

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Author's declaration

"This project is my work except where indicated. All text, Figures, tables, data or results, which are not my work, are indicated, and the sources acknowledged. Also, I confirm that the hardcopy and the e-submission are identical."

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Publications

Duggan, S., Laabei, M., **Alnahari, A. A.,** O'Brien, E. C., Lacey, K. A., Bacon, L., … Massey, R. C. (2020). A small membrane stabilizing protein critical to the pathogenicity of *Staphylococcus aureus*. *Infection and Immunity*, *88*(9), 1–20. https://doi.org/10.1128/IAI.00162-20

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LIST OF SYMBOLS

µg	microgram
µl	microliter
agr	accessory gene regulator
AIP	autoinducing thiolactone-containing cyclic peptide
APS	ammonium persulfate
Arg	arginine
Asp	aspartic acid
BiFC	bimolecular fluorescence complementation
BiFC-FRET	BiFC-fluorescence resonance energy transfer
bp	base pair
CaCl2	calcium chloride
CA-MRSA	community-associated MRSA
CHIPS	chemotaxis inhibitory proteins of staphylococci
ClfA	clumping factor A
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
E. coli	*Escherichia coli*
FBS	fetal bovine serum
FnBPA	fibronectin binding protein A
FW	forward
GFP	green fluorescence protein
GlcNAc	*N-acetylglucosamine*
Gly	glycine
GWAS	genome-wide association study
h	hours
H_2O_2	hydrogen peroxide
HA	hyaluronic acid
Hla	alpha toxin
HrtAB	heme regulated transporter
IgG	immunoglobulin G
JE2	*Staphylococcus aureus* wild type

Kb	kilobase
kDa	kilodalton
LB	Luria Bertani
LTA	lipoteichoic acid
M	molar
mg	milligram
MgCl	magnesium chloride
MH	Muller Hinton
ml	milliliters
mM	millimolar
mRNAs	messenger RNAs
MRSA	methicillin-resistant *Staphylococcus aureus*
MspA	membrane stabilizing protein A
MSSA	methicillin susceptible *S. aureus*
MurNAc	*N-acetylmuramic* acid
NaCl	sodium chloride
NETs	neutrophil extracellular traps
NTML	Nebraska Transposon Mutant Library
OD	optical density
PBP2a	penicillin-binding protein 2
PBPs	penicillin Binding Proteins
PCR	polymerase chain reaction
PG	peptidoglycan
pRMC2	plasmid
PSM	phenol-soluble modulins
PVL	Panton-Valentine leukocidin
QS	quorum-sensing
RboP	ribitol-phosphate
RNA	ribonucleic acid
RNase	ribonuclease
ROS	reactive oxygen species
RT-qPCR	quantitative reverse transcription PCR

RV	reverse
S. aureus	*Staphylococcus aureus*
S. epidermidis	*Staphylococcus epidermidis*
S. saprophyticus	*Staphylococcus saprophyticus*
SAGs	Bacterial superantigens
SCCmec	Staphylococcal cassette chromosome *mec*
SDM	site-directed mutagenesis
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS–polyacrylamide gel electrophoresis
sec	second
SEs	staphylococcal enterotoxins
SH1000	*Staphylococcus aureus* wild type
SNP	single-nucleotide polymorphism
SOD	superoxide dismutase
SSSS	*Staphylococcus* scalded skin syndrome
TBE	Tris-borate-EDTA
TEA	Tris-acetate-EDTA
TEM	transmission electron microscope
TEMED	tetramethyl ethylenediamine
TSA	tryptic soy agar
TSB	tryptic soy broth
TSST	toxic shock syndrome toxin
UV	ultraviolet
V	volt
vWF	von Willebrand factor
WTA	wall teichoic acid

1. Introduction

1.1 Overview and basic microbiology

Staphylococcus aureus is a Gram-positive that is coccus commonly found in the upper respiratory tract, and on the skin of humans, mainly in the anterior nares, axilla, perineum, and vagina. It is a member of the Micrococcaceae family of bacteria (Lowy, 1998), and is a part of the natural flora of a large proportion of humans, where carriers are at increased risk of infection. It can also cause nosocomial infections, where it is transmitted through healthcare workers either from their natural reservoir of *S. aureus* or from interaction with infected patients elsewhere in the hospital (Lowy, 1998; Kent et al, 2009). *S. aureus* is the causative agent of a wide range of localized, systemic, hospital-acquired, and community-acquired diseases (Thomas and Percival, 2009, González-García et al., 2021). *S. aureus,* often known as *"golden staph" and "oro staphira"* (Rub and Sasikumar, 2016), is a nonmotile bacterium that does not produce spores (Turnidge et al., 2008). It appears as staphylococci (grape-like clusters) under a microscope and when grown on blood agar plates, as spherical, golden-yellow colonies, typically with haemolysis (Habib et al., 2015). Binary fission is used by *S. aureus* to reproduce asexually. *S. aureus* autolysin mediates complete separation of the daughter cells, and in the absence of it or specific inhibition, the daughter cells remain linked to one another and appear as clusters (Turnidge et al., 2008). *S. aureus* can also produce catalase, an enzyme that breaks down hydrogen peroxide (H_2O_2) into water and oxygen. Staphylococci are sometimes distinguished from enterococci and streptococci via catalase activity assays (Turnidge et al., 2008). Coagulase-positivity is a common feature of *S. aureus*, where coagulase is an enzyme that converts fibrinogen to fibrin. Coagulase-negative species such as *S. epidermidis* or *S. saprophyticus* can be detected by a negative coagulase test (Turnidge et al., 2008, Becker et al., 2020).

1.2 *S. aureus* infection

While it is normally a commensal bacterium that colonizes around 30% of the world population asymptomatically, *Staphylococcus aureus* can occasionally cause disease (Sakr et al., 2018). *S. aureus* is a significant cause of skin, soft tissue, bone, and respiratory infections. These include endovascular disorders, such as bacteraemia, endocarditis (infection of the heart valves), and pneumonia, which are all risks associated

with joint replacements (Saginur and Suh, 2008), sepsis, metastatic infections and toxic shock syndrome (Lowy, 1998;Petti and Fowler, 2002). Infections occur when the bacteria enter the human body through damage to the skin or mucosal barrier, such as by an indwelling catheter, shaving, or during surgery. These events allow the bacteria to access from outside into underlying tissue or directly into the bloodstream (Lowy, 1998; Goetghebeur et al., 2007;Mohanty et al., 2018). In 1997 in the United States methicillin resistant *Staphylococcus aureus* MRSA infections were one of the most common causes of skin and soft tissue infection (Klevens et al., 2007; Zecconi & Scali, 2013). In the period between 1990 and 1992, MRSA was the most common cause of hospital-acquired pneumonia and wound infection (Lowy, 1998). In England, where all cases of bacteraemia are reported to Public Health England, the rates of MRSA bacteraemia have remained unchanged at 1.5 cases per 100,000 persons between the period of 2014 and 2017. However, the rate of methicillin susceptible *S. aureus* (MSSA) bacteraemia has increased every year since mandatory reporting began in 2011, resulting in an overall increase of 24.5% in *S. aureus* bacteraemia (Gever et al., 2013). On top of this, *S. aureus* biofilms represent a major concern, being one of the most common causes of infection from orthopaedic implants, although they can also be discovered on cardiac implants, vascular grafts, catheters, and cosmetic surgical implants (Table 1.1) (Veerachamy et al., 2014).

Table 1.1. Infections caused by *staphylococcus aureus* (Levinson, 2020).

Source of infection	Disease
Skin and soft tissue	Impetigo, boils, carbuncles, abscesses, cellulitis, fasciitis, pyomyositis, surgical and traumatic wound infections
Foreign body-associated	Intravascular catheter, urinary catheter
Intravascular	Bacteraemia, sepsis, septic thrombophlebitis, infective carditis
Bone and joints	Septic osteomyelitis, septic arthritis
Respiratory	Pneumonia, empyema, sinusitis, otitis media
Other invasive infections	Meningitis, surgical space infection
Toxin-mediated disease	Staphylococcal toxic shock, food poisoning, staphylococcal scalded skin syndrome, bullous impetigo, necrotizing pneumonia, necrotizing osteomyelitis

1.4 Cell wall structure

The cell wall of *Staphylococcus aureus* contains peptididoglycan (PG), which is an important structural component. PG is a component of the cell wall that forms the sacculus by creating a single macromolecule that surrounds the cytoplasmic membrane (Vollmer and Holtje, 2004). PG is made up of glycan chains of overlapping *N-acetylmuramic* acid (MurNAc) and *N-acetylglucosamine* (GlcNAc) residues linked by short peptides attached to the MurNAc residues (Bern et al., 2017). L-alanine, D-isoglutamine, L-lysine with a penta-glycine linked towards the epsilon amino group, and a terminal D-alanine-D-alanine make up the nascent stem peptides in *S. aureus* PG (Sutton et al., 2021, Dmitriev et al., 2004). Crosslinks in PG produce a three-dimensional network that is rigid and robust (Sutton et al., 2021) (See Fig. 1.1). Up to 80%–90% of the stem peptides in *S. aureus* are connected by such a 3–4 crosslink via the pentaglycine bridge (Dmitriev et al., 2004). *S. aureus* PG displays lower crosslinking in the stationary phase than in the exponential phase when cultivated *in vitro* on synthetic medium, which could be related to glycine depletion (Sutton et al., 2021). *O-acetylation* of MurNAc residues can also be used to modify PG after it has been synthesized (Bernard et al., 2011).This leads to lysozyme resistance and virulence in *S. aureus* (Bernard et al., 2011, Brott and Clarke, 2019) (Fig 1.1).
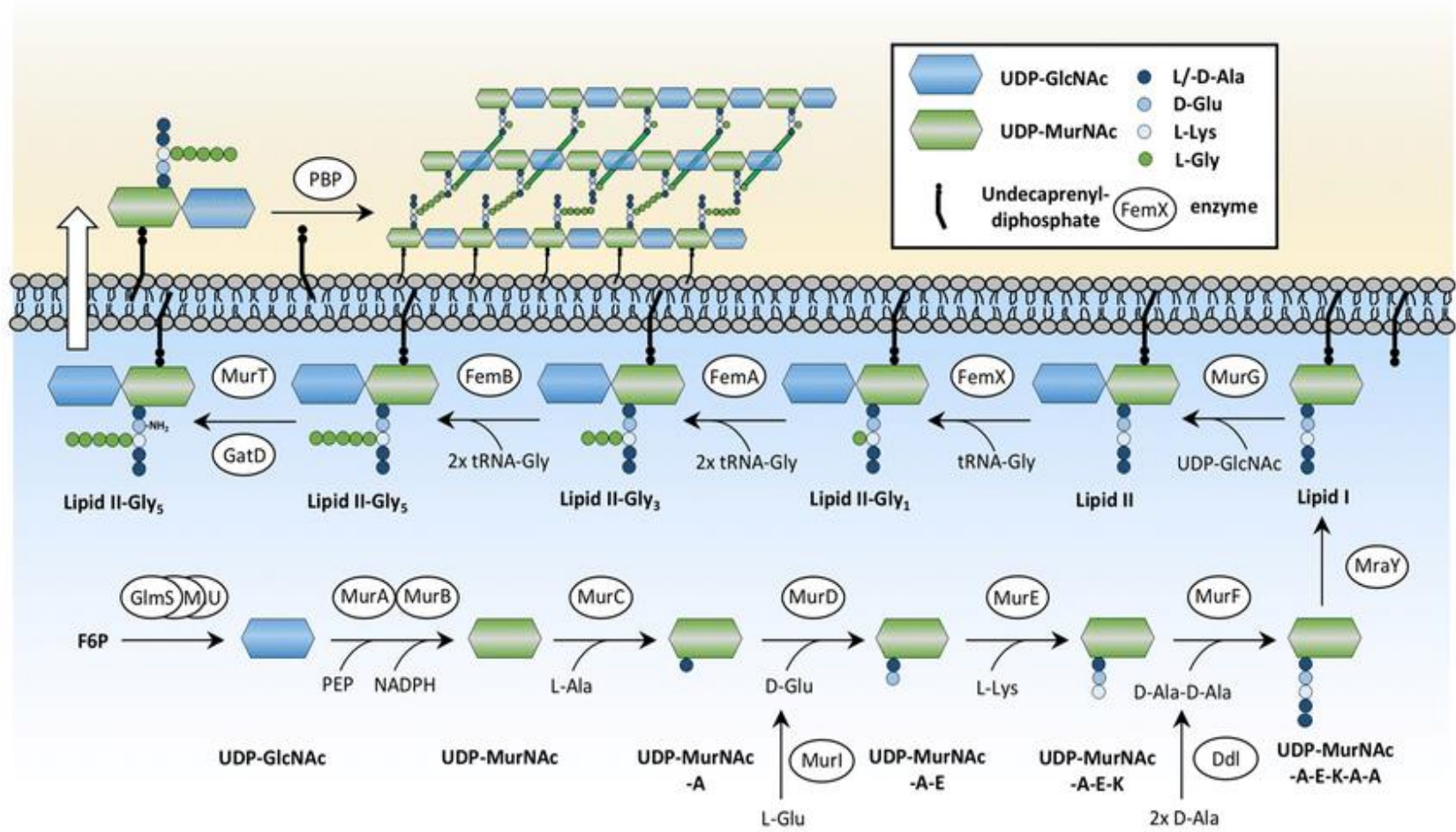
Fig. 1.1: Peptidoglycan synthesis pathway of *S. aureus* taken from (Jarick et al., 2018)

Penicillin binding proteins (PBPs) complete the last stages of PG synthesis by carrying out the glycosyltransferase and/or transpeptidase activities required to manufacture nascent PG and integrate it into the sacculus (Pazos and Vollmer, 2021, Sauvage and Terrak, 2016). *S. aureus* possesses four native PBPs. PBP1 is a transpeptidase that is required for the development of septa during growth and division (Pereira et al., 2007, Pereira et al., 2009). PBP2 is required for survival and functions as a glycosyltransferase and/or a transpeptidase (Reed et al., 2015). PBP3 is a non-essential monofunctional transpeptidase that interacts with RodA to correct mid-cell localization and PG insertion at places other than the septum (Shaku et al., 2020). The strong cross-linkage throughout PG of *S. aureus* is due to PBP4 (Maya-Martinez et al., 2019). In community-acquired MRSA bacteria, PBP4 is essential for β-lactam resistance (Memmi et al., 2008). MRSA strains also carry the non-native *mecA* gene, which encodes PBP2A and is responsible for low-level or high-level lactam antibiotic resistance depending on whether particular *rpoB* and *rpoC* mutations are present (Panchal et al., 2020). Once all native PBPs are inhibited by the inclusion of β-lactam antibiotics, *S. aureus* producing PBP2A produces PG with a low amount of crosslinking, comparable to *pbp4* mutants (Srisuknimit et al., 2017). PBP2A can make a small amount of PG cross-links to assist cells survive in the presence of β-lactams, but it cannot compensate for PBP4 absence, resulting in poor PG cross-linking (Sutton et al., 2021).

Teichoic acids are another key component of Gram-positive bacterial cell walls (Romaniuk and Cegelski, 2018). Wall teichoic acid (WTA) is made up of D-alanine and *N-acetylglucosamine* (GlcNAc) residues added to up to 40 ribitol-phosphate (RboP) subunits. Lipoteichoic acid (LTA) is anchored in the plasma membrane, while wall teichoic acid is covalently attached to PG (Hendriks et al., 2021, van Dalen et al., 2020) (Fig. 1.2).
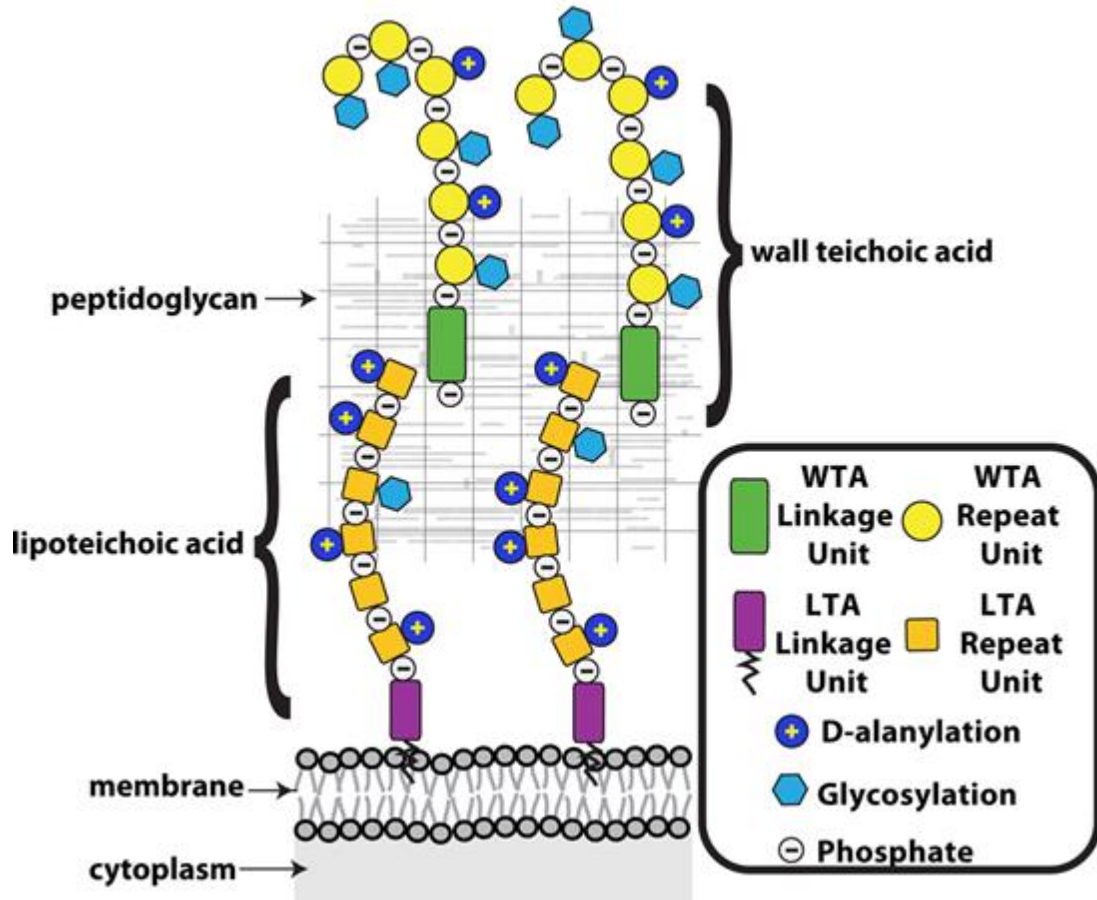
Fig 1.2: The Gram-positive cell wall contains teichoic acid polymers. WTAs are covalently anchored to PG and LTAs are connected to the membrane. WTAs can reach beyond the PG layer, although fully expanded LTAs may not be able to reach the PG layer. WTA- wall teichoic acid , LTA- lipoteichoic acid. Taken from (Brown et al., 2013).

1.5 *Staphylococcus aureus* virulence factors

The ability of *S. aureus* to cause disease can be categorised into three main strategies, where the bacteria can utilise one or two and sometimes all three strategies to cause disease. These are adhesion, which is the ability of these bacteria to bind and enter host cell and tissue; secondly, toxicity, which is the secretion of proteins that cause damage to human tissue and cells; and finally, evasion, which is the ability of *S. aureus* to interfere with the protective activity of the human immune system (Foster et al., 2014; Seilie and Wardenburg, 2017). These strategies for infection are described in detail below.
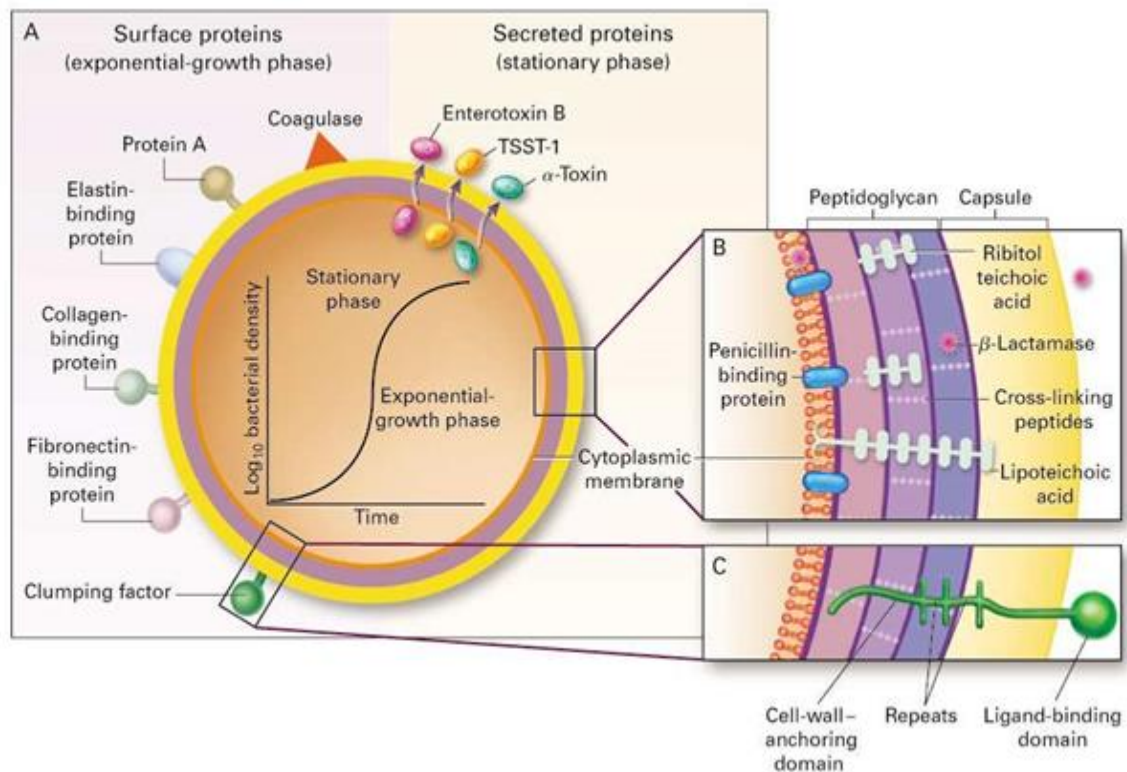
Fig. 1.3: *S. aureus* cell structure and virulence factors. A: An illustration of some of the cell surface expressed adhesion proteins *S. aureus* used to attach to host tissues. On the right-hand side, some of the secreted toxins are also illustrated. B: An illustration of the components of the *S. aureus* cell wall, including the membrane, the peptidoglycan layer, and its capsule. C: An illustration of how the surface proteins are anchored within the cell wall taken from (Lowy, 1998).

1.5.1 Adhesion

After the bacteria gain access to the human body, they adhere to human cells using specific cell surface proteins. *S. aureus* has many groups of surface proteins, the largest group of which is called microbial surface component recognising adhesive matrix molecules (MSCRAMMs). Three examples of such proteins will now be described.

1.5.1.1 Protein A

Protein A (also know as SpA), a 49-kDa surface protein encoded by the *spa* gene, is regulated by DNA topology, cell osmolarity, and the ArlS-ArlR two-component system (Cheung and Zhang, 2002, Cheung et al., 2004, Novick, 2006). It has been the focus of many biochemical studies due to its ability to bind immunoglobulins (Cheung and Zhang, 2002, Cheung et al., 2004, Novick, 2006). It is made up of five Ig-binding domains that wrap into a three-helix bundle. Each domain can bind proteins from a variety of mammalian species, including IgGs (Atkins, 2006). Protein A is both located on the bacterial surface and freely released into the extracellular environment, and is one of many molecules produced by *S. aureus* that can contribute to immune evasion. Protein A binds to the Fc regions of antibodies and the Fab portions of B-cell receptors, preventing opsonophagocytosis and resulting in B-cell mortality in vitro (Kobayashi and DeLeo, 2013) (Fig 1.4).

S. aureus uses Protein A, as well as a wide variety of other proteins and surface components, to enhance survival and pathogenicity. Protein A has a variety of functions.It binds the Fc region of antibodies, making them inaccessible to opsonins and preventing bacterial phagocytosis via immune cell attack (Ukpanah and Upla, 2017). Also, it can help *S. aureus* attaches to human von Willebrand factor (vWF)-coated surfaces, boosting the bacterium infectiousness at the site of skin infection (Thomer et al., 2016, Pietrocola et al., 2017). This protein has been demonstrated to impair humoral (antibody-mediated) immunity, allowing individuals to be infected with *S. aureus* multiple times because humans are unable to build a significant antibody response (Pauli, 2015). Protein A has been found to enhance the production of biofilms both in solution and when covalently attached to the bacterial cell wall (Parastan et al., 2020) and works as an immunological mask and aids to suppress phagocytic engulfment (De Jong et al., 2019).
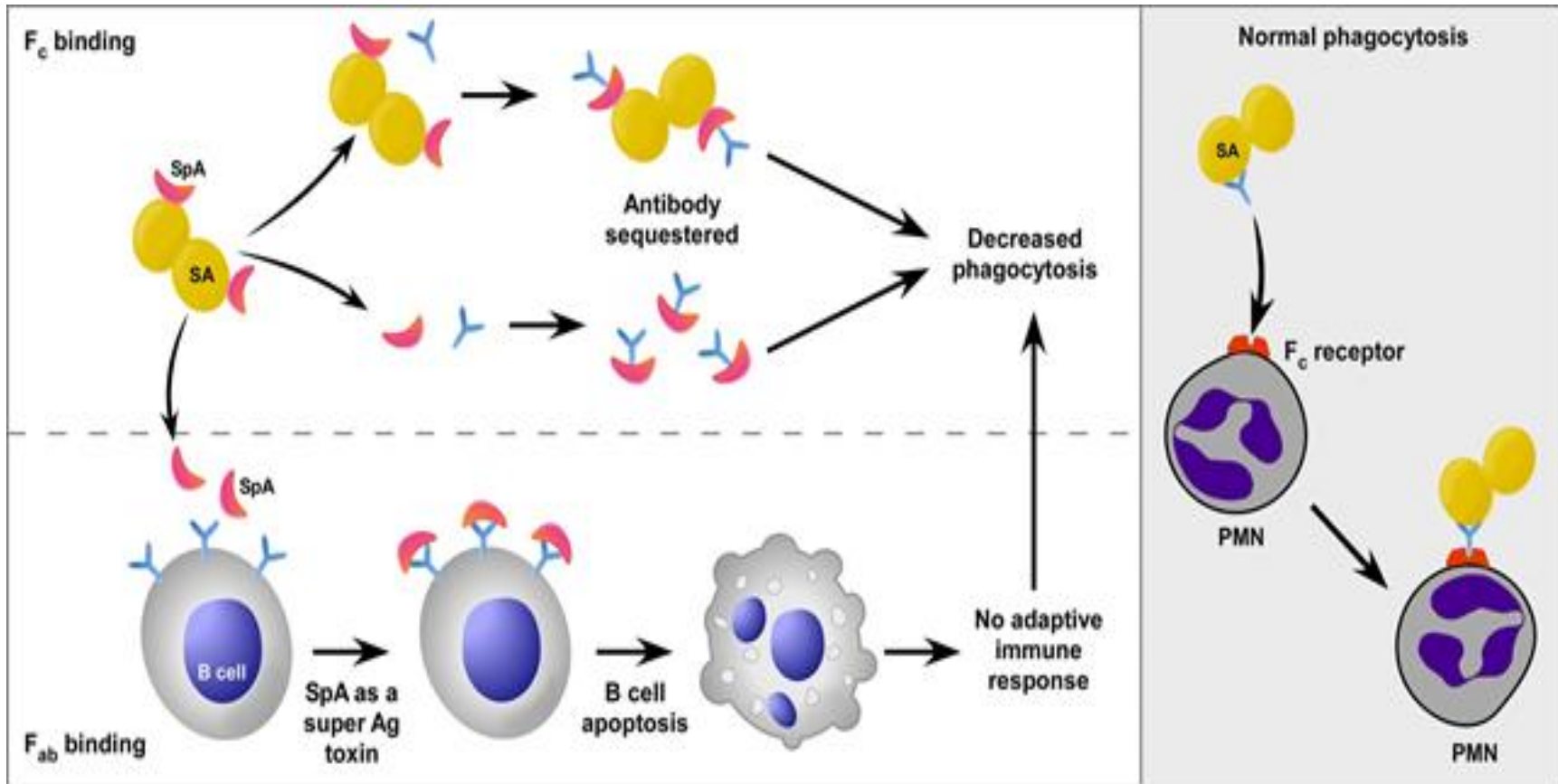
Fig 1.4: SpA-mediated evasion of phagocytosis. Specific antibody binding to the Fc region mediated suppression of opsonophagocytosis as indicated**.** PMN, polymorphonuclear leukocyte. Taken from (Kobayashi and DeLeo, 2013).

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1.5.1.2 Clumping factor A

Clumping factor A (ClfA) is a cell-wall-anchored protein of *S. aureus* that allows the bacteria to adhere to fibrinogen. It is a virulence factor in a number of infections and facilitates colonisation of protein-coated biomaterials (Herman-Bausier et al., 2018). ClfA has an N-terminal secretory signal peptide, followed by a 520-residues region A that comprises the ligand-binding domain, an R region that predominantly consists of dipeptide repeats, and a C-terminal domain that permits cell wall attachment. ClfA enhances bacterial adherence to fibrinogen and blocks phagocytosis in blood plasma (Flick et al., 2013, Higgins et al., 2006). *S. aureus* is one of the few bacteria capable of clotting blood, and it also generates a number of fibrinogen-binding proteins that help in the clumping process. Clump formation has been associated with *S. aureus* pathogenicity and immune evasion as it is characterised as massive, tightly packed groups of cells held together by fibrinogen. Due to a fibrinogen coat that functions as a shield, clusters of bacterial cells are able to resist identification by the host's immune system, and the size of the clumps facilitates phagocytosis avoidance. Furthermore, clumping could be a crucial first stage in the development of infections involving tight clusters of cells trapped in host matrix proteins, including soft tissue abscesses and endocarditis (Crosby et al., 2016, Miljković-Selimović et al., 2015). (Fig 1.5)
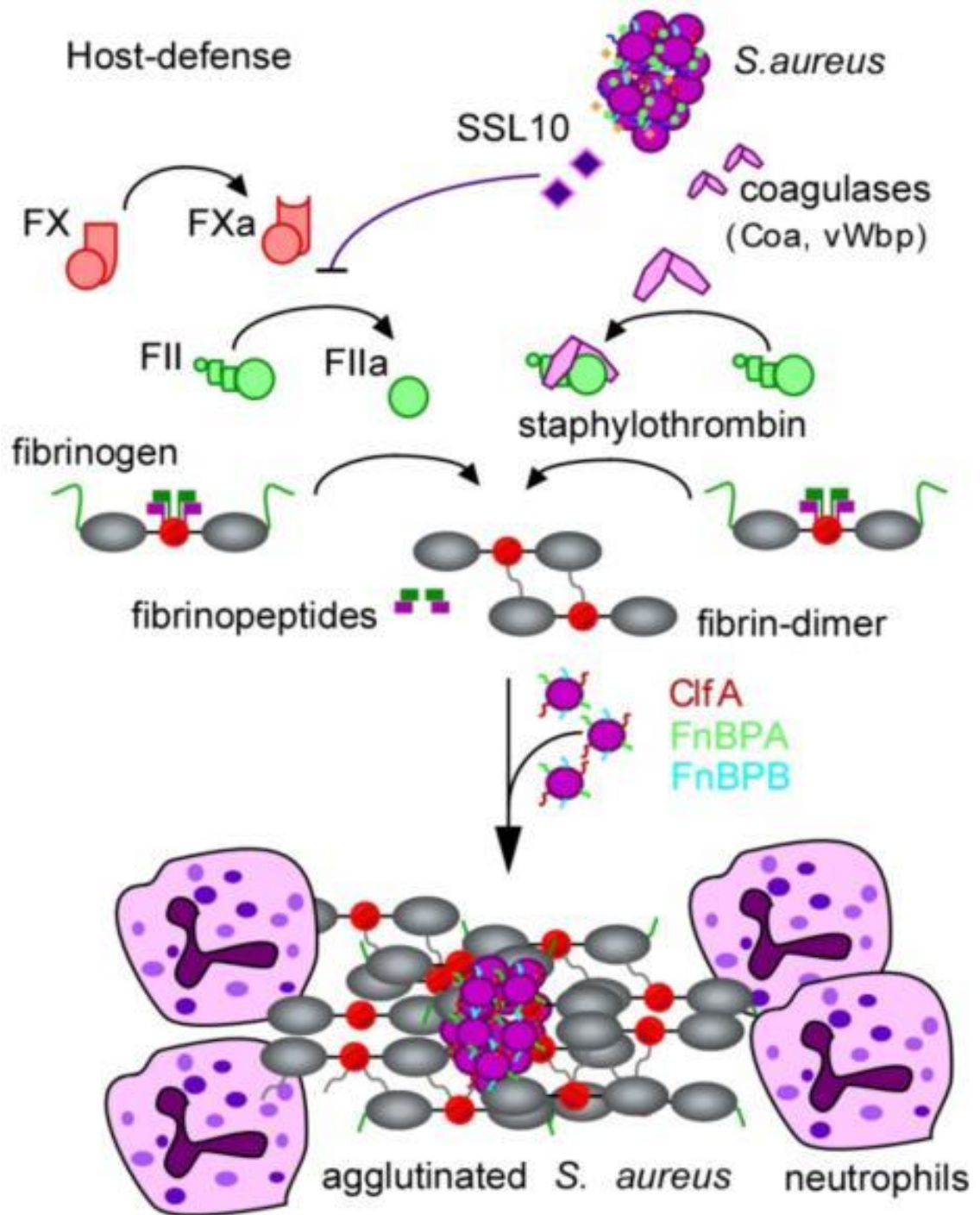
Fig 1.5: Agglutination of *Staphylococcus aureus* with fibrin gives protection against phagocytes. Taken from (Thammavongsa et al., 2015).

1.5.1.3 Fibronectin binding protein A (FnBPA)

FnBPA is a protein found on the cell wall surface that interacts to both fibronectin and fibrinogen. There are two fibronectin binding domains; one in the C-terminal D repeat region and the other in the N-terminal A region. FnBPA also has the ability to bind to fibronectin in the extracellular matrix (Williams et al., 2002, Piroth et al., 2008). Similarly, the integrin found on host cells links to fibronectin to form a connection to its actin cytoskeleton, binding via fibronectin's Arg-Gly-Asp (RGD) motif (Xu and Mosher, 2011). Fibronectin can operate as a 'bridge' across *S. aureus* and the host cells, allowing both to bind at either end of the molecule, making adhesion easier (Fig 1.6) (Massey et al., 2001, Foster, 2016). FnBPA is associated osteomyelitis as it is the most prevalent adhesin for adhesion to osteoblasts, a type of bone cell present in large numbers. In the absence of FnBPA, fewer *S. aureus* cells are internalized into osteoblasts (Josse et al., 2015). FnBPs are required for CA-MRSA strain LAC to produce biofilms (McCourt et al., 2014).
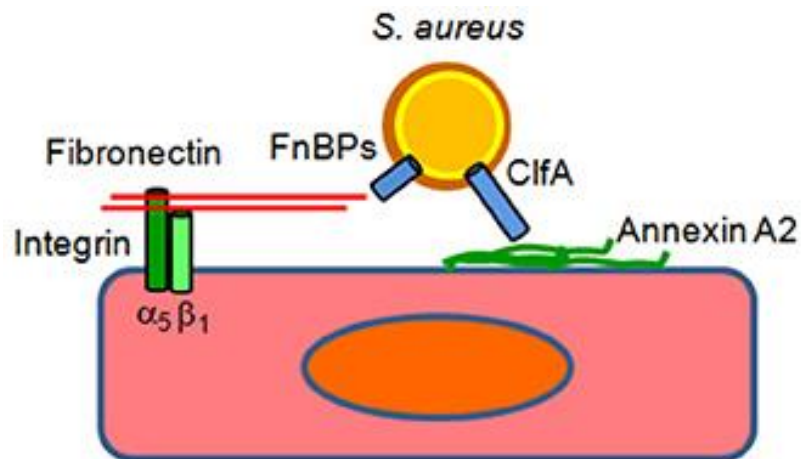
Fig 1.6: The role of *S. aureus* to adhere and invasion of epithelial cell. Fibronectin-binding proteins (FnBPs) bind to fibronectin in the extracellular matrix, which interacts with integrin $51\alpha_5\beta_1$ on the surface of epithelial cells to cause cell invasion. Clumping factor A (ClfA) bind to annexin A2 and this help the to interact into epithelial cell surface, taken from (Thomer et al., 2016, Pietrocola et al., 2017).

1.5.2 Toxicity

S. aureus produces many toxins, which are grouped depending on their mechanisms of action. Cytotoxins cause pore formation in mammalian cells, such as the α-toxins and bicomponent leukocidins (e.g., Panton-Valentine leukocidin) (Seilie & Wardnburg, 2017). Enterotoxins also have superantigenic activity and when ingested they result in food poisoning. *Staphylococc*al scalded skin syndrome (SSSS) is caused by exfoliative toxins (e.g., epidermolysis toxins A and B) (Mishra et al., 2016). the toxic shock syndrome (TSS) toxin (Larkin et al., 2009). *S. aureus* also secretes a number of other enzymes that cause host damage that are not typically referred to as toxins, but act in a similar manner. Extracellular enzymes such as proteases, hyaluronidase, lipase, and nuclease, as well as membrane-damaging toxins that produce cytolytic effects on host cells and tissue damage, and superantigens that contribute to the symptoms of septic shock, can be secreted by the organism (Foster, 2005, Bohach, 2006, Dinges et al., 2000).

One of the important toxins that is produced by *S. aureus* is alpha toxin (Hla), which is a 33 kDa protein. It is cytolytic and causes pore-formation in host cells, distrupting the host cell membrane, and can lead to the lysis of erythrocytes to provide iron for the bacteria. This toxin is encoded by the *hla* gene and causes the pore in the host cell membrane by binding as a monomer to specific receptors on the host cell membrane. These then form a heptameric complex by oligomeric structure to generate the pore. As well, this toxin causes Ca^{2+} influx in platelets, and that causes procoagulatory factor release. Calcium is essential for actin-myosin filaments(Paller et al., 2019).

Hyaluronidases are bacterial enzymes that break the β-1,4 glycosidic link in hyaluronic acid (HA) which is a component of the extracellular matrix in host cells, a high-molecular-weight polymer made up of repeated *N-acetylglucosamine* and glucuronic acid disaccharide units (Hynes and Walton, 2000). The first reports of *S. aureus* hyaluronidase were published in 1933 by Duran-Reynals, who discovered what they called a 'spreading' component in invasive *S. aureus* strains' spent medium. *S. aureus* also produces deoxyribonuclease, an enzyme that can breakdown neutrophil extracellular traps (NETs), making *S. aureus* resistant to NET-mediated death. Nuclease-deficient *S. aureus* were much more vulnerable to extracellular death by neutrophils in a murine mouse model, while nuclease expression resulted in bacterial clearance being delayed and increased mortality. *In vitro* experiments have further shown that the NET degradation product 2'-

deoxyadenosine can trigger death in macrophages, further enhancing *S. aureus* immune cell evasion (Berends et al., 2010, Thammavongsa et al., 2013, Block and Zarbock, 2021). Described below are some of the toxins that help *S. aureus* to cause disease. (Fig 1.7).
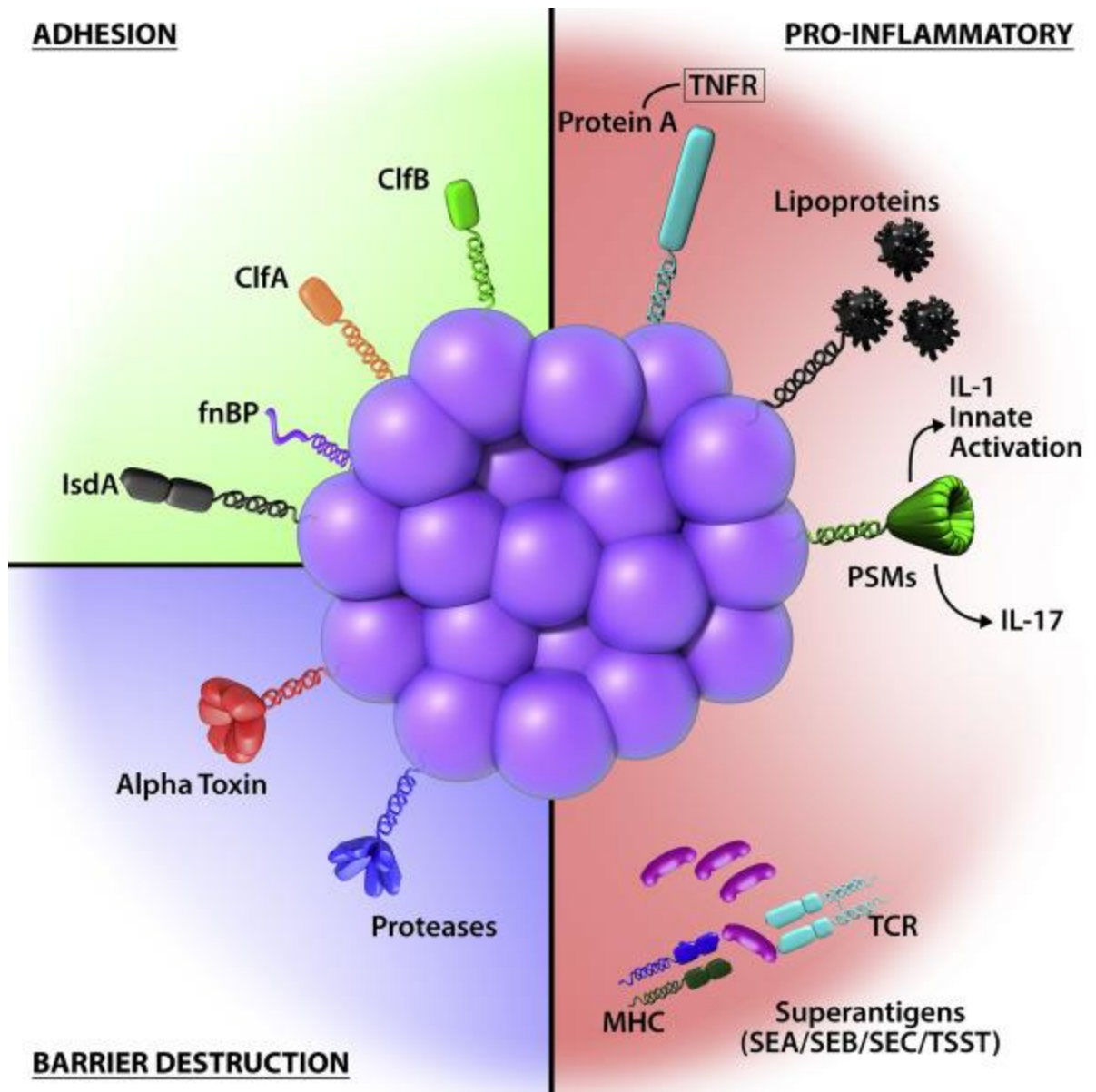
Fig 1.7: Overview of the some of the virulence factors produced by *S. aureus* (taken from Paller et al., 2019).

1.5.2.1 Superantigens

This group includes the 25 staphylococcal enterotoxins (SEs) that have been found and called alphabetically from A to Z (SEA - SEZ), including enterotoxin type B and the toxic shock syndrome toxin TSST-1, which causes TSS when tampons are mis-used (Larkin et al., 2009). Fever, erythematous rash, low blood pressure, shock, multiple organ failure, and skin peeling are all symptoms of TSS. The development of TSS is aided by a lack of anti-TSST-1 antibodies (Brosnahan and Schlievert, 2011). Bacterial superantigens (SAGs) released by *S. aureus* cause this disease by attaching to class II major histocompatibility complex components on antigen-presenting cells and to certain variable areas of the T-cell antigen receptor. In doing so SAGs bypass conventional antigen presentation and stimulate T cells at a far higher level than antigen-specific stimulation, resulting in enormous cytokine release, which is thought to be responsible for the most severe TSS symptoms(McCormick et al., 2001). Some *S. aureus* strains can produce an enterotoxin, which is the cause of a kind of gastroenteritis. This kind of gastroenteritis is self-limiting, with vomiting and diarrhea beginning 1–6 hours after administration of the toxin and lasting 8–24 hours. Nausea, vomiting, diarrhea, and severe stomach pain are some of the symptoms.(Seo and Bohach, 2012, Bennett and Monday, 2003).

1.5.2.2 Exfoliative toxins

Exfoliative toxins are exotoxins linked to the staphylococcal scalded skin syndrome (SSSS), a disease that primarily affects newborns and children, that has been responsible for out breaks in hospital nurseries. The exfoliative toxins' protease activity induces skin peeling, as seen with SSSS (Mishra et al., 2016). The exfoliative toxins have a highly charged N-terminal and a peculiar orientation of a key peptide bond that inhibits the active site of the toxins, preventing them from having any enzymatic activity in their natural state. Desmoglein-1, a desmosomal glycoprotein that plays a vital role in maintaining cell-to-cell contact in the superficial epidermis, has recently been identified as the toxin's target. The binding of the N-terminal to desmoglein-1 is thought to cause a conformational shift in the toxin, allowing it to split the extracellular domain of desmoglein-1 between the third and fourth domains, causing intercellular adhesion to be disrupted and superficial blisters to form and sheets of skin to be released in a manner similar to scalding, hence the name scalded skin syndrome (Bukowski et al., 2010, Ladhani, 2003).

1.5.2.3 Panton-Valentine leukocidin (PVL),

PVL, a bicomponent toxin made up of two subunits, LukS-PV and LukF-PV, is linked to severe necrotizing pneumonia (Kaneko and Kamio, 2004). The capacity to produce pores in human neutrophil cell membranes and cause the production of chemotactic molecules is unrivalled. (Alonzo III and Torres, 2014). PVL associated necrotizing pneumonia caused by CA-MRSA strains is becoming more common in many countries and has a high fatality rate of 56–63 (David and Daum, 2010, Mandell and Wunderink, 2012).

1.5.2.4 Phenol-soluble modulins (PSMs)

Phenol-soluble modulins (PSMs) are a group of amphipathic, α-helical structured proteins(Cheung et al., 2014). This family of toxins have many biological roles that help the bacteria to cause diseases. For example, the expression of proinflammatory cytokines can be stimulated by PSMs, and they can lyse neutrophils and erythrocytes (Shu et al., 2014). PSMs can be divided into six groups: PSMα, PSMβ, PSMγ, PSMδ, PSMε and PSM-mec, each with a unique amino acid sequence (Wang et al., 2007). PSMs groups are very small. For example, PSMα has 22 amino acids (aa), PSMβ has 44 aa and PSMγ has 25 aa (Mehlin et al., 1999);(Queck et al., 2008); (Peschel and Otto, 2013); (Cheung et al., 2014). PSMs are involved in staphylococcal pathogenesis and have a wide range of biological activities. *S. aureus* PSM group lyses white and red blood cells effectively (Wang et al., 2007); (Cheung et al., 2010). PSMs have the ability to act as both cytotoxins and pro-inflammatory agents (Mehlin et al., 1999) and can help to build biofilms and spread bacteria during infection (Periasamy et al., 2012). (Fig 1.8).
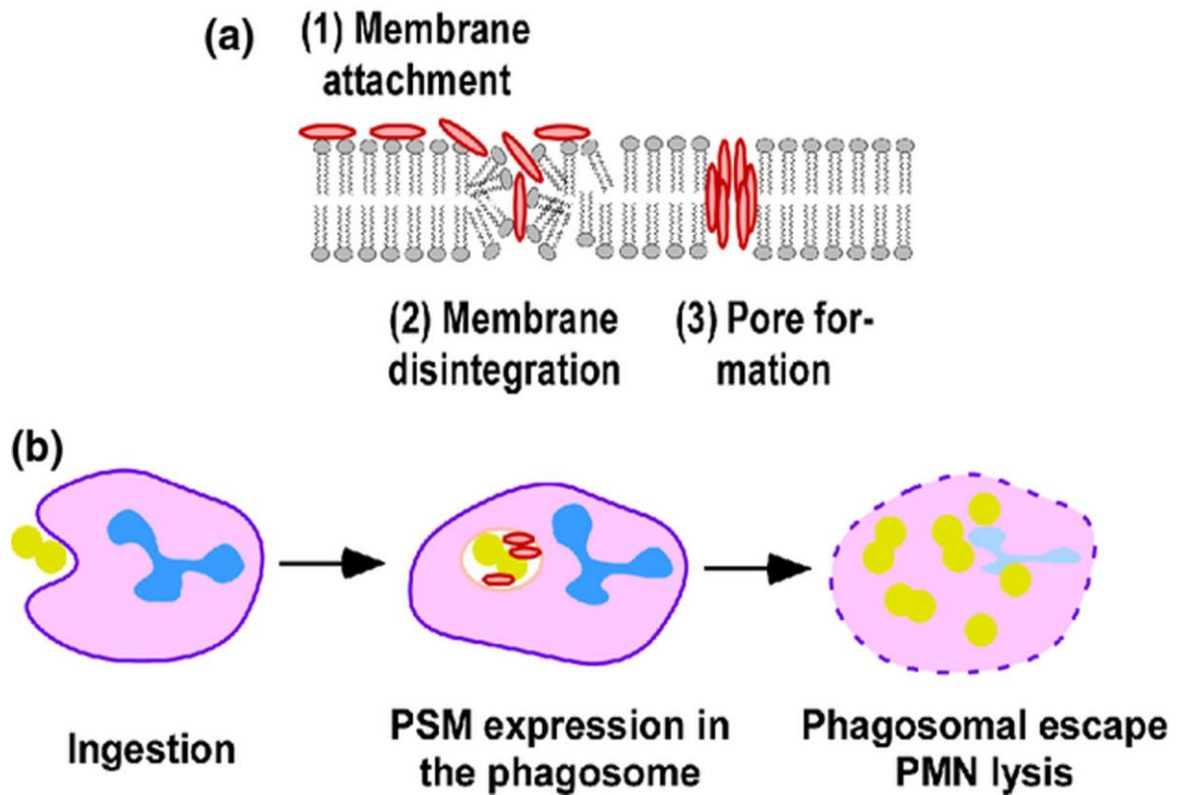
Fig 1.8: The role of PSM in cytolysis activity a: the mechanism of pore formation by PSM (Verdon et al., 2009) b. the mechanism of killing intracellular neutrophil by PSM. Taken from (Surewaard et al., 2013, Cheung et al., 2014).

1.5.3 Evasion

The immune system of a healthy adult is usually sufficient to protect against and limit infections caused by *S. aureus*. However, the bacteria have evolved many strategies to evade this. Some of the proteins involved also play a role in adhesion and toxicity. For example, Protein A interferes with immunoglobulin activity by binding to these molecules, and superantigens interfere with the normal T cell immune response to antigen presentation. Protein A in *S. aureus* can function both in adhesion and evasion because it binds to Fc region of immunoglobulin molecules, on the one hand, and protects the bacteria from phagocytosis, on the other hand.

S. aureus can also evade the immune system by using toxins to lyse neutrophils and prevent phagocytosis. Furthermore, the bacteria express many proteins to inhibit neutrophil chemotaxis or complement activation, for example the chemotaxis inhibitory proteins of staphylococci CHIPS, staphylokinase, aureolysin and pore-forming leukotoxins (Foster, 2005). *S. aureus* has also developed a number of compounds that

allow it to avoid the complement cascade, immunoglobulins, and phagocytic components. Because these components evade, the immune response is delayed or reduced, allowing the bacterium to survive and multiply within its host. (Fig 1.9).
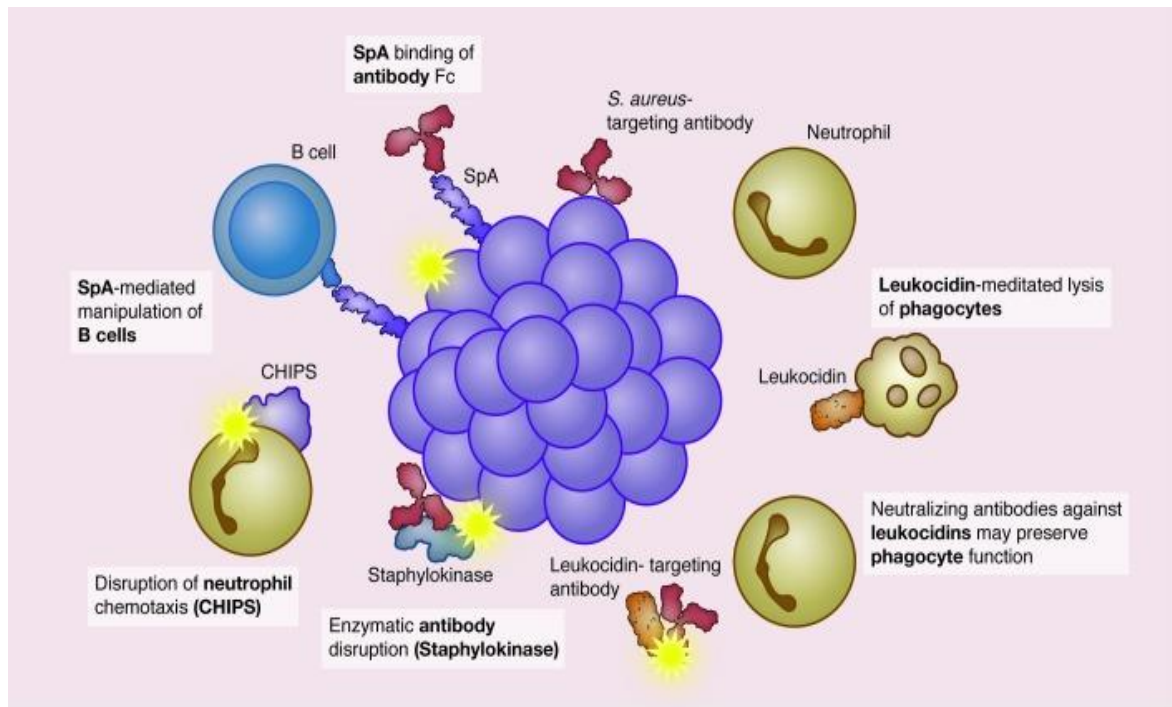
Fig 1.9: Some virulence factors that are used by *S. aureus* to evade immune response. Taken from (Thomsen and Liu, 2018).

1.5.3.1 Staphyloxanthin

Staphyloxanthin is a membrane-bound carotenoid produced by *Staphylococcus aureus*. Until colourless strains were discovered, the orange pigmentation of *S. aureus* was employed as a species-identifiable trait (Kashef and Hamblin, 2017, Walter and Strack, 2011, Pelz et al., 2005). Chemical analysis of *S. aureus* pigments and 17 intermediate products revealed triterpenoid carotenoids with a C30 chain rather than the C40 carotenoid structure observed in most other organisms (Pelz et al., 2005). The major pigment, staphyloxanthin, was discovered as glucopyranosyl 1-O-glucopyranosyl (4,4-diaponeurosporen- 4-oate)-6-O-(12-methyltetradecanoate). With its conjugated double bonds, staphyloxanthin scavenges free radicals (Clauditz et al., 2006). Because staphyloxanthin is found in the cell membrane, it most likely protects lipids, but it could also protect proteins and DNA (Clauditz et al., 2006). Although enzymes like catalase and superoxide dismutase (SOD) are likely to play a larger role in cell survival throughout stress and the host response, staphyloxanthin has a secondary role in protecting cells

from reactive oxygen species (ROS) damage, hence increasing their virulence and fitness (Clauditz et al., 2006). (Fig 1.10).
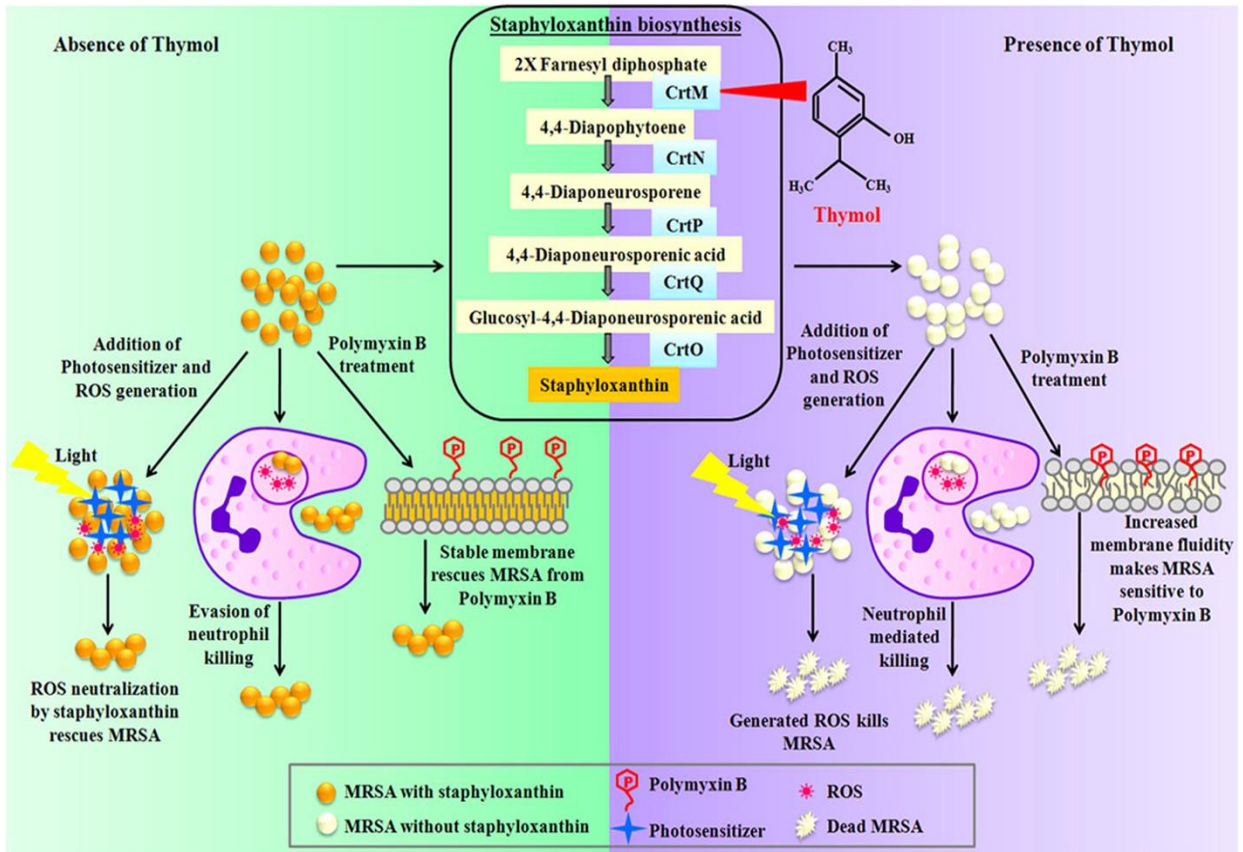
Fig 1.10: The effect of staphyloxanthin inhibition on *S. aureus.*Taken from (Valliammai et al., 2021).

1.6 Iron acquisition for *S. aureus* growth and pathogenesis

Iron is an essential nutrient for most bacteria to survive and grow such that the ability of bacteria to take up iron is often considered as a determinant of its virulence. Iron required for cellular functions such as oxygen carriage and electron transport (Stojiljkovic and Perkins-Balding, 2002). In the human body, iron is bound to haemoproteins such that invading bacteria require specific mechanisms to gain access to it (Skaar et al., 2004). *S. aureus* can gain iron through siderophore mechanisms, which remove iron from host sources such as lactoferrin and transferrin. *S. aureus* also produces cell wall-associated protein that allows the bacteria to uptake iron from host protein directly (Park et al., 2005). For example, *S. aureus* can produce 42 kilodalton cell wall-associated protein, which

binds human transferrin (Modun et al., 1994). *S. aureus* can even utilise free heme including haemoglobin as a source of iron (Mazmanian et al., 2003) (Crossley et al., 2009, Friedman et al., 2006). Therefore, iron is an important factor in host-bacterial interactions. In environments with high levels of heme, *S. aureus* evades heme toxicity by switching on the heme regulated transporter (HrtAB) system that pumps excess heme out of the cell (Torres et al., 2007)(Fig. 1.11). When the level of heme becomes very low, *S. aureus* moves from using respiration to generate energy to fermentation and two proteins, IsdI and IsdG (heme oxygenases), are expressed. All of these expression changes in response to iron levels are mediated by the ferric uptake regulator (Fur) (Lojek et al., 2018).
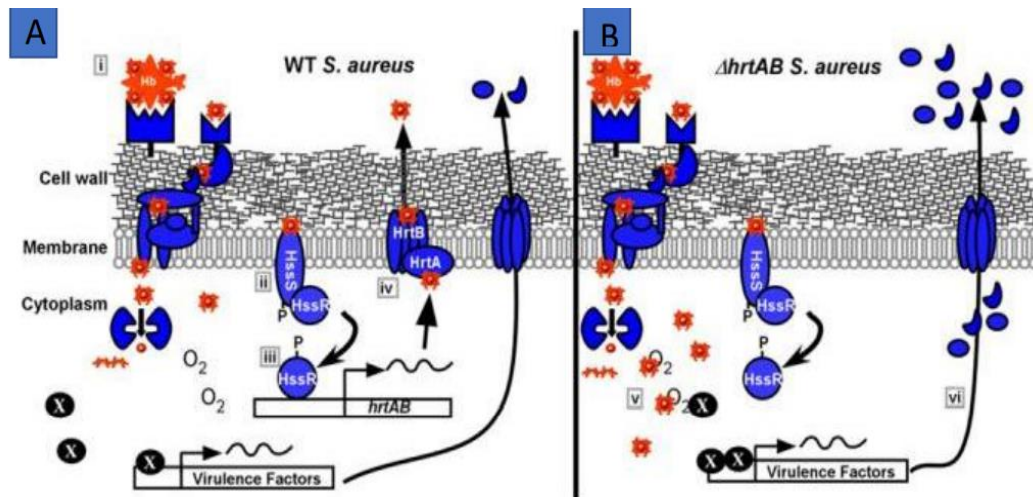
Fig. 1.11: The role of HrtAB in *S. aureus*. A: In *S. aureus*, heme toxicity controlled via cell wall anchored proteins HssS sensing heme and activating HssR. After that, HssR binds to the promoter region upstream of *hrtAB*, and that leads to increased expression and preparation of the HrtAB efflux pump. Then HrtAB pumps exec cytoplasmic heme out of the cell. **B:** When *hrtAB* is absent of increases cellular stressoccur of the cytoplasmic accumulation of heme (Torres et al., 2007)

S. aureus has developed sophisticated mechanisms for obtaining the iron it requires to thrive in vertebrates. Heme accounts for 80% of the iron in the host and *S. aureus* prefers to steal iron from heme (Skaar et al., 2004). Considering the robustness of the hemoglobin-haptoglobin complex, *S. aureus* is able to connect to it and use it as an iron source(Hammer and Skaar, 2011). *S. aureus* changes its protein expression pattern considerably in response to the host's iron-restricted environment. The iron-dependent ferric uptake regulator is responsible for this alteration in protein expression and Fur binds to the fur box, a consensus DNA sequence located upstream of Fur-

regulated genes, in the presence of iron (Friedman et al., 2006). Fur is liberated from the DNA when iron becomes scarce, easing Fur-mediated transcriptional repression (Baichoo et al., 2002, Beasley et al., 2009). Through the formation of siderophores, *S. aureus* takes iron from lactoferrin or transferrin in an indirect manner (Conrou et al., 2019). *S. aureus* produces two siderophores, staphyloferrin A and staphyloferrin B, which have similar characteristics and are controlled by Fur (Lee et al., 2008, Beasley et al., 2011). Siderophores are tiny secreted molecules with a high affinity for iron that host iron binding proteins (Wilson et al., 2016, Conrou et al., 2019). Recognized by homologous receptors on the bacterial surface, siderophore-iron complexes allow the stealing of iron from lactoferrin or transferrin (Cheung et al., 2009).

1.6.1 Iron as an essential and toxic nutrient

Iron is a vital nutrient to humans, and its deficiency results in the cause of diseases like anaemia (Gardner and Kassebaum 2020; Pasricha et al., 2021). In the case of Fe deficiency, medical implications can be problematic as supplemented iron is only partially absorbed by the human body, which makes iron exists at high levels in the human gut microbiome (DeLoughery 2019; Finlayson-Trick et al., 2020). Microbiomes are considered extra organs in the human body that participate in many essential functions. Therefore, when metal compounds exist at unrequired levels, dysbiosis occurs and survival/reproduction of gut commensal bacteriome declines, which promotes several human health diseases like diabetes, inflammatory bowel diseases (IBD), Parkinson's disease (PD), etc. (Glassner et al., 2020, Sheldon and Skaar 2019; Zaky et al., 2021). Intravenous Fe supplementation has proven lately to be the best solution to cope with the problem (Macdougall 2020; Schaefer et al., 2020). Such an approach allows blood-borne pathogens, ex., *Staphylococcus aureus*, to be exposed to excess iron. The methicillin-resistant (MRSA) strains of the latter pathogen are considered a global human health challenge (David and Daum 2010; DeLeo et al., 2010; Ganz et al., 2020). Interestingly, the molecular form of iron in blood influences the level of uptake by *S. aureus*, as several reports indicated that *S. aureus* grows better in the presence of heme iron form than in the presence of transferrin-bound iron (BTI) form (Barton Pai et al., 2006; Cross et al., 2015; Suffredini et al., 2017; Skaar et al., 2004).

1.6.2 Iron homeostasis in humans

More recently, *S. aureus* was investigated to detect the influence of either excess host iron or iron-limiting condition on the bacteria during infection to promote the

nutritional immunity approach of treatment (Van Dijk et al., 2022). Bacteria tend to maintain certain intracellular concentrations of different nutrients, including iron, to promote their growth, a process called nutrient homeostasis. It is speculated that bacteria harbour sensors that measure the level of intracellular iron, and a control system(s) that manipulate the expression of genes encoding certain proteins and enzymes to maintain a target nutrient level. This complex regulation(s) is tuned at transcriptional, post-transcriptional, translational and/or post-translational levels. These transcriptional levels respond through several genetic circuits to the calls for iron influx or efflux, based on necessity and the surrounding environment. Forms of iron include free iron (Fe), which is essential for life, while Fe^{2+} form generates toxic reactive oxygen species (ROS) and Fe^{3+} is insoluble under neutral aqueous conditions, and bacteria cannot benefit from it unless it is converted to Fe with Fe^{2+} production as an intermediate step (Fig 1.12).
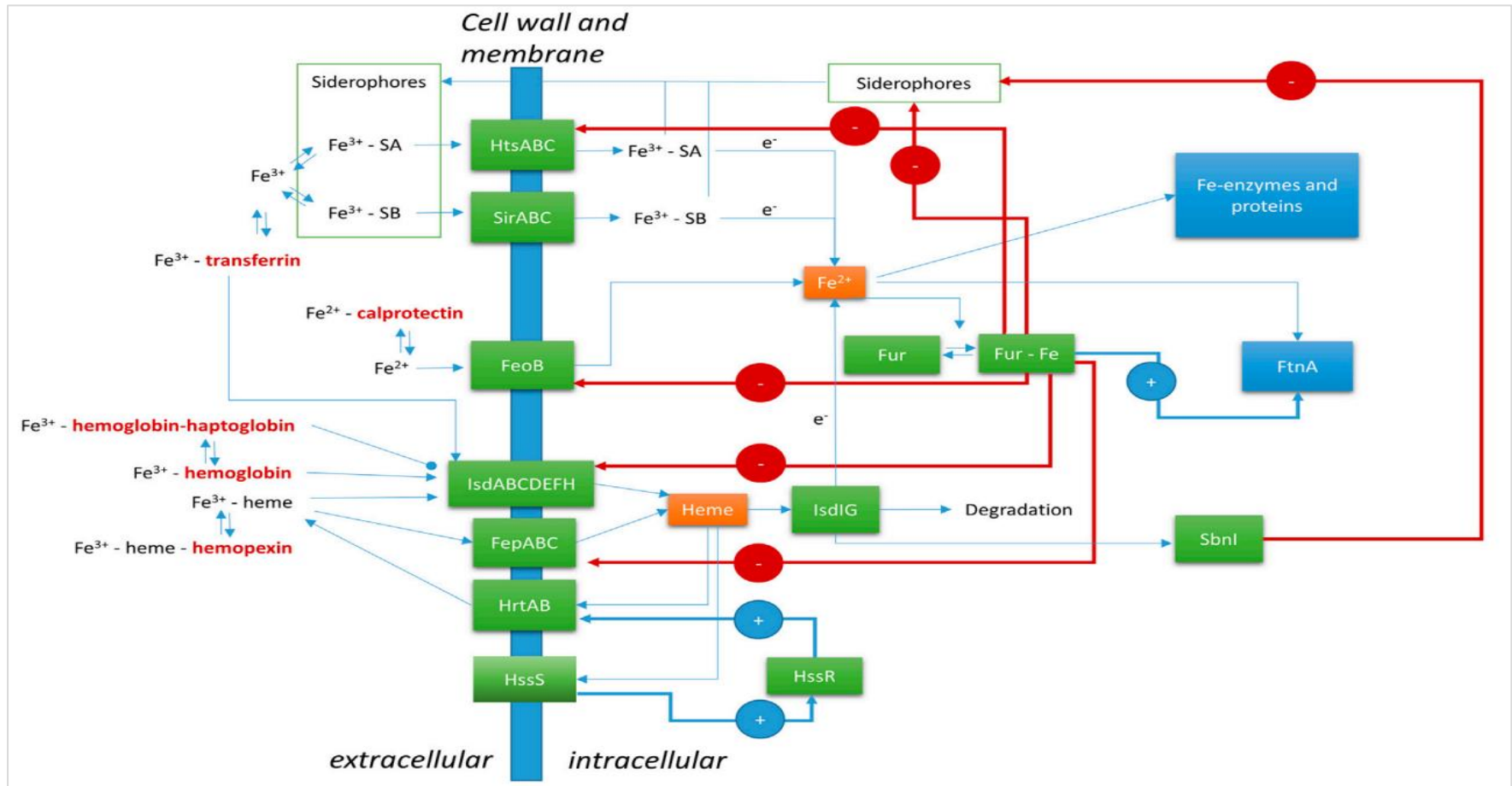
Fig 1.12: Free iron (Fe) regulation by *Staphylococcus aureus* at the host-microbe interface. FeoB = ferrous Fe transporter; FepABC = Fe dependent peroxidase transporter; FtnA = ferritin; Fur = ferric uptake regulator; HrtAB = heme regulator transporter efflux pump; HssR = heme sensing two-component regulator regulatory protein; HssS = heme sensing two-component regulator sensor protein; HtsABC = heme transport system involved in Fe-SA uptake; IsdABCDEFGHI = iron-regulated surface HssS, heme sensing two-component regulator sensor protein; HtsABC = heme transport system involved in Fe-SA uptake; IsdABCDEFGHI = iron-regulated surface in bold and dark red font. Taken with permission after Van Dijk et al. (2022).

1.6.2.1 Regulation of free iron uptake and efflux

Ferric uptake regulator (Fur) is the major control system for iron homeostasis, e.g., influx/effux, utilization, storage/maintenance (Figure 1.12). The homodimeric Fur protein has two domains, of which a metal ion binding site exists between the two domains (Price and Boyd 2020). Fur in *S. aureus* was described to bind two separate Fe2+ ions in the hinge regions between the two domains. In the dimeric form, the DNA binding part of Fur becomes a transcriptional repressor of a number of genes related to iron homeostasis, including those encoding Fe transporters. When Fur binds to the Fur-box existing upstream of some of these genes, virulence factors involved in disease processes are produced. Ferrous iron uptake by transporter FeoB can be blocked by antibacterial agent or inhibitors that can be considered an important medical implication as novel antibiotics against MRSA (Shin et al., 2021). In addition, the FepABC (Fe-dependent peroxidase) transporter system is implicated in Fe and possibly heme uptake. The transporter consists of FepA which acts as a Fe binding protein, peroxidase FepB which can bind heme and protoporphyrin IX (heme without Fe) (Turlin et al., 2013). Interestingly, heterologous expression of FepAB in *S. aureus* likely allows heme utilization (Turlin et al., 2013). Siderophores are encoded by two gene clusters, both are transcriptionally repressed by Fur. They play an essential role in *Staphylococcus* biofilm formation to ensure Fe availability (Johnson et al., 2005; Oliveira et al., 2021). However, the heme-sensitive regulator of siderophore production, namely SbnI (Laakso et al., 2016; Verstraete et al., 2019), produces a precursor to siderophore production. When the protein binds to DNA and is dimerized, a heme-binding domain is generated. As soon as heme is transferred from IsdI to SnbI, it can help control the production of siderophores to allow heme utilization rather than free iron uptake.

1.6.2.2 Regulation of hemic iron uptake and efflux

During infection, *S.* aureus obtains heme from human red blood cells (RBCs) as the primary source of iron (Skaar et al., 2004). When heme is presented at high levels inside the bacteria, it becomes detrimental as it provokes the formation of reactive oxygen species (ROS). Thus, bacteria should use a regulatory mechanism to prevent such a condition. S. aureus, in particular, uses the heme-sensing two-component regulator HssRS (Figure 1.12) to manipulate the intracellular free heme level (Price and Boyd 2020; Stauff, Torres, and Skaar 2007; Stauff and Skaar 2009). When HssS regulator is activated, it phosphorylates the histidine of HssR, which in turn, acts as a transcriptional

activator of the heme efflux transporter HrtAB (Heme regulator transporter efflux pump) (Price and Boyd 2020). On the other hand, when bacteria are required to uptake heme, it induces a cell wall system called Isd (Iron-regulated surface determinant system) (Skaar and Schneewind 2004; Grigg et al., 2010b; Mazmanian et al., 2003). This system involves nine different proteins, four of which, e.g., IsdA, IsdB, IsdC, and IsdH, are bound to the cell wall, two, e.g., IsdE and IsdF, constitute an ABC-transporter for the heme cofactor, two, e.g., IsdI and IsdG, are soluble intracellular heme degrading enzymes, while IsdD is a transmembrane protein of unknown function. IsdB and IsdH firstly bind to free heme, then, IsdC and IsdA are involved in heme transport through the cell wall to the ligand binding component of the ABC-transporter IsdE. Then, heme is translocated to the cytoplasm and the cofactor IsdE is degraded by the heme-degrading enzymes IsdI and IsdG to release Fe from the cofactor (Grigg et al., 2010b). Fur regulates the expression of genes encoding IsdA, IsdB, IsdC and IsdH. The latter four proteins are anchored to the cell wall by the action of a gene encoding the enzyme sortase B (SrtB), which is also regulated by Fur.

1.7 Mechanism of Antibiotic Resistance *S. aureus*

Antibiotic resistance is a major global health problem, and *S. aureus* has evolved many mechanisms to facilitate this. Penicillin, the first antibiotic treatment for *S. aureus* infection, was introduced in the 1940s. However, by the beginning of 1942, new strains emerged that were resistant to penicillin due to the release of the enzyme β-lactamase that inactivates the penicillin molecule. A β-lactamase resistant version of penicillin, methicillin, was synthesised in the 1950s, however by 1961 the first methicillin-resistant *Staphylococcus aureus* (MRSA) was reported. Vancomycin is an effective MRSA treatment, however, in 1996, the first MRSA resistant to vancomycin was isolated from a Japanese patient who was undergoing long-term therapy with vancomycin due to post-operative wound infection (Hiramatsu, 2001). History, therefore, suggests that if we are to avoid a return to the pre-antibiotic era, where people died from relatively simple infections, we need both a greater understanding of how this bacterium becomes resistant to antibiotics, as well as a steady pipe-line of new drugs.

S. aureus can also be multidrug-resistant, with strains circulating that resist a variety of antibiotics, including β-lactams, vancomycin, and aminoglycosides (India et al., 2013). MRSA are genetically unique *S. aureus* strains (Hiramatsu et al., 2002) in humans, and are responsible for a number of infections that are difficult to cure (Otto, 2012). The

first MRSA isolates were found in Britain in 1961, and from 1961 to 1967, there were isolated hospital infections in Western Europe and Australia (Ayliffe, 1997). Other antibiotic-resistant strains of *S. aureus* have been discovered. In 1996, Japan was the first country to report vancomycin resistance (Hiramatsu, 1998). MRSA outbreaks have been reported to spread from hospital to hospital in many places (Cuny et al., 2006). By 1995, the prevalence of MRSA had grown to 22%, and by 1997, MRSA-related hospital infections accounted for 50% of all *S. aureus* infections (Haddadin et al., 2002).

The first incidence of community-associated MRSA (CA-MRSA) was reported in 1981, and in 1982, a significant outbreak of CA-MRSA among intravenous drug users was observed in Detroit, Michigan (Rybak and LaPlante, 2005). More CA-MRSA outbreaks were subsequently observed in the 1980s and 1990s. In the mid-1990s, the first CA-MRSA outbreak was documented in children in the United States (David and Daum, 2010). Hospital-acquired methicillin-resistant *Staphylococcus aureus* HA-MRSA rates stabilised between 1998 and 2008, however, CA-MRSA rates continued to rise. Over two periods (1993–1995 and 1995–1997), research released by the University of Chicago Children's Hospital found a 25-fold rise in the risk of MRSA hospitalizations among children in the United States (Zhang et al., 2013). In 1999, the University of Chicago reported the first invasive MRSA mortality in the United States among apparently healthy newborns (Weber, 2005).

1.7.1 *mecA* gene

The *mecA* gene is used as a biomarker for methicillin and oxacillin resistance. It must be incorporated and localized in the *S. aureus* chromosome after it has been acquired (Lee et al., 2018). Penicillin-binding protein 2a (PBP2a), encoded by *mecA* gene, is a penicillin-binding protein that varies from other PBPs in that its active site does not bind methicillin or other β-lactam antibiotics (Fishovitz et al., 2014). As a result, even in the presence of antibiotics, PBP2a can continue to catalyze the transpeptidation event necessary for peptidoglycan cross-linking, allowing cell wall formation (Macheboeuf et al., 2006). Two regulatory genes, *mecI* and *mecR1* (Fig. 1.13), are in charge of *mecA* gene (Petinaki et al., 2001). MecI is a repressor that is generally linked to the *mecA* promoter (Petinaki et al., 2001, Arêde et al., 2012). MecR1 generates a signal transduction cascade that results in the transcriptional activation of *mecA* in the presence of a β-lactam antibiotic (Petinaki et al., 2001, Arêde et al., 2012, Fuda et al., 2005). This is accomplished by MecR1-mediated MecI cleavage, which relieves MecI repression

(Petinaki et al., 2001, Meng et al., 2006). Two co-repressors, are also involved in the control of *mecA* (Black et al., 2011, Hou et al., 2011). The genes *blaI* and *blaR1* are similar to *mecI* and *mecR1*, respectively, and generally serve as regulators of *blaZ*, the gene that causes penicillin resistance (Black et al., 2011, Hou et al., 2011, Niemeyer et al., 1996, Stapleton and Taylor, 2002).
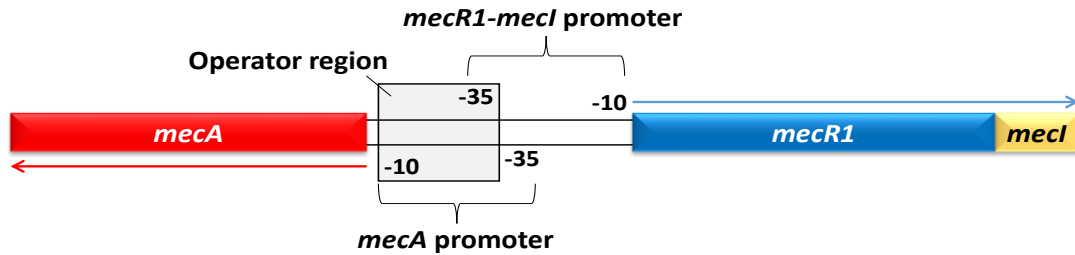
Fig. 1.13. Schematic representation of the *mecA-mecR1-mecI* coding region. Arrows indicate the relative directions of transcription of the *mecA* and *mecR1-mecI* genes. Drawn after Stapleton and Taylor (2002). (Stapleton and Taylor, 2002)

1.7.2 Staphylococcal cassette chromosome *mec* (SCC*mec*)

The complete genomic sequencing of numerous MRSA strains has provided insight into the scattering of mobile genetic elements in the bacterial chromosome that code for antibiotic resistance and virulence (Kuroda et al., 2001). The antibiotic resistance gene *mecA* is included within SCC*mec,* a genomic island of unknown origin (Ito et al., 2003). SCC*mec* is believed to have evolved in the closely adjacent Coagulase Negative Staphylococci (CoNS) species and been horizontally transmitted to *S. aureus* (Hanssen et al., 2004). SCC*mec* comprises genes other than *mecA*, such as *psm-mec*, a cytolysin gene that may modulate virulence in HA-acquired MRSA strains (Queck et al., 2009). SCC*mec* also comprises the genes *ccrA* and *ccrB* (Fig. 1.14), which encode recombinases that mediate the SCC*mec* element's site-specific integration and excision from the *S. aureus* chromosome (Wang and Archer, 2010).
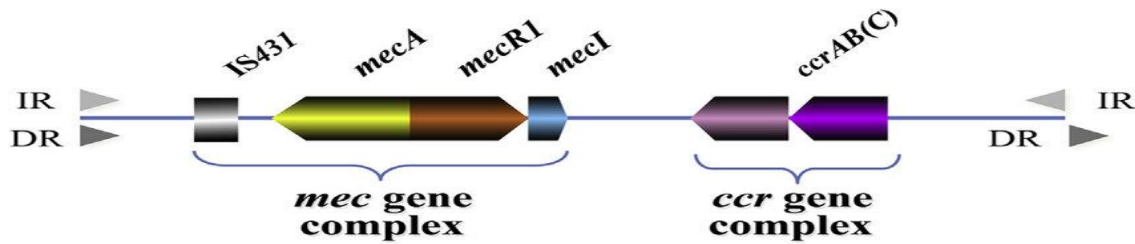
Fig. 1.14: The SCC*mec* structure. SCC*mec* is made up of two gene complexes that are required for its function. The *mec*-gene complex encodes methicillin resistance (*mecA* gene) and its regulators (*mecI* and *mecR1* genes), while the *ccr*-gene complex encodes the mobility of the complete SCC element (chromosome integration and precise excision). IR stands for inverted repeat; DR stands for direct repeat. (Hiramatsu et al., 2014).

Eleven distinct SCC*mec* types have been found so far, with sizes ranging from ~23 to 68 kb (Fig. 1.15) (Zhang et al., 2009, Elements, 2009, Hiramatsu et al., 2013). They are classified as classes I–XI, with differences in the *mec* and *ccr* gene complexes distinguishing them (Elements, 2009). Because of the size of the SCC*mec* element and the limitations of horizontal gene transfer, MRSA infections are assumed to be disseminated by at least five clones, with clonal complex (CC) 8 being the most common (Smith et al., 2021). SCC*mec* genotypes confer various microbiological features, such as antibiotic resistance rates and infection types (Mohammadi et al., 2014, McManus et al., 2015, Park et al., 2009). Types I–III SCC*mec* are big elements found in HA-MRSA strains that frequently contain additional resistance genes (Fig. 1.15). CA-MRSA, on the other hand, is linked to types IV and V, which are smaller and lack other resistance genes than *mecA* (Daum et al., 2002, Ma et al., 2002). Carriage of a big or small SCC*mec* element is linked to differences in bacterial fitness. Carriage of big elements, such as SCC*mecI*–III, is costly to bacteria, resulting in a reduction in virulence expression as a compensation (Collins et al., 2010). MRSA thrives in hospital environments, where antibiotic resistance is high, but their pathogenicity is low. The propensity of immunocompromised patients in hospitals is such that despite being less virulent HA-MRSA can still cause significant disease. However, as greater levels of virulence and toxicity are necessary to infect an otherwise healthy hosts, CA-MRSA has evolved to carry the smaller, lower-fitness costing SCC*mec* elements (Bal et al., 2016, Knight et al., 2013, Eshlak, 2019).
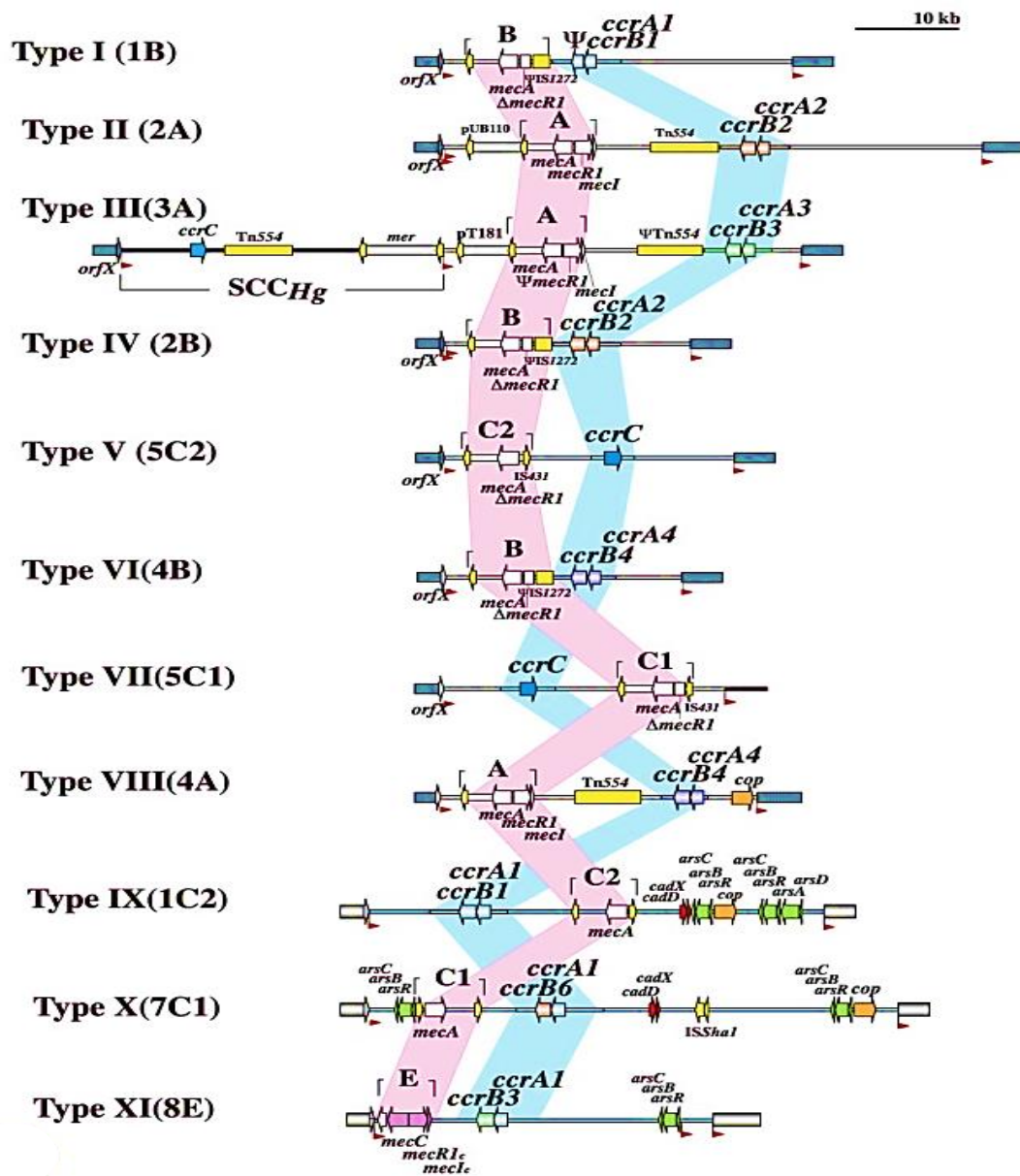
Fig. 1.15: Structures of 11 types of SCC*mec* based on nucleotide sequences deposited in the DDBJ/EMBL/GenBank databases. Type I (strain NCTC10442, locus AB033763 = 39,332 bp); type II (strain N315, locus D86934 = 58,237 bp); type III (strain 85/2082, locus AB037671 = 68,256 bp); type IV (strain CA05, locus AB063172 = 26,090 bp); type V (strain WIS or WBG8318, locus AB121219 = 28,612 bp); type VI (strain HDE288, locus AF411935 = 23,293 bp); type VII (strain JCSC6082, locus AB373032 = 33,261 bp); type VIII (strain C10682, locus FJ390057 = 33,371 bp); type IX (strain JCSC6943, locus AB505628 = 44355 bp); type X (strain JCSC6945, locus AB505630 = 51483 bp) and type XI, LGA251 (FR821779.1 = 29,419 bp, newly identified). (Hiramatsu et al., 2013).

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SCC*mec* acquisition in methicillin-resistant *S. aureus* (MRSA) results in a variety of genetically distinct MRSA lineages (Mediavilla et al., 2012). MRSA has been divided into more than five phylogenetically different lineages, indicating many historical transfer events (Table 1.2). MSSA acquired the *mecA* gene on 20 or more separate occasions, according to phylogenetic research, and then converted to MRSA (Hiramatsu, 2004, Hiramatsu et al., 2002, Robinson and Enright, 2004). Variability in virulence and related MRSA infections may be explained by genetic differences among MRSA strains (Holden et al., 2004). SCC*mec* and ST250-MSSA integration resulted in the first MRSA strain, ST250 MRSA-1. Historically, HA-MRSA infections were caused by the MRSA clones ST2470-MRSA-I, ST5-MRSA-II, ST239-MRSA-III and ST5-MRSA-IV (Enright et al., 2002). The most frequent MRSA strains in the UK are EMRSA15 and EMRSA16 (Moore and Lindsay, 2002). The ST36:USA200 strain, which exists in the United States, has been discovered to be identical to EMRSA16 (MRSA252), which carries the SCC*mec* type II, enterotoxin A, and toxic shock syndrome toxin 1 genes (Moore and Lindsay, 2002, Umaru et al., 2011, Diep, 2005). In Asia, EMRSA 15 strain is one of the most frequent MRSA strains (Ko et al., 2005). ST5:USA100 and EMRSA 1 strains are two other frequent strains (Otto, 2012). Phylogenetically, CA-MRSA differs from HA-MRSA (Thurlow et al., 2012) and genome analysis of CA-MRSA and HA-MRSA suggests that novel MRSA strains have independently integrated SCC*mec* into MSSA (Boyle-Vavra and Daum, 2007). MRSA acquired in the community is easier to treat and more virulent than MRSA acquired in hospitals (HA-MRSA) (Boyle-Vavra and Daum, 2007, Xie et al., 2016).

Table 1.2. Global distribution of MRSA SCC*mec* lineages. Taken from (Mediavilla et al., 2012).

ST	SCC*mec*	Country
ST1	IV	Abu Dhabi, Australia, Brazil, Canada, China, Denmark, Egypt, Finland, France, Germany, Greece, Ireland, Italy, Japan, Pakistan, Romania, Samoa, Singapore, South Korea, Switzerland, United Kingdom, United States
ST5	I	France, Iceland, South Africa
	II	Iceland, Japan
	IV	Algeria, Argentina, Australia, Austria, Azores, Canary Islands, China, France, Germany, Iceland, Italy, Morocco, Samoa, Senegal, Spain, Switzerland, United Kingdom
	V	Australia, Cameroon, Egypt, Japan, Nigeria
	VI	Azores, Spain
ST6	IV	Japan, Malaysia
ST8	IV	Abu Dhabi, Argentina, Australia, Austria, Belgium, Brazil, Bulgaria, Cameroon, Canada, Canary Islands, China, Colombia, Costa Rica, Cuba, Czech Republic, Denmark, Ecuador, Finland, France, French Polynesia, Gabon, Germany, Greece, Hong Kong, Iceland, India, Iraq, Ireland, Israel, Italy, Japan, Madagascar, Mexico, Netherlands, New Zealand, Nigeria, Norway, Pakistan, Peru, Poland, Portugal, Romania, Russia, Samoa, South Korea, Spain, Sweden, Switzerland, Trinidad & Tobago, United Kingdom, United States, Uruguay, Venezuela
	V	Germany, Nigeria
	VI	Portugal
ST22	IV	Australia, Azores, Canary Islands, Germany, India, Ireland, Japan, Netherland, Singapore, United Kingdom
	V	Germany
ST30	IV	Abu Dhabi, Australia, Austria, Brazil, Canary Islands, China, Czech Republic, Denmark, Egypt, Finland, France, French Polynesia, Germany, Hong Kong, Ireland, Italy, Japan, Kuwait, Latvia, Malaysia, Netherlands, New Zealand, Pakistan, Peru, Philippines, Poland, Romania, Russia, Samoa, Singapore, South Korea, Spain, Sweden, Switzerland, Taiwan, Turkey, United Kingdom, United States, Uruguay
	V	China, Madagascar
ST45	IV	Australia, Azores, Belgium, Germany, Hong Kong
	V	Australia, China, Hong Kong
	VI	Switzerland
ST59	IV	Australia, China, Denmark, Finland, Germany, Hong Kong, Netherlands, Singapore, Sweden, Taiwan, United Kingdom, United States, Vietnam
	V	Australia, China, Germany, Hong Kong, Japan, Poland, Singapore, Sweden, Taiwan, United Kingdom, United States, Vietnam
ST72	IV	Abu Dhabi, Czech Republic, Germany, Portugal, South Korea, Sweden, United States
	V	United States
ST75	IV	Australia
ST78	IV	Australia
ST80	IV	Abu Dhabi, Algeria, Australia, Austria, Belgium, Bulgaria, Croatia, Czech Republic, Denmark, Egypt, Finland, France, Germany, Greece, Ireland, Israel, Italy, Jordan, Kuwait, Lebanon, Libya, Malta, Netherlands, Norway, Poland, Portugal, Romania, Singapore, Slovenia, Spain, Sweden, Switzerland, Tunisia, United Kingdom
ST88	IV	Abu Dhabi, Angola, Cameroon, China, Gabon, Italy, Japan, Madagascar, Mali, Netherlands, Niger, Nigeria, Portugal, Senegal, Spain, Sweden, United Kingdom
	V	China, Italy, Madagascar, Sweden
ST91	II	Japan
	IV	Japan
	V	Japan
ST93	IV	Australia, Czech Republic, Finland, Italy, Netherlands, Samoa, United Kingdom
ST97	IV	Abu Dhabi, Canary Islands, Denmark, Germany, Netherlands
	V	Egypt, Kuwait, United Kingdom
ST121	V	Australia, Cambodia
ST152	V	Austria, Canary Islands, Denmark, Germany, Kosovo, Macedonia, Slovenia, Sweden, Switzerland
ST377	V	Australia, France, Greece, Netherlands, Switzerland
ST398	II	Hong Kong
	IV	Austria, Belgium, China, Denmark, Germany, Hong Kong, Italy, Netherlands
	V	Austria, Belgium, Canada, China, Denmark, Germany, Hong Kong, Italy, Netherlands, Norway, Portugal, Spain, Sweden, United States
	NT	Belgium, Denmark, Germany, Netherlands
ST772	IV	India
	V	Abu Dhabi, Australia, Bangladesh, Finland, Germany, Hong Kong, India, Ireland, Italy, Japan, Netherlands, United Kingdom

1.8 Accessory gene regulator (*agr*)

The *agr* locus is a five-gene complex that encodes a quorum sensing system that regulates *S. aureus* virulence (Sakoulas, 2006). The Agr system is a two-part transcriptional quorum-sensing (QS) system that is activated by an autoinducing thiolactone-containing cyclic peptide (AIP) (Sakoulas, 2006, Novick, 2006).

1.8.1 Basic architecture of the *agr* autoinduction circuit

The *agr* operon (*agr*BDCA) system has two principal transcripts, RNAII and RNAIII, which are driven by P2 and P3 promoters, respectively (Fig. 1.16) (Novick et al., 1995, Novick et al., 1993). AgrB, AgrD, AgrC, and AgrA are the elements of the *agr* system encoded by RNAII. The active 8-amino-acid (may range from 7–9) pheromone, with a distinctive thiolactone ring structure, dubbed autoinducing peptide, is produced by processing AgrD to a propeptide or autoinducing peptide (AIP) (Yarwood and Schlievert, 2003, Ji et al., 1995, Novick et al., 1995, Novick, 2006, Novick et al., 1993). AgrB is involved in the processing of the propeptide AgrD, secretion of the AIP signal, and conversion of the AIP to its functionally active structure. AgrC is a phosphorylated histidine protein kinase, while AgrA is a response regulator in the cell. AIP is produced during the log phase of growth, and the AIP becomes more concentrated in a manner that is directly proportional to cell density, which triggers activation of the Agr system (Yarwood and Schlievert, 2003, Ji et al., 1995, Novick et al., 1995, Novick, 2006, Novick et al., 1993) (Fig 1.16 and 1.17).
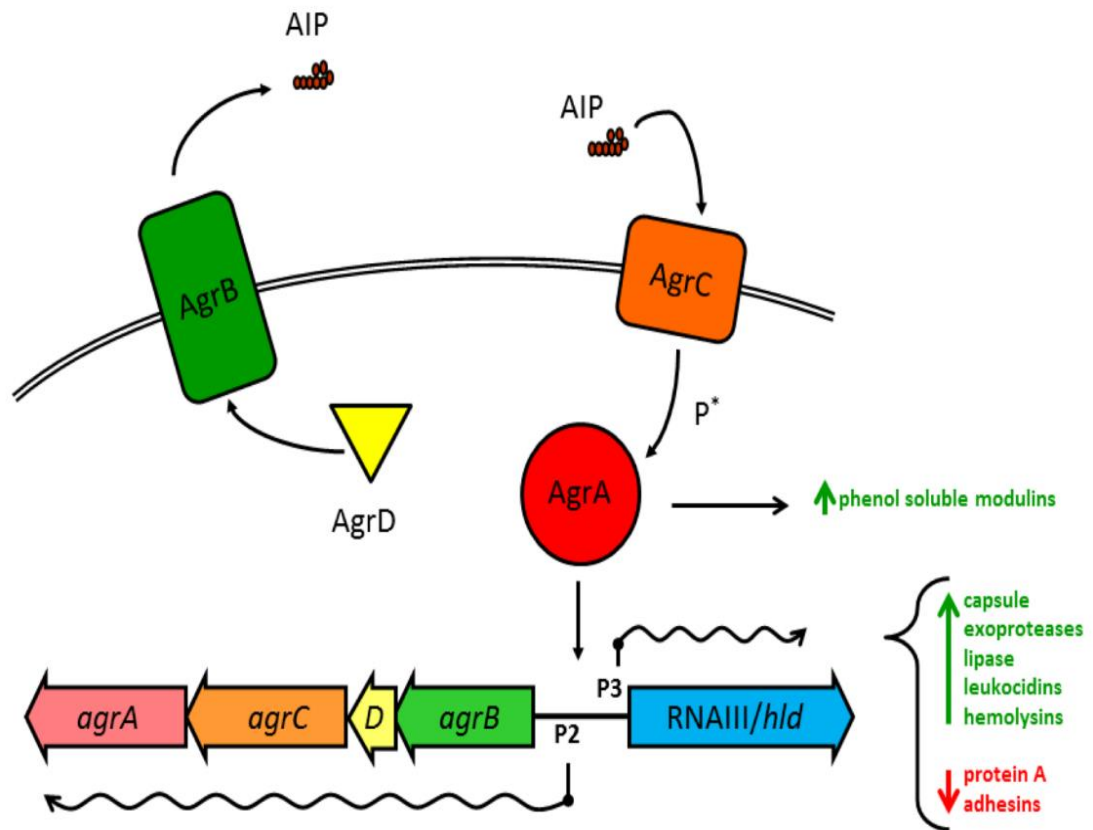
Fig. 1.16: Regulation of *agr* operon. AgrB is a multifunctional endopeptidase and chaperone protein that links to AIP. AgrD is a propeptide that AgrB converts to AIP, a tiny thiolactone. AgrC is a membrane sensor that is part of a two-part regulatory system. AgrA is a transcription factor response regulator that works in tandem with AgrC to upregulate *agr* genes and RNAIII expression, as well as several other transcriptional targets, by acting on the divergent P2/P3 promoter. The regulatory RNA molecule RNAIII modulates gene expression through post-transcriptional regulation by acting on a variety of gene transcripts. (Gray et al., 2013).
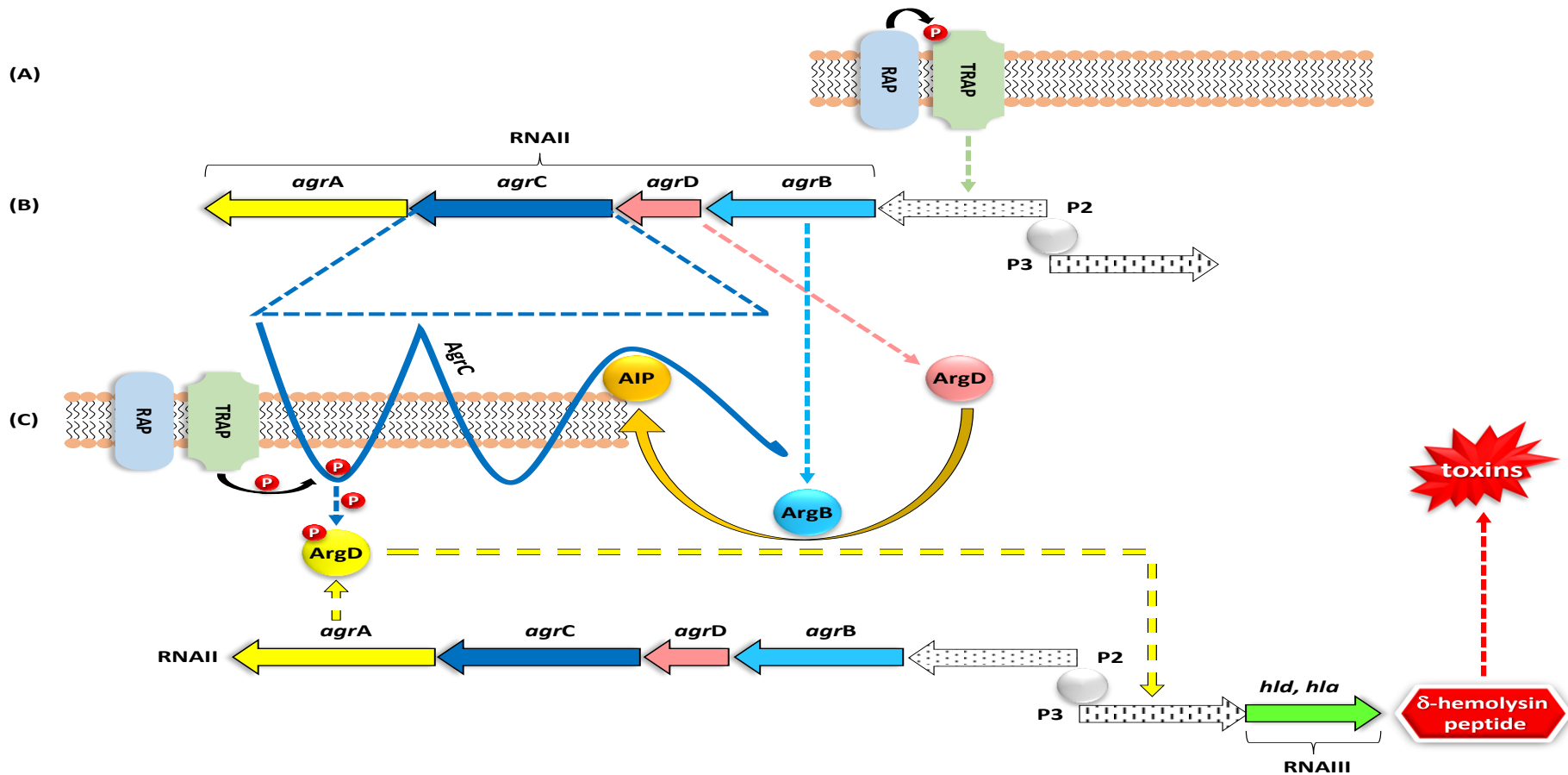
Fig. 1.17: The role of Agr activity in *S. aureus*. Interaction of RAP (autoinducer)-dependent phosphorylated TRAP and AgrC (A), to produce RNAII and phosphorylate AgrC by the action of AIP (B), that consequently phosphorylate AgrA (C) that acts as a transcription activator of P3 leading to the production of RNAIII. RNAIII causes the expression of toxic exomolecules resulting in dissemination, and toxin production (eg. Hla) and in disease. Drawn from (Balaban et al., 2001).

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1.8.2 *agr*-mediated regulation of virulence factors in MRSA

Agr is a significant virulence regulator in staphylococci, where the QS system is predominantly involved in invasiveness, and its principal target genes include surface and secreted virulence factors (Yarwood and Schlievert, 2003, Archer et al., 2011). Agr is thought to modulate the host immunological response by upregulating secreted virulence factors (Fig. 1.18) and downregulating cell surface proteins in *S. aureus* (Yarwood and Schlievert, 2003, Mack et al., 2007, Dassy et al., 1993, Luong et al., 2002, van Wamel et al., 2002, Vuong et al., 2000, Batzilla et al., 2006, Vuong et al., 2004).
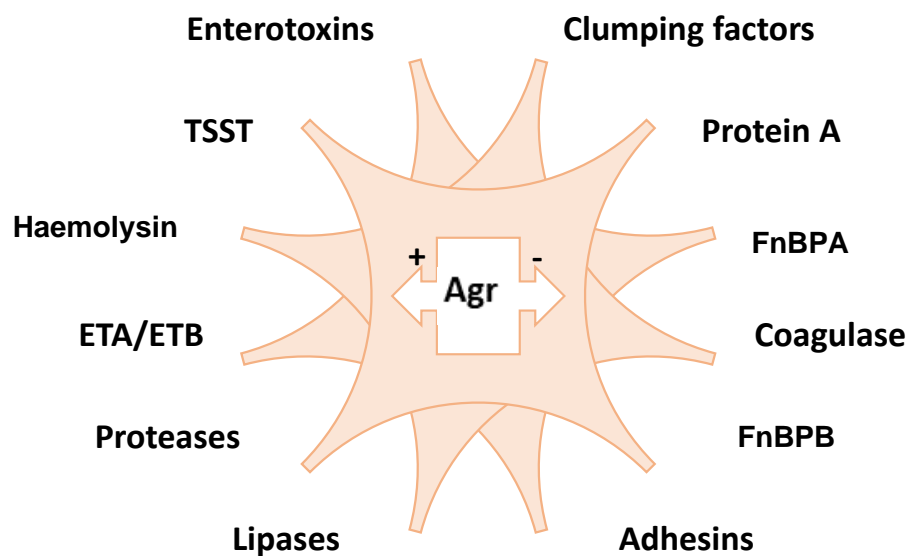
Fig. 1.18: Schematic representation of *agr*-regulated virulence factors of *staphylococcus aureus.* (Williams et al., 2002, Piroth et al., 2008), (Xu and Mosher, 2011) (McCourt et al., 2014); (Josse et al., 2015),(Foster, 2016).

Typically, in two-component systems (TCS), an external signal activates the membrane-associated histidine kinase by autophosphorylation, which leads to subsequent phosphorylation of the response regulator. The latter binds to a specific DNA sequence motif, which results in the alteration of target gene expression. *S. aureus* strains encode 16 different TCS (Somerville and Proctor, 2009- Villanueva et al., 2018), of which one is essential (WalKR) (White et al., 2014- Matsuo et al., 2010). TCSs such as agrAC, saeRS and arlRS participate in *S. aureus* virulence and regulate a plethora of host-impacting secreted proteins. The accessory gene regulator (agr) is considered the model system, which encodes a quorum-sensing (QS) system that represents the master virulence regulator (Recsei et al., 1986). In addition, *S. aureus*

withstands the host environment using a number of other important cytoplasmic regulators (Somerville and Proctor, 2009) such as the SarA protein family of transcriptional regulators (e.g., SarA, Rot, MgrA, etc.), and the alternative sigma factors (SigB and SigH).

1.8.3 Interaction of Agr system and environmental factors and other regulators

The agr system has a crucial role in pathogenesis. Therefore, *S. aureus* utilizes many strategies to promote the expression of *agr* genes under different environmental conditions. These agr factors sense environmental host-derived stimuli. Among the environmental factors that influence the agr system is pH, as acidic pH, which occurs due to the catabolism of glucose or glucose deprivation, inhibits agr (Regassa et al., 1992- Weinrick et al., 2004), while alkaline pH represses RNAIII transcription. Accordingly, agr system performs maximum activity when pH is near neutral (Regassa and Betley ,1992). In addition, reactive oxygen species (ROS) generated due to oxidative stress can influence agr activity and reduce the pathogenicity of the bacteria. The latter type of stress can also make AgrA incapable of binding its gene promoters due to the formation of disulfide bond (Sun et al , 2012). ROS can also indirectly regulate agr activity by altering the TCS activity of AirSR (Sun et al., 2012) and SrrAB (Yarwood et al., 2001). AirSR was proven to mediate resistance against ROS via transcriptional regulation of Staphyloxanthin production (Hall et al., 2017). *S. aureus* can also modulate its susceptibility to H2O2 by SrrAB activity, and thus can coexist with H2O2-producing oral streptococci (Oogai et al., 2016). Regarding the human immune system, neutrophils are among the fast responders to *S. aureus* infectivity as they destroy bacteria with oxidative killing mechanisms.

1.8.4 Toxin gene expression and secretion

AgrA can regulate the expression of RNAII and RNAIII and genes encoding phenol-soluble modulins (PSMs) involved in *S. aureus* virulence (Peschel and Otto, 2013 – Cheung et al., 2014 – Wang et al., 2007). AgrA can also bind to the promoters of the alpha- and beta-PSM encoding operons (Queck et al., 2008). RNAIII affects QS in *S. aureus* and induces upregulation of genes encoding several virulence factors, including alpha-toxin (Hla), cysteine proteases (ScpA, SspB), gamma-hemolysin (Hlg), and lipase (Geh) (Dunman et al., 2001- Novick et al., 1993 – Cheung et al., 2011 – Morfeldt et al., 1995). On the contrary, RNAIII induces downregulation of some surface proteins including protein A (Spa) and cell wall secretory protein (IsaA) (Dunman et al., 2001- Cheung et al., 2011 – Morfeldt et al., 1995). Among these surface proteins, protein A stands out due to its dominant role in pathogenicity in

several different types of *S. aureus* infections, including pneumonia (Bubeck et al., 2007 – Heyer et al., 2002), bloodstream infections (Date et al., 2014), and septic arthritis (Palmqvist et al., 2002). The action of RNAIII can be at the transcriptional level by modulating transcription initiation, or at the post-transcriptional level by interacting with mature transcript (Novick et al., 1993). Alpha-toxin (hla) is an example of positive regulation by RNAIII as hla forms a hairpin loop to prevent the ribosome from accessing the ribosome-binding site, while RNAIII bind to the hla mRNA to relieve the hairpin loop structure and allows the ribosome to recognize the binding site to start translation (Morfeldt et al., 1995).

Toxin production in *S. aureus* is a task that requires the regulation of large number of genes within the context of several regulatory mechanisms in the cell. These mechanisms secure balancing energy consumed for toxin production and several physiological processes in the cell (Joo et al., 2016). PSMs, for example, are produced at high levels, reaching ~60% of the total secreted protein in *S. aureus* (Chatterjee et al., 2013). This action poses large stress on the bacteria to compensate the shortage of energy required for other important physiological processes. Thus, bacteria had to put the process of toxin production under strict regulatory framework(s). This raises the necessity of controlling the accessory gene regulator (agr) quorum-sensing system to manipulate such required metabolic adaptations (Cheung et al., 2011). Thus, PSM synthesis is strictly regulated by agr mechanism by binding the AgrA response regulator to *psm* operon promoters (Queck et al. 2008). Regulation of PSM expression and secretion is a challenging task as it, not only, is important as the major offensive mechanism required for the lifetime of staphylococci, but also becomes deleterious when secreted in large amounts in the cytosol (Chatterjee et al., 2013; Peschel and Otto 2013; Cheung et al., 2014). In addition, accumulated cytosolic PSMs regulate their own export by binding to a DNA repressor, PmtR, to construct a PSM/PmtR-controlled transcriptional unit. This unit disrupts the pmt promoter-PmtR repressor complex, leading to transcription of the pmtR and exporter pmtABCD genes that enable PSM secretion (Joo et al., 2016). This three-party interaction for regulating toxin production and secretion is summarised in Fig 1.19.
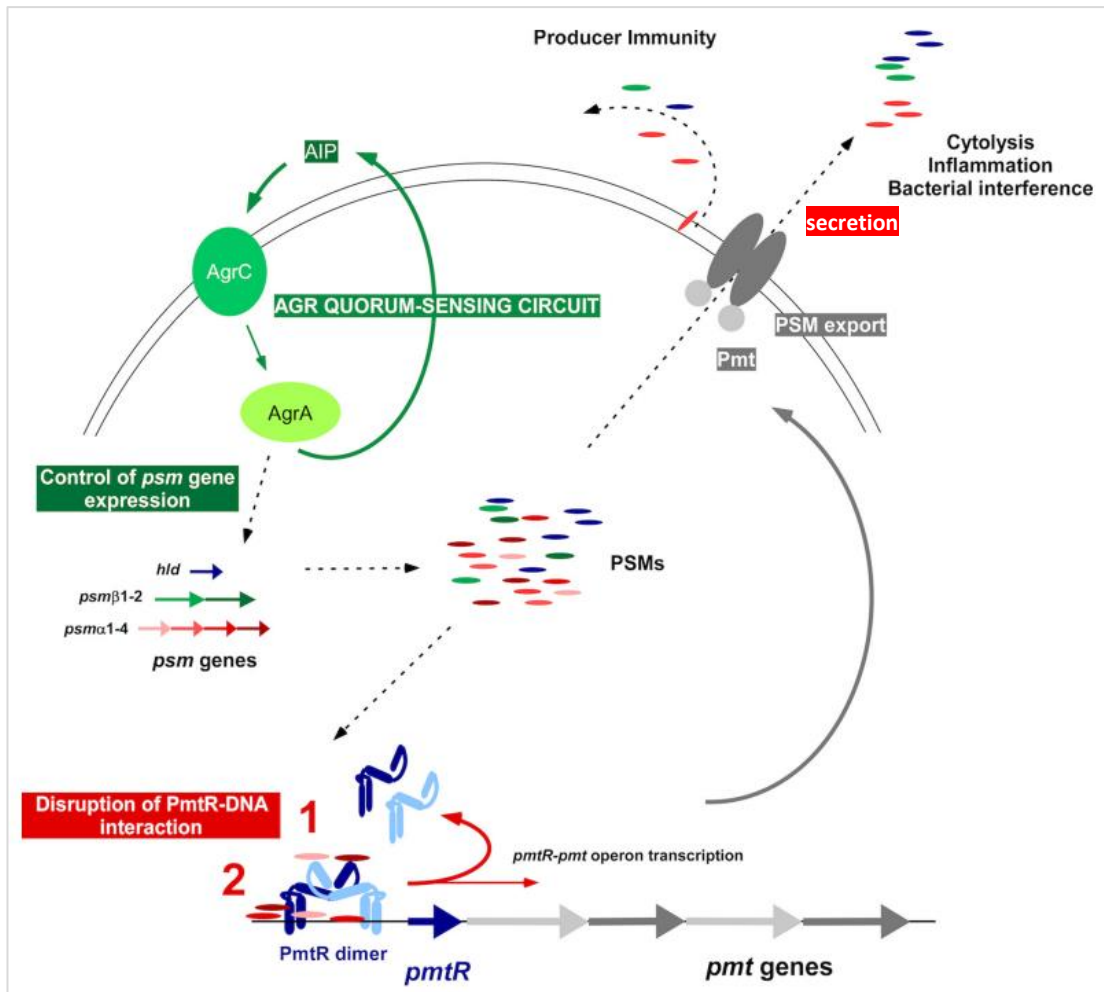
Fig. 1.19: Mechanisms of PSM regulation, interaction with pmt and export. Expression *psm* genes is under the control of the agr quorum-sensing system due to binding of AgrA to psm promoters. When PSMs are accumulated, they disrupt the pmt promoter-PmtR repressor complex, which leads to expression of *pmtR* and downstream *pmtABCD* genes and subsequent transport of PSMs. Taken after Joo et al. (2016).

1.9 Genome-wide association study (GWAS) and the prediction of the novel MspA protein

A genome-wide association study (GWAS) is a statistical test for associations between specific loci in the genome and the phenotype of interest. Given the impact these types of GWAS approaches have had on understanding the genetic basis of human disease, it has recently been applied to study bacterial traits. For example, Prof. Massey's group has adopted this approach to studying pathogenicity, where they applied GWAS using the genome sequences of 90 MRSA clinical isolates to identify new genes and proteins that affect the ability of *S. aureus* to produce cytolytic toxins (toxicity). They measured the toxicity of each of these 90 isolates, then performed statistical tests on the genome sequence and toxicity data to identify loci that are associated with changes in toxicity of the isolates. While doing this, the group found several genes that positively correlated with toxicity (Laabei et al., 2014; Recker et al., 2017).

The emergence of epidemic MRSA variants like USA300 and ST239 is due to differences in toxin or adhesin expression (Li et al., 2010, Li et al., 2012, Otto, 2010). As a result of the high incidence of the extremely toxic USA300 clone, guidelines exist that urge treating suspected infections with vancomycin and a second antibiotic such as clindamycin or linezolid to minimize toxin expression and illness severity. An early indication whether an infecting strain is highly toxic could help doctors adjust treatment techniques and raise their index of anticipation for illness consequences in infected people. To tackle this, Laabei and colleagues used GWAS and a prediction model to see if the genome sequences of the 300 *S. aureus* bacteraemia isolates could be used to predict virulence. They showed how genotypes responsible for biofilms and secreting cytolytic toxins can be utilized to infer complicated phenotypes from whole genome sequence data for collections of strains to find genetic signatures associated with a given characteristic (Laabei et al., 2014, Duggan et al., 2020).

Membrane stabilizing protein A, or MspA, is one of these toxicity associated genes, and it is predicted to be a small membrane-bound protein. The protein is the focus of the thesis, where it has been found to have an impact on a wide range of *S. aureus* activities mediated by membrane proteins. Here we show that MspA inactivation affects toxin generation, innate immune resistance, and iron homeostasis, leading to full pathogen attenuation in both a superficial and invasive infection scenario. As a result, it is a prospective therapeutic target that has to be explored further (Duggan et al., 2020, Laabei et al., 2014).

1.10 Aims of the study

- Verify the contribution of the MspA protein to the toxicity of *S. aureus.*

- Characterise the mechanism by which MspA protein affects *S. aureus* toxicity and investigate whether there is any relation between iron efflux/influx and MspA protein.

- Characterise β-lactam resistance when *mspA* gene of *S. aureus* was knocked out.

- Detect expression of PBP genes and autolytic activity when *mspA* gene of *S. aureus* was knocked out and influence of the mutation on cell wall thickness.

- Detect the bacterial response to changing conserved amino acids of *mspA* gene in *S. aureus* via alanine-scanning mutagenesis as well as to manipulating protein's domain and loop structures in terms of toxicity, defensive capabilities against human immune system via staphyloxanthin production (carotenoid biosynthesis) and resistance to oxacillin.

2. Materials and methods

2.1. Materials

A list of all the materials used in this project is found in Table 2.1

Table 2.1: Detailed description of all materials used in this study.

Material	Description	Supplier
Ethanol	Solvent	ThermoFisher Scientific
Dimethyl sulfoxide	Solvent	Sigma
Ampicillin	β-lactam antibiotic targeting bacterial cell wall	Sigma
Oxacillin sodium salt monohydrate	β-lactam antibiotic targeting bacterial cell wall	Sigma
Oxacillin 5 µg antibiotic disc	Antibiotic disc for disc diffusion method	Sigma
Antibiotic blank disc	Disc for disc diffusion method	Sigma
Erythromycin	Macrolide antibiotic to inhibit the bacterial protein synthesis	Sigma
Chloramphenicol	Protein synthesis inhibitor antibiotic	Sigma
Anhydrous tetracycline	To induce *mspA* gene expression from in the expression PRMC2 plasmid	Sigma
Streptonigrin	Antibiotic that breaks DNA strands in the presence of iron	Insight Biotechnology
Hemin	Used as a source of iron	MedChem Express
RPMI 1640	Roswell Park Memorial Institute cell culture medium	Sigma
L-glutamine penicillin streptomycin solution	Used for cell culture as a supplement and antibiotic	Sigma
Fetal bovine serum	Used as a supplement for cell culture	Gibco
PBS	Phosphate buffered saline	ThermoFisher Scientific
Acetone	Solvent	Sigma
Agarose	DNA / RNA gels for electrophoresis	Sigma
APS	Ammonium persulfate	Sigma
TEMED	Tetramethyl ethylamine	Sigma
Bromophenol blue	Protein loading dye	Sigma
β-mercaptoethanol	Used to prepare the sample for SDS-PAGE gel	Sigma
Cell freezing media	To store THP-1 cells	Sigma

0.2 cm electroporation cuvette	Used for electroporation of competent bacterial cells	Invitrogen
SYBR-Safe	DNA gel stain	ThermoFisher Scientific
Phusion master mix	High-Fidelity PCR Master Mix with HF Buffer	ThermoFisher Scientific
qScript® cDNA SuperMix	Used to generat cDNA	Quantabio
Quick-RNA™Fungal/Bacterial Miniprep Kit	RNA extraction kit	Zymo Research
Isopropanol	Solvent	ThermoFisher Scientific
Lysostaphin	Glycyl-glycine endopeptidase	MeRck
Skimmed milk	Blocking reagent	Sigma
Lysozyme	Used for DNA extraction	Sigma
MgSO4	Magnesium chloride	Sigma
NaCl	Sodium chloride	Sigma
SDS	Sodium dodecyl sulfate	ThermoFisher Scientific
Sucrose	Used to prepare competent cell	Sigma
TAE	Tris-acetate EDTA buffer	ThermoFisher Scientific
TBE	Tris borate EDTA buffer	ThermoFisher Scientific
TCA	Trichloroacetic acid	ThermoFisher Scientific
Gene ruler	1kb or 50bp DNA ladder	ThermoFisher Scientific
GeneJet Gel extraction kit	Used to extract the DNA from agarose gel	ThermoFisher Scientific
GeneJet PCR purification kit	Used to purify PCR product	ThermoFisher Scientific
GeneJet Plasmid purification kit	Used for plasmid purification	ThermoFisher Scientific
Glycerol	Used to prepare bacterial freezer stocks	ThermoFisher Scientific
BHI	Brain heart infusion broth	Oxoid
6X DNA loading dye	Used to dye samples for gel electrophoresis	ThermoFisher Scientific
KpnI	Restriction enzyme	New England Biolabs
SacI	Restriction enzyme	New England Biolabs
DpnI	Restriction enzyme	Promega
FastRead counting chamber	Cell counting slides	Kova International
GoTaq 2X green master mix	PCR master mix	Promega
LB	Luria Bertani broth and agr	ThermoFisher Scientific
Virkon	Disinfectant	ThermoFisher Scientific
TURBO™ DNase	DNAse digestion kit	Invitrogen

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Triton X-100	Detergent	Sigma
Trypan blue	Cell stain	Sigma
TSA	Tryptic soy agar	Oxoid
TSB	Tryptic soy broth	Oxoid
Tween-20	Detergent	Sigma
Rabbit anti-*hla*	Antiserum against α-haemolysin	Sigma
Bocillin™FL penicillin,sodium salt	Fluorescent version of penicillin	ThermoFisher Scientific
DEPC treated water	DNase- and RNase-free water	ThermoFisher Scientific
Opti-4CN substrate kit	Used to visualized protein sample	Bio-Rad
Page Ruler™ Plus Pre-stained	Protein ladder	ThermoFisher Scientific
96-well plate	Used for measuring turbidity	Costar/Sigma
96 well plate, black/clear bottom.	Used for fluorescence assays	Appleton Woods Ltd
Trans-Blot Turbo	Mini Nitrocellulose Transfer Packs	Bio-Rad
Urea	Used to resuspend protein extraction	Sigma
T-25	Tissue culture flask	Corning
T-75	Tissue culture flask	Corning
SimplyBlue SafeStain	SDS-PAGE gel stain	ThermoFisher Scientific
SOC medium	Super Optimal broth with Catabolite repression	ThermoFisher Scientific
Mueller Hinton agar cation adjusted	Used for antibiotic disc assay	ThermoFisher Scientific
2.5% glutaraldehyde in cacodylate buffer (0.1M)	Fixative buffer used to prepare my samples for Transmission Electron Microscopy (TEM)	TEM unit
RNAprotect	To stabilize RNA in the cell	Qiagen
KAPA SYBR mix	Used for highest performance in real-time PCR	Roche
Nitrocefin	For detecting the presence of β-lactamase enzyme	Oxoid
Methanol	Solvent	Sigma
T4 DNA ligase	Used for ligation	Bio Labs

2.2. Bacterial strains

The transposon mutants used in this study were obtained from the Nebraska Transposon Mutant Library (NTML), which has a collection of 1,952 *Staphylococcus aureus* transposon mutants in strain USA300 (JE2 background). We obtained certain JE2 *mspA::tn* mutants from the NTML in the chromosome region between SAUSA300_2212 and SAUSA300_2213 (Diep et al., 2006; Duggan et al., 2020). The USA300 JE2 wild type was used as a control in all experiments of this study (Bae et al., 2008; Fey et al., 2013). All strains and mutants used in this study are listed in Table 2.2.

Table 2.2: *Staphylococcus aureus* strains used in this study.

Strain	Description	Reference
JE2	USA300; community acquired-MRSA, lacking plasmids p01 and p03, type IV SCC*mec,* wild type strain	Fey et al., 2013
JE2 *mspA*::*tn*	*mspA* transposon mutant of JE2	Duggan et al., 2020
JE2 *mspA*::*tn* (p*mspA*)	*mspA* transposon mutant of JE2 that has been complemented with the gene in pRMC2 expression plasmid	Duggan et al., 2020
SH1000 *mspA*::*tn* (p*mspA*)	*mspA* transposon mutant of SH1000 that has been complemented with the gene in pRMC2 expression plasmid	This study
SH1000	Wild-type strain methicillin-sensitive *Staphylococcus aureus* (MSSA) Laboratory strain 8325-4 with a repaired *rsbU* gene;SigB positive	Horsburgh et al., 2002
SH1000 *mspA*::*tn*	*mspA* mutant of SH1000	This study
JE2 *fur::tn* SAUSA300_1514	*fur* mutant of JE2	Fey et al., 2013
JE2 *hrtB::tn* SAUSA300_2307	*hrtB* mutant of JE2	Fey et al., 2013
JE2 *hrtA::tn* SAUSA300_2306	*hrtA*	Fey et al., 2013
JE2 NE42 SAUSA300_2212	Conserved hypothetical protein	Fey et al., 2013
JE2 NE627 SAUSA300_2211	Putative membrane protein	Fey et al., 2013
JE2 NE866 SAUSA300_2213	AcrB/AcrD/AcrF family protein which functions as transmembrane transporter	Fey et al., 2013
SH1000 NE42 SAUSA300_2212	NE42 conserved hypothetical protein transposon mutant in SH1000 via φ11	This study
SH1000 NE627 SAUSA300_2211	NE627 putative membrane protein transposon mutant in SH1000 via φ11	This study

SH1000 NE866 SAUSA300_2213	NE866 transmembrane transporter protein transposon mutant in SH1000 via φ11	This study
JE2 *atl::tn* SAUSA300_0955	Autolysis deficient strain	Fey et al., 2013
JE2 RNAIII:*gfp*	JE2 transformed with a GFP-tagged RNAIII	This study
JE2 *crtM::tn* SAUSA300_2499	Dehydrosqualene desaturase mutant in JE2	Fey et al., 2013
JE2 *floA::tn* SAUSA300_1533	Flotillin mutant in JE2	Fey et al., 2013
S. aureus RN4220	*S. aureus* lacking restriction enzymes	This study
E. coli DH5α	Competent cell	This study
E. coli MachI	Competent cell	This study
E. coli DH5α (p*mspA*)	(pRMC2 *mspA*) in *E. coli* DH5α strain	This study
JE2 *mspA::tn* pRMC2-*mspA*(MspA-L31)	*mspA* transposon mutant of JE2 that has been complemented with the gene in pRMC2 expression plasmid and had substituted amino acid number 31 in MspA protein which is Leucine (L) with Alanine (A)	This study
JE2 *mspA::tn* pRMC2-*mspA*(MspA-R32)	*mspA* transposon mutant of JE2 that has been complemented with the gene in pRMC2 expression plasmid and had substituted amino acid number 32 in MspA protein which is Arginine (R) with Alanine (A)	This study
JE2 *mspA::tn* pRMC2-*mspA*(MspA-I34)	*mspA* transposon mutant of JE2 that has been complemented with the gene in pRMC2 expression plasmid and had substituted amino acid number 34 in MspA protein which is Isoleucine (I) with Alanine (A)	This study
JE2 *mspA::tn* pRMC2-*mspA*(MspA-M35)	*mspA* transposon mutant of JE2 that has been complemented with the gene in pRMC2 expression plasmid and had substituted amino acid number 35 in MspA protein which is Methionine (M) with Alanine (A)	This study

JE2 *mspA::tn* pRMC2-*mspA*(MspA-L38)	*mspA* transposon mutant of JE2 that has been complemented with the gene in pRMC2 expression plasmid and had substituted amino acid number 38 in MspA protein which is Leucine (L) with Alanine (A)	This study
JE2 *mspA::tn* pRMC2-*mspA*(MspA-L39)	*mspA* transposon mutant of JE2 that has been complemented with the gene in pRMC2 expression plasmid and had substituted amino acid number 39 in MspA protein which is Leucine (L) with Alanine (A)	This study
JE2 *mspA::tn* pRMC2-*mspA*(MspA-V42)	*mspA* transposon mutant of JE2 that has been complemented with the gene in pRMC2 expression plasmid and had substituted amino acid number 42 in MspA protein which is Valine (V) with Alanine (A)	This study
JE2 *mspA::tn* pRMC2-*mspA*(MspA-P51)	*mspA* transposon mutant of JE2 that has been complemented with the gene in pRMC2 expression plasmid and had substituted amino acid number 51 in MspA protein which is Proline (P) with Alanine (A)	This study
JE2 *mspA::tn* pRMC2-*mspA*(MspA-W55)	*mspA* transposon mutant of JE2 that has been complemented with the gene in pRMC2 expression plasmid and had substituted amino acid number 55 in MspA protein which is Tryptophan (W) with Alanine (A)	This study
JE2 *mspA::tn* pRMC2-*mspA*(MspA-W56)	*mspA* transposon mutant of JE2 that has been complemented with the gene in pRMC2 expression plasmid and had substituted amino acid number 56 in MspA protein which is Tryptophan (W) with Alanine (A)	This study
JE2 *mspA::tn* pRMC2-*mspA*(MspA-V57)	*mspA* transposon mutant of JE2 that has been complemented with the gene in pRMC2 expression plasmid and had substituted amino acid number 57 in MspA protein which is Valine (V) with Alanine (A)	This study
JE2 *mspA::tn* pRMC2-*mspA*(MspA-F58)	*mspA* transposon mutant of JE2 that has been complemented with the gene in pRMC2 expression plasmid and had substituted amino acid number 58 in MspA protein which is Phenylalanine (F) with Alanine (A)	This study
JE2 *mspA::tn* pRMC2-*mspA*(MspA-V60)	*mspA* transposon mutant of JE2 that has been complemented with the gene in pRMC2 expression plasmid	This study

	and had substituted amino acid number 60 in MspA protein which is Valine (V) with Alanine (A)	
JE2 *mspA::tn* pRMC2-*mspA*(MspA-L61)	*mspA* transposon mutant of JE2 that has been complemented with the gene in pRMC2 expression plasmid and had substituted amino acid number 61 in MspA protein which is Leucine (L) with Alanine (A)	This study
JE2 *mspA::tn* pRMC2-*mspA*(MspA-L62)	*mspA* transposon mutant of JE2 that has been complemented with the gene in pRMC2 expression plasmid and had substituted amino acid number 62 in MspA protein which is Leucine (L) with Alanine (A)	This study
JE2 *mspA::tn* pRMC2-*mspA*(MspA-63)	*mspA* transposon mutant of JE2 that has been complemented with the gene in pRMC2 expression plasmid and had substituted amino acid number 63 in MspA protein which is Leucine (L) with Alanine (A)	This study
JE2 *mspA::tn* pRMC2-*mspA*(MspA-V68)	*mspA* transposon mutant of JE2 that has been complemented with the gene in pRMC2 expression plasmid and had substituted amino acid number 68 in MspA protein which is Valine (V) with Alanine (A)	This study
JE2 *mspA::tn* pRMC2-*mspA*(MspA-E69)	*mspA* transposon mutant of JE2 that has been complemented with the gene in pRMC2 expression plasmid and had substituted amino acid number 69 in MspA protein which is Glutamic acid (E) with Alanine (A)	This study
JE2 *mspA::tn* pRMC2-*mspA*(MspA-T71)	*mspA* transposon mutant of JE2 that has been complemented with the gene in pRMC2 expression plasmid and had substituted amino acid number 71 in MspA protein which is Threonine (T) with Alanine (A)	This study
JE2 *mspA::tn* pRMC2-*mspA*(MspA-F73)	*mspA* transposon mutant of JE2 that has been complemented with the gene in pRMC2 expression plasmid and had substituted amino acid number 73 in MspA protein which is Phenylalanine (F) with Alanine (A)	This study
JE2 *mspA::tn* pRMC2-*mspA*(MspA-K74)	*mspA* transposon mutant of JE2 that has been complemented with the gene in pRMC2 expression plasmid and had substituted amino acid number 74 in MspA protein which is Lysine (K) with Alanine (A)	This study

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JE2 *mspA::tn* pRMC2-*mspA*(MspA-K77)	*mspA* transposon mutant of JE2 that has been complemented with the gene in pRMC2 expression plasmid and had substituted amino acid number 77 in MspA protein which is Lysine (K) with Alanine (A)	This study
JE2 *mspA::tn* pRMC2-*mspA*(MspA-D79)	*mspA* transposon mutant of JE2 that has been complemented with the gene in pRMC2 expression plasmid and had substituted amino acid number 79 in MspA protein which is Aspartic acid (D) with Alanine (A)	This study
JE2 *mspA::tn* pRMC2-*mspA*(MspA-K81)	*mspA* transposon mutant of JE2 that has been complemented with the gene in pRMC2 expression plasmid and had substituted amino acid number 81 in MspA protein which is Lysine (K) with Alanine (A)	This study
JE2 *mspA::tn* pRMC2-*mspA*(MspA-L86)	*mspA* transposon mutant of JE2 that has been complemented with the gene in pRMC2 expression plasmid and had substituted amino acid number 86 in MspA protein which is Leucine (L) with Alanine (A)	This study
JE2 *mspA::tn* pRMC2-*mspA*(MspA-N87)	*mspA* transposon mutant of JE2 that has been complemented with the gene in pRMC2 expression plasmid and had substituted amino acid number 87 in MspA protein which is Asparagine (N) with Alanine (A)	This study
JE2 *mspA::tn* pRMC2-*mspA*(MspA-Domain 1)	*mspA* transposon mutant of JE2 that has been complemented with the gene in pRMC2 expression plasmid with domain number 1 removed	This study
JE2 *mspA::tn* pRMC2-*mspA*(MspA-Domain 2)	*mspA* transposon mutant of JE2 that has been complemented with the gene in pRMC2 expression plasmid withdomain number 2 removed	This study
JE2 *mspA::tn* pRMC2-*mspA*(MspA-Domain 3)	*mspA* transposon mutant of JE2 that has been complemented with the gene in pRMC2 expression plasmid with domain number 3 removed	This study
JE2 *mspA::tn* pRMC2-*mspA*(MspA-Domain 4)	*mspA* transposon mutant of JE2 that has been complemented with the gene in pRMC2 expression plasmid with domain number 4 removed	This study

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JE2 *mspA::tn* pRMC2-*mspA*(MspA-Loop1sG)	*mspA* transposon mutant of JE2 that has been complemented with the gene in pRMC2 expression plasmid and had substituted the amino acids in loop number 1 with Glycine	This study
JE2 *mspA::tn* pRMC2-*mspA*(MspA-Loop3sloop1)	*mspA* transposon mutant of JE2 that has been complemented with the gene in pRMC2 expression plasmid and had substituted the amino acid in loop number 3 with the same amino acid in loop number 1	This study

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2.3. Experiments conducted in this study

All experiments were performed by the Ph.D. candidate unless otherwise stated. The reason for having experiments performed commercially, or by collaborators of is because of the need for their resources and/or technical expertise. For example, sequencing was done by Eurofins in Germany, tandem mass tagging mass spectroscopy was done by the proteomic unit in the University of Bristol and generating images was undertaken by the electron microscopy unit in the University of Bristol.

2.3.1. Bacterial growth media and antibiotic concentrations

In this study, *Staphylococcus aureus* strains were routinely grown tryptic soy agar (TSA) or in tryptic soy broth (TSB). In antibiotic sensitivity tests, cation-adjusted Muller Hinton (MH) broth and agar were used. Information about the antibiotics and other materials incorporated into the media is as follows:

- Erythromycin (5 µg/ ml) was added to select for the transposon.
- The complemented strain was grown on media containing chloramphenicol (10 µg/ml).
- Tetracycline (50-200 ng/ml) was used to induce the expression of the *mspA* gene in the pRMC2 expression plasmid.
- Streptonigrin (200 µg/ml) was used as an indirect measure for intracellular iron.
- Hemin (100 µg/ml) was used as a source of iron in the media.
- Ampicillin (100 µg/ml) was used to select for *E. coli* transformants.

Freshly streaked plates were used to inoculate fresh broth and then grown for 18 h at 37°C with shaking (180 rpm). Before starting the assays, optical density (OD_{600}) was normalized to a given number of OD_{600} in each experiment.

2.3.2. SDS-PAGE gel preparation

In this study, SDS-PAGE at 10% acrylamide was performed. Resolving gel was prepared by add 1.5 ml 40% acrylamide stock to 1.5 ml separating buffer (1.5 M Tris-HCl, pH 8.8, 0.4% SDS), 3.0 ml dH_2O, 35 µl 10% ammonium persulfate (APS), and 4 µl tetramethyl ethylenediamine (TEMED). The resolving gel was left to polymerise for approximately 20 min before the stacking gel was added. The stacking gel (5% acrylamide) was prepared by adding 0.5 ml 40% acrylamide stock, 1.0 ml stacking buffer, 2.5 ml dH_2O, 35 µl 10% APS, and 4 µl TEMED. The protein samples were loaded after mixing with loading dye (bromophenol blue), and the pre-stained protein

ladder (Page Ruler™ Plus) was used as a standard. Finally, the gel was run in 1x running buffer (0.025 mM Tris, 0.192 mM glycine, 0.01% SDS) at 100 V for 90 min.

2.3.3. Tissue culture

THP-1 cells were used in this study. These cells represent a monocyte-like cell line derived from a leukaemia patient (Bosshart & Heinzelmann, 2016). THP-1 cells are sensitive to many toxins produced by *Staphylococcus aureus* cells (Laabei et al., 2014). These cells were sub-cultured for two to four days in Roswell Park Memorial Institute medium (RPMI 1640). RPMI was supplemented with fetal bovine serum (FBS 10%) and a solution of L-glutamine at 200 mM, penicillin (10,000 units) and streptomycin at 10 mg/ml (Sigma).

2.3.4. Competent cell preparations

2.3.4.1. *E. coli* competent cells

Overnight bacterial cultures diluted by adding 1 ml into 100 ml LB in a 500-ml flask. The diluted culture was grown at 37°C with shaking (180 rpm) until reaching an OD_{600} of 0.4. The cells were divided into two 50-ml falcon tubes and left on ice for 20 min. The cells then harvested by centrifugation at 4100 rpm for 10 min. The pellet was resuspended in 25 ml cold 100 mM $CaCl_2$. Again, the cells were harvested by centrifugation at 4100 rpm for 10 min. Finally, cells were resuspended in 10 ml cold 100 mM $CaCl_2$ with 10% glycerol, and aliquoted in Eppendorf tubes to be incubated overnight at 4°C. Next day, the cells were vortexed and stored at -80°C.

2.3.4.2. *Staphylococcus aureus* competent cells

Overnight cultures were grown 37°C with shaking (180 rpm). Cells were transferred into a 2 L flask containing 200 ml TSB. The cells were grown again to mid-log phage (OD_{600} = 0.5). Then, they were transferred into a falcon tube and collected by centrifugation at 5000 *xg* for 15 min. The supernatant was discarded, and the cell pellet was suspended in 40 ml of ice-cold sterile 0.5 M sucrose dissolved in deionized water. The cell suspension was transferred to a pre-chilled 50 ml sterile centrifuge tube and kept on ice. The cells were collected by centrifugation at 8000 *xg* for 10 min at 4°C. Then, again supernatant was discarded, and the cell pellet was suspended in 20 ml of the ice-cold 0.5 M sucrose. Then, cells were collected by centrifugation at 8,000 *xg* for 10 min at 4°C. The last two final steps were repeated twice, before the cells were transferred, in 100 µl aliquots of the prepared electrocompetent cells, into microcentrifuge tubes chilled on ice to be stored at -80°C.

2.3.5. Preparation of whole cell lysate

Overnight cultures were grown in 37°C with shaking at 180 rpm. The cells were harvested by centrifugation at 4000 rpm for 10 min. The pellet was washed in 1 ml PBS and 10 µl lysostaphin (10 mg/ml) was added to the cells that were shaken at 180 rpm for 1 h at 37°C. Finally, 110 µl SDS (20%) was added to reach a final concentration of 2% and the samples were incubated at 98°C for 10 min.

2.3.6. Toxicity assay

To determine the toxicity of *Staphylococcus aureus*, the effect of bacterial supernatants on THP-1 cells was investigated. In this assay, *S. aureus* strains were grown in 5 ml TSB for 18 h at 37°C, where erythromycin (5 µg/ml) was added to select the transposon mutants, chloramphenicol (10 µg/ml) was used to maintain the pRMC2 plasmid, and tetracycline (50-200 ng/ml) was used to induce the expression of the *mspA* gene in the knockout mutant. First, 1 ml from the bacterial cultures was taken and centrifuged for 1 min at 10,000 rpm and the supernatant was harvested. Then, the supernatant was diluted to 30% in fresh TSB for the SH1000 strain, or to 10% for the JE2 strain. Then, the THP-1 cells were harvested by centrifugation and washed by resuspending the pellet in phosphate buffered saline (PBS) and adjusted to a working cell density of 100-150 cells per 1 µl. Finally, to determine the toxicity, 15 µl bacterial diluted supernatant was added to 15 µl of washed THP-1 cells and incubated for 12 min at 37°C. Then, the samples were stained with 15 µl trypan blue (which was excluded from live cells) and the number of dead cells was counted in a haemocytometer slide (Laabei et al., 2014).

2.3.8. Genetic manipulation and transduction

We have complemented the *mspA* knocked out mutation pRMC2 plasmid harbouring the JE2 *mspA* wild type gene, while transduction was SH1000 background. In our previous work (e.g., Duggan et al., 2020), the *mspA* gene was amplified by PCR from JE2 using Phusion high-fidelity DNA polymerase and the following forward and reverse primers:

Primer name	Primer sequence
MspAFW	CGGGTACCGAACCCTTTGAAACG
MspARV	GCGAGCTCGTTGCAATTATGTTATTGC

Complementation of the *mspA* gene in the mutant with *mspA* gene knocked out was attempted using the tetracycline-inducible plasmid vector pRMC2, as it contains an inducible promoter to control the transcription of the complemented gene inserted in restriction sites *KpnI* and *SacI* of the plasmid. The recombinant plasmid was electroporated into *Staphylococcus aureus* RN4220 and subsequently into JE2. In addition, DNA from JE2*mspA*::Tn mutant was transduced into wild-type SH1000 by phage lysate Φ11. Transposon insertion of *mspA* gene in SH1000*mspA*::Tn was verified by PCR using the above *mspA* primers. Another transduction was performed using phage lysate (ϕ11) to move the transposon from the JE2 strain to lines NE42, NE627, and NE866 in SH1000 strain background. The latter lines were recovered from the NTML with transposons in the genes neighbouring *mspA*.

Overnight cultures were grown with shaking at 37ºC for 18 h. Next day, the cultures were diluted to 1:100 in TSB (12.5 ml) and incubated for 1 h at 37°C with shaking. Then, the samples were centrifuged at 4,500 *xg* for 10 min, and the pellet was resuspended in 250 µl TSB. Then, bacteriophage and 20 µl of CaCl$_2$ (10 mg/ml) were added and the culture was incubated at room temperature for 10 min, then at 30°C in a static incubator for 35 min. Then, 2.5 ml TSB was added, and the mixture was centrifuged at 4,500 *xg* for 10 min. The pellet was resuspended in 5 ml TSB, and 125 µl 0.2 M sodium citrate was added and the mixture was incubated for 90 min at 37°C with shaking. Finally, the samples were centrifuged at 4,500 *xg* for 10 min and the pellet was resuspended in 500 µl TSB. Then, 100 µl from each sample was plated on TSA and incubated at 37°C for 24-48 h (Krausz, 2016).

To confirm the transductions, two methods were performed. First, the samples were grown on erythromycin and separately on oxacillin as the transductants have an inserted cassette containing an erythromycin resistance gene, which we used to select for knockout transformants. Oxacillin sensitivity was used to ensure that none of the original mutants within the JE2 background survived during the phage lysate process. Second, PCR was performed, and the recovered linear band was visualized by doing 1.5% agarose gel electrophoresis followed by SYBR-safe staining. The DNA samples were isolated by using a DNA extraction kit (High-Pure PCR template preparation kit) following the manufacturer's instructions.

2.3.8.1. PCR to detect transposon

PCR (25 µl volume) included 12.5 µl GoTaq MasterMix, 9.5 µl PCR grade water, 1 µl template DNA, 10 mM (2 µl) from each of the following primer sequences. To confirm the mutated strains, we used the Buster and/or Upstream primers which are

the primers made by NTML to verify occurrence, orientation and location of the inserted transposon in the bacterial chromosome.

Primer name	Primer sequence
Buster RV	GCTTTTTCTAAATGTTTTTTAAGTAAATCAAGTAC
Upstream FW	CTCGATTCTATTAACAAGGG
NE42 RV	TTAATCTTGCATTTGAGCACGAACG
NE627 FW	ATGATTATTTTATTAATTGCGCTTGGTTAC
NE866 FW	GTGATAAAAAGCTATTACAATTTTCTTTAG

NTML designed these primers to use one in conjunction with either the forward (FW) or reverse (RV) primer of the gene of interest in the three mutants NE42, NE627 and NE866. The Use of Buster or Upstream primer is dependent on the mutant orientation. If the transposon was inserted on the plus orientation, so we will use the Upstream primer, but if it was inserted on the minus orientation, we will use the Buster primer. In the case of NE627 and NE866 lines, Buster primer was used as the reverse primer, while forward primers were generated from these two genes of interest. In the case of the NE42 mutant, the Upstream primer was used as a forward primer, while reverse primer was generated from the third gene of interest. The PCR reactions started by heating to 95°C for 2 min. After that, reactions were subjected to a further 35 cycles of 95°C for 50 sec, 55°C for 30 sec and 72°C for 1 min, with a final 5 min extension step at 72°C.

2.3.9. Phenol-soluble modulins (PSMs) extractions

Overnight cultures were grown at 37°C with shaking at 180 rpm for 18 h. The cultures were centrifuged for 10 min at 3000 rpm, and the supernatants were moved to a new tube. Then, trichloroacetic acid was added to a final concentration of 20% and the samples were put on ice for 1 h, then, centrifuged for 10 min at 3000 rpm. The pellets, which contain the PSMs, were washed three times in ice-cold acetone. Finally, the pellets were resuspended with 100 µl urea (8 M) and run on SDS-PAGE (10%) for 90 min at 100V. The bands were visualised after staining the gel overnight with GelCode™ Blue-Safe Protein stain (Stevens et al., 2017).

2.3.10. Proteomic analysis

Staphylococcus aureus proteins (whole cell lysates) were harvested prior to analysis by tandem mass tagging mass spectroscopy. Proteins were extracted from triplicate cultures of the wild-type strain JE2 and its isogenic *mspA* knockout mutant,

and from an *agrB* mutant in JE2 background used as a control to avoid any overlap between the *mspA* and *agrB* phenotypes. To extract the proteins, *S. aureus* strains were grown overnight for 18 h in 2 ml TSB, then, the strains were centrifuged for 1 min at 13000 rpm, and the pellets were resuspended in 200 µl protein extraction buffer (2.5 ml Tris, 1.5 ml NaCl, 250 µl MgCl2, 45.75 ml distilled water). To lyse the cells, 5 µl lysostaphin, 5 µl lysozyme, 2 µl DNase 2 µl RNase were added to the pellet and incubated at 37ºC for 30 min. Un-lysed cells and insoluble debris were pelleted by centrifugation for 1 min at 13000 rpm, and the supernatant containing the extracted proteins was extracted and quantified by using a NanoDrop. The concentration of total protein samples was adjusted to 2 mg/ml and separated on SDS-PAGE gel to confirm good quality. Then, all samples were brought to Dr. Kate Heasom in the Bristol Proteomics Suite for quantification of different proteins in each sample.

2.3.11. RNAIII activity assay

This assay was performed to determine the level of activity of the toxin regulating the Agr quorum sensing system in the JE2*mspA::tn* mutant compared to the JE2 wild-type. The wild-type and the mutant were transformed with a plasmid containing RNAIII promoter fused to GFP (green fluorescence protein): JE2 RNAIII:*gfp*. A single colony was incubated overnight in TSB at 37°C with shaking at 180 rpm. The samples were diluted with TSB 1:1 into a black fluorescence 96-well plate and the readings were taken at a range of excitation of 470 nm: emission of 515 nm) to detect GFP.

2.3.12. Hla Western blotting

In this assay, we used the cell lysate (described previously) and the supernatant. The protein concentration was calculated by using the NanoDrop and normalised across comparative samples to 2 mg/ml. An amount of 10 µl of the sample was mixed with 10 µl loading dye (2x) and boiled for 10 min at 100ºC. Then, 15 µl of the sample and protein ladder were loaded to SDS-PAGE (10%) and run at 150 V for 60 min. Then, separated proteins were transferred onto nitrocellulose blotting membrane by using a Trans-Blot Turbo (BIO-RAD). Then, the membrane was washed using PBS + Tween 20 and incubated overnight with blocking buffer (Skimmed milk + PBS and Tween 20). Then, primary antibody (rabbit anti-*hla*, 1:2000 dilution, Sigma) was added to the membrane that was incubated for 40 min and washed 3 times with PBS + Tween 20. Each incubation time was 5 min. Then, secondary antibody was added (anti-rabbit IgG, 1:1000 dilution, Invitrogen) and incubated again for 40 min and

washed 3 times as for the previous step. Then, the blot was visualized by using an Opti-4CN detection kit.

2.3.13. Streptonigrin resistance assay

Streptonigrin is an antibiotic that is used as an indirect measure for intracellular iron in bacteria (Dyer et al., 1987). It works also as an anti-tumor drug that causes RNA damage and breaks DNA strands in the presence of iron. As such, quantifying the level of sensitivity of a bacterium to this antibiotic can be used as an indication of the relative amount of iron present in the bacterial cytosol. The interaction between streptonigrin hydroquinone and O_2 in the presence of iron results in the generation of O_2 radicals, which causes damage to DNA or RNA (Bolzán & Bianchi, 2001). Two methods were used to determine the relative streptonigrin sensitivity of the *mspA* mutant as described below.

2.3.13.1. Disk diffusion test

The antibiotic was suspended in methanol. *Staphylococcus aureus* strains were grown overnight for 18 h at 37ºC. Then, suspensions were diluted 1:10 with TSB and incubated for 1 h at 37ºC. Once an optical density of OD_{600} 0.1-0.2 was reached, 200 µl of the culture was spread onto the surface of a Muller-Hinton agar plate. Sterile filter paper discs soaked in 40 µl streptonigrin (200 µg/ml) were placed in the middle of the plate and incubated for 18 h at 37ºC. Then, the diameter of the inhibition zone was recorded.

2.3.13.2. Serial dilution test

Staphylococcus aureus strains were grown in TSB for 18 h at 37ºC. Overnight cultures were normalized and diluted 1:1000, and 5 µl of this was used to inoculate 9ml of TSB with different concentrations of streptonigrin from 0 to 0.586 µg/ml . Finally, in the 96-well plate the bacterial growth was quantifying OD_{600} after 8 h at 37°C using the plate reader (Spectrostar Nano, BMG labtech). .

2.3.14. Hemin adaptation assay

A single bacterial colony was inoculated into TSB containing either hemin (10 µM) or no hemin. Cultures were then incubated at 37°C for 18 h with shaking at 180 rpm. To start the assay, falcon tubes were prepared with different hemin concentrations serial dilution was prepared from (0 to 40 µM hemin). In the 96 well plate 200 µl from the previous dilution was added. After that, 50 µl from the samples with normalized OD_{600} at 0.1 were inoculated in each well with different concentrations of hemin. Finally, the 96-well plate was incubated in the plate reader

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(Spectrostar Nano, BMG labtech) to measure the OD$_{600}$ after 8 h at 37°C (Fig. 2.1) (Torres et al., 2007).

2.3.15. Hemin toxicity assay

Staphylococcus aureus wild type (JE2 and SH1000) strains were grown in TSB with different hemin concentrations (0 to 40 µM) for 18 h at 37ºC with shaking at 180 rpm. In the next day, we performed the toxicity assay and detected the level of PSM production (explained previously).

2.3.16 Carotenoid pigmentation analysis (staphyloxanthin)

Bacteria were grown for 20-24 h at 37°C with shaking, then, cells were harvested by centrifugation at 10,000 *xg* for 3 min. The pllet was suspended with 100% methanol, and cells were heated for 3 min at 55°C. To remove the cell debris, the cell was centrifuged at 10,000 *xg* for 2 min and absorbance was measured at 435 nm.

2.3.17. Population analysis profiling to compare the area under the curve (PAP-AUC assay)

To determine if the mutant *mspA* a mixed (heterogeneous) population for the level of resistance to oxacillin, we applied (PAP) assay. In this method, the bacterial population was exposed to a gradient of antibiotic concentrations. The strains were grown overnight at 37°C with shaking for 18 h. Then, the strains were normalized at OD$_{600}$. to 0.01 and inoculated in the 96-well plate that contain media with 2-fold dilutions oxacillin (16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.01325, 0.015625, 0.007813, 0 µg/ml). The readings at OD$_{600}$ were taken after 24 h (El-Halfawy & Valvano, 2015; Wootton et al., 2001).
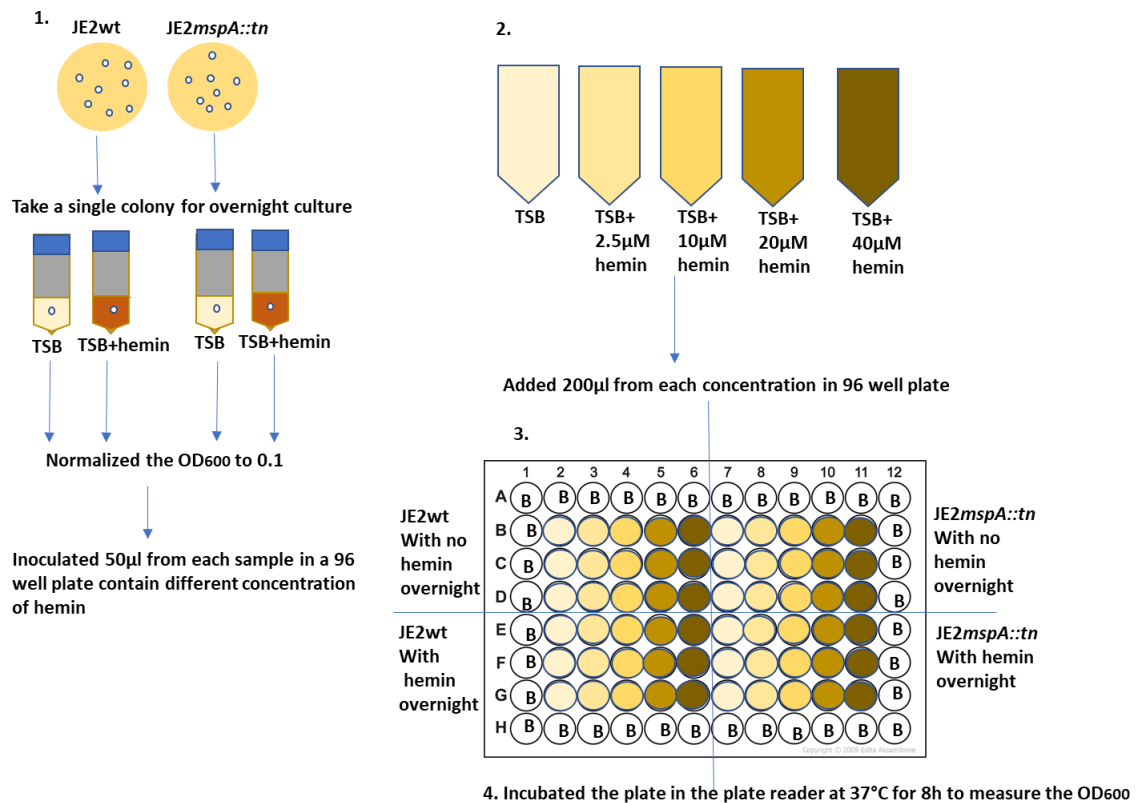
Fig. 2.1: Hemin adaptation assay.

2.3.18. Disk diffusion assay

Oxacillin antibiotic discs (5 µg/ml) used for this assay. *Staphylococcus aureus* strains were grown overnight for 18 h at 37ºC, and colonies were suspended in PBS using a sterile cotton swap to reach the OD$_{600}$ of 0.1. The suspension was spread on the Muller Hinton cation adjusted agar plate. Finally, an oxacillin disc was put in the middle of the plate and incubated overnight for 18 h at 37ºC, then, the diameter of the inhibition zone was measured.

2.3.20. Autolysis assay

Bacterial overnight culture 500 µl 20 ml of fresh TSB and was grown to reach an OD$_{600}$ of 0.6-0.8. Then, 1 ml of the bacterial culture was washed in cold PBS and resuspended in PBS containing 0.1% Triton X-100 and 200 ul from the suspension was transferred into 96-well plates. OD$_{600}$ was measured using the plate reader at 37ºC every 30 min for 6.5 h with 500 rpm shaking before each reading cycle.

2.3.21. RNA extraction

Overnight culture was grown in TSB at 37ºC for 18 h. Samples were treated with RNAprotect, then total RNA was extracted using Quick-RNA Fungal/Bacterial Miniprep kit according to the manufacturer's instructions. To eliminate the

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contamination by genomic DNA, the samples were treated with RNase-free TURBO™ DNase.

2.3.22. Quantitative reverse transcriptase

This experiment was performed to quantify the expression of the *pbp* genes in the mutant strain compared with the wild type. First, cDNA was generatedfollowing primers were then used to perform RT-qPCR of the five genes.

Gene	Forward primer	Reverse primer
*PBP*1	TGCATCACACTTAATTGGTAGAG	TAGTATTTGGTGCGATATATCCC
PBP2	ATAATGGTGGGAAATCCAAC	CTTTGCAGGAATCGGATC
PBP3	TTACATCTCTACCTAAGTCTCCACC	ACGGAGAGCCAAGAGTTAAC
PBP4	TGCTAATCCAGCGACAAG	ACGCGGACTATCCAAGAG
PBP2a	CCGTTCTCATATAGCTCATCATCA	CAAGGAGAAACTGGCAGAC

RT-qPCR was by adding 10 µl KAPA SYBR mix (2x), 1 µl each from forward and reverse primers (10 µM), 5 µl cDNA and RNase-free water to reach a volume of 20 µl. Finally, we used the following conditions for 35 PCR cycles:

Cycle	Temperature/time
Initial denaturation	95ºC / 2 min (followed by 35 cycles of denaturation, annealing and extension)
Denaturation	95ºC / 30 sec
Annealing	55ºC / 30 sec
Extension	72ºC / 30 sec

We calculated the number gene transcripts using the $2^{-(\Delta\Delta ct)}$ method.

2.3.23. Bocillin binding assay

Bacterial overnight culture (500 µl) was used to inoculate 20 ml of fresh TSB in a flask and bacteria were left to grow to reach an OD_{600} of 0.6. Then, 10 µl bocillin FL penicillin sodium salt was added (1 mg/ml) to 1 ml of culture. The culture was incubated for 1 h at 35ºC in the dark without shaking and 1 ml of the bacterial culture was washed 3 times with PBS. Then, 200 ul of the culture was transferred to a fluorescence 96-well plate and analysed using a CLARIOstar plate reader (excitation of 477 nm: emission of 525 nm).

2.3.24. Nitrocefin assay

Nitrocefin is a substrate that is used to detect the presence of β-lactamase enzyme in resistant bacteria (O'Callaghan et al., 1972). In this assay, one colony was transferred to 100 µl nitrocefin (0.5 mg/ml) and the colour visually inspected after 30 min. If the strain produced β-lactamase enzyme the colour changed from yellow to red.

2.3.25. Bacterial growth curve with and without exposure to oxacillin

Strains were grown overnight in TSB for 18 h at 37ºC. In the following day, strains were normalised to reach OD_{600} of 0.01. Then, in 96-well plate , 20 µl from the bacterial suspension was added to 180 µl of fresh TSB containing 0, 0.25 and 0.5 µg/ml oxacillin and measurements of the OD_{600} were taken every 30 min for 24 h at 37ºC using the plate reader Spectrostar Nano (BMG).

2.3.26. Fixation of the samples for transmission electron microscope (TEM)

Strains were grown overnight in TSB for 17 h at 37ºC with 0 and 0.5 µg/ml oxacillin. In Eppendorf tubes, strains were spun down, and the pellets at the bottom of the tube with a depth of <1 mm depth were resuspended with 2.5% glutaraldehyde in cacodylate buffer (0.1 M) at 4°C. The samples were , then , sent to the TEM unit at the University of Bristol for imaging.

2.3.27. Site-directed mutagenesis

This method was used to determine the active amino acid(s), domain(s) and loop(s) in the MspA protein that promote(s) its function. Amino acid sequence alignment was done between MspA protein of *Staphylococcus aureus* and its analogues in other bacteria to detect conserved amino acids to be substituted to the nonpolar, aliphatic α-amino acid (α-aa) alanine via site-directed mutagenesis. MspA has 105 aa comprising four domains and three loops. Based on alignment results, we have chosen 26 aa from the protein sequence and substituted them to alanine, one at a time, to recover 26 different variants of this protein. Concurrently, we have removed each of the four domains and made substitutions for 2 loops to understand the effects at the domain and loop levels. In this assay, the plasmid pRMC2-*mspA* was extracted using the plasmid extraction kit GeneJET Plasmid Miniprep kit according to the manufacturer's instructions. The *mspA* gene, encoding a protein with a change of a single amino acid, domain or loop, was amplified using Phusion high-fidelity DNA polymerase and the primers described in Tables 2.3 and 2.4 were used.

In Table 2.3, the primers were designed to substitute a single amino acid to alanine, one at a time. In Table 2.4, the primers were designed to remove every domain (domain 1 to domain 4, one at a time) and to substitute loop 1 entirely with aa glycine (G) and to substitute loop 3 with the amino acids originally presented in loop 1. Schematic representation to describe the steps followed to generate amino acid-based variants are shown in Fig. 2.2, while those to describe the steps followed to generate domain- or loop-based variants are shown in Fig. 2.3.

The reactions started by heating at 98°C for 30 sec, then, subjected to a further 35 cycles of 98°C for 10 sec, 59°C for 30 sec and 72°C for 4 min, with a final extension step for 7 min and 5 sec at 72°C. For the melting temperature, most of the primers required 59°C, but we did a gradient PCR for all of them between 59 and 62°C. The only PCR product that required further cutting with a restriction enzyme was that used for removing domain 4, as the forward primer was very close to the end of the gene. In this situation, the PCR product was cut with *SacI* (10 μl DNA, 5 μl cut smart buffer, 2 μl *SacI*, 33 μl water) at 37°C overnight. Next day, the ligation reaction was performed by adding 1 μl T4 DNA ligase buffer, 1 μl T4 ligase, and 8 μl DNA sample. This reaction was left overnight at 16°C before digesting with *Dpn*I and performing the bacterial transformation. After PCR for all the variants and cut/ligation of domain 4, the recovered products were digested with *Dpn*I to remove all the methylated nucleotides in the original plasmid (Fig. 2.4) before this PCR product was transformed in to *E. coli* strains DH-5α and Mach1. During the last step*,* plasmid and competent cells were mixed and left on ice for 30 min, then, heat shock was performed by exposure at 42°C for 2 min, then the samples were moved to ice again for 3 min. Then,cells were cultured in Soc prewarmed media or LB broth and incubated for 1 h at 37°C with shaking.

pRMC2-*mspA*^{wt} miniprep

↓

PCR using primer to generate domain- or loop-based variants

Exception: Removal of domain 4 was done by restriction digestion

↓

Digest with *DpnI*

↓

Transform pRMC2-*mspA*^{mut} into *E. coli* DH5α

↓

pRMC2-*mspA*^{wt} miniprep

↓

PCR with pRMC2 primers

↓

Gel electrophoresis of amplicon

↓

Check *mspA*^{mut} gene by sequencing

↓

Transform pRMC2-*mspA*^{mut} into *S. aureus* RN4220

↓

pRMC2-*mspA*^{wt} miniprep

↓

Transform pRMC2-*mspA*^{mut} into JE2*mspA*::tn to generate CS^{mut}

↓

Check *mspA*^{mut} by PCR

Fig. 2.2: Schematic representation to describe the steps followed to generate amino acid-based variants. pRMC2-*mspA*^{wt} is the pRMC2 plasmid with wild type version of *mspA* gene. pRMC2-*mspA*^{mut} was generated from PCR with specific primers (see Table 2.3) to produce AA variants and pRMC2-*mspA*^{wt} was used as the template. CS^{mut} is the knockout *mspA* mutant strain complemented with different pRMC2-*mspA*^{mut} plasmids.

pRMC2-*mspA*^{wt} miniprep

⬇

PCR using primers to generate domain-or loop-based variants

Exception: Removal of domain 4 was done by restriction digestion

⬇

Digest with *DpnI* (see Figure 5.5 for details)

⬇

Transform pRMC2-*mspA*^{mut} into *E. coli* DH5α

⬇

pRMC2-*mspA*^{mut} miniprep

⬇

PCR with pRMC2 primers

⬇

Gel electrophoresis of amplicon

⬇

Check *mspA*^{mut} gene by sequencing

⬇

Transform pRMC2-*mspA*^{mut} into *S. aureus* RN4220

⬇

pRMC2-*mspA*^{mut} miniprep

⬇

Transform pRMC2-*mspA*^{mut} into JE2*mspA*::tn to generate CS^{mut}

⬇

Check *mspA*^{mut} by PCR

Fig. 2.3: Schematic representation to describe the steps followed to generate domain-or loop-based variants. pRMC2-*mspA*^{mut} is the pRMC2 plasmid with wild type version of *mspA* gene. pRMC2-*mspA*^{mut} was generated from PCR with specific primers (see Table 2.4) and pRMC2-*mspA*^{wt} was used as the template. CS^{mut} is the knockout *mspA* mutant strain complemented with different pRMC2-*mspA*^{mut} plasmids. Domain 4 was removed by *SacI* restriction digestion then the plasmid was left to self-ligate.

Table 2.3: Primers to substitute original amino acids to alanine. Nucleotides in red in forward primers refer to alanine codon preferred in *Staphylococcus aureus*, while those in reverse primers refer to their complementary sequences. Note that forward and reverse primers of a given variant have complementary DNA sequences.

Variant	Original aa	Forward primers	Reverse primers
MSPA-L31	L	GTATTTACTCGCATTGCAAGAATTATTATGGGTG	CACCCATAATAATTCTTGCAATGCGAGTAAATAC
MSPA-R32	R	TTTACTCGCATTTTGGCAATTATTATGGGTGTG	CACACCCATAATAATTGCCAAAATGCGAGTAAA
MSPA-I34	I	CGCATTTTGAGAATTGCAATGGGTGTGTTGTTA	TAACAACACACCCATTGCAATTCTCAAAATGCG
MSPA-M35	M	CATTTTGAGAATTATTGCAGGTGTGTTGTTATTA	TAATAACAACACACCTGCAATAATTCTCAAAATG
MSPA-L38	L	GAATTATTATGGGTGTGGCATTATTATTCGTCTTAG	CTAAGACGAATAATAATGCCACACCCATAATAATTC
MSPA-L39	L	ATTATGGGTGTGTTGGCATTATTCGTCTTAGC3	GCTAAGACGAATAATGCCAACACACCCATAAT
MSPA-V42	V	GTGTTGTTATTATTCGCATTAGCATTAACGACG	CGTCGTTAATGCTAATGCGAATAATAACAACAC
MSPA-P51	P	CGACGATGAGTTTTGCAAAAGAGAATTGGTGG	CCACCAATTCTCTTTTGCAAAACTCATCGTCG
MSPA-W55	W	GTTTTCCAAAAGAGAATGCATGGGTATTTATCGTC	GACGATAAATACCCATGCATTCTCTTTTGGAAAAC
MSPA-W56	W	CCAAAAGAGAATTGGGCAGTATTTATCGTCTTA	TAAGACGATAAATACTGCCCAATTCTCTTTTGG
MSPA-V57	V	CAAAAGAGAATTGGTGGGCATTTATCGTCTTATTAC	GTAATAAGACGATAAATGCCCACCAATTCTCTTTTG
MSPA-F58	F	GAGAATTGGTGGGTAGCAATCGTCTTATTACTC	GAGTAATAAGACGATTGCTACCCACCAATTCTC
MSPA-V60	V	GGTGGGTATTTATCGCATTATTACTCTTAGTC	GACTAAGAGTAATAATGCGATAAATACCCACC
MSPA-L61	L	GGGTATTTATCGTCGCATTACTCTTAGTCGG	CCGACTAAGAGTAATGCGACGATAAATACCC
MSPA-L62	L	GTATTTATCGTCTTAGCACTCTTAGTCGGTAATG	CATTACCGACTAAGAGTGCTAAGACGATAAATAC
MSPA-L63	L	TTTATCGTCTTATTAGCATTAGTCGGTAATGTC	GACATTACCGACTAATGCTAATAAGACGATAAA
MSPA-V68	V	CTCTTAGTCGGTAATGCAGAAGTGACAGGATTT	AAATCCTGTCACTTCTGCATTACCGACTAAGAG
MSPA-E69	E	CTCTTAGTCGGTAATGTCGCAGTGACAGGATTT	AAATCCTGTCACTGCGACATTGACACCTAAGAG

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MSPA-T71	T	GGTAATGTCGAAGTGGCAGGATTTAAAATGCTT	AAGCATTTTAAATCCTGCCACTTCGACATTACC
MSPA-F73	F	GAAGTGACAGGAGCAAAAATGCTTAAAAAAGAT	GATCTTTTTTAAGCATTTTTGCTCCTGTCACTTC
MSPA-K74	K	GAAGTGACAGGATTTGCAATGCTTAAAAAGATC	GATCTTTTTTAAGCATTGCAAATCCTGTCACTTC
MSPA-K77	K	GGATTTAAAATGCTTGCAAAAGATCTAAAAGGC	GCCTTTTAGATCTTTTTGCAAGCATTTTAAATCC
MSPA-D79	D	AAAATGCTTAAAAAAGCACTAAAAGGCGTAAAC	GTTTACGCCTTTTAGTTGCTTTTTTAAGCATTTT
MSPA-K81	K	CTTAAAAAAGATCTAGCAGGCGTAAACATCTTG	CAAGATGTTTACGCCTGCTAGATCTTTTTTAAG
MSPA-L86	L	AAAGGCGTAAACATCGCAAATTTAATGTCATTA	TAATGACATTAAATTTGCGATGTTTACGCCTTT
MSPA-N87	N	GGCGTAAACATCTTGGCATTAATGTCATTATTT	AAATAATGACATTAATGCCAAGATGTTTACGCC

88
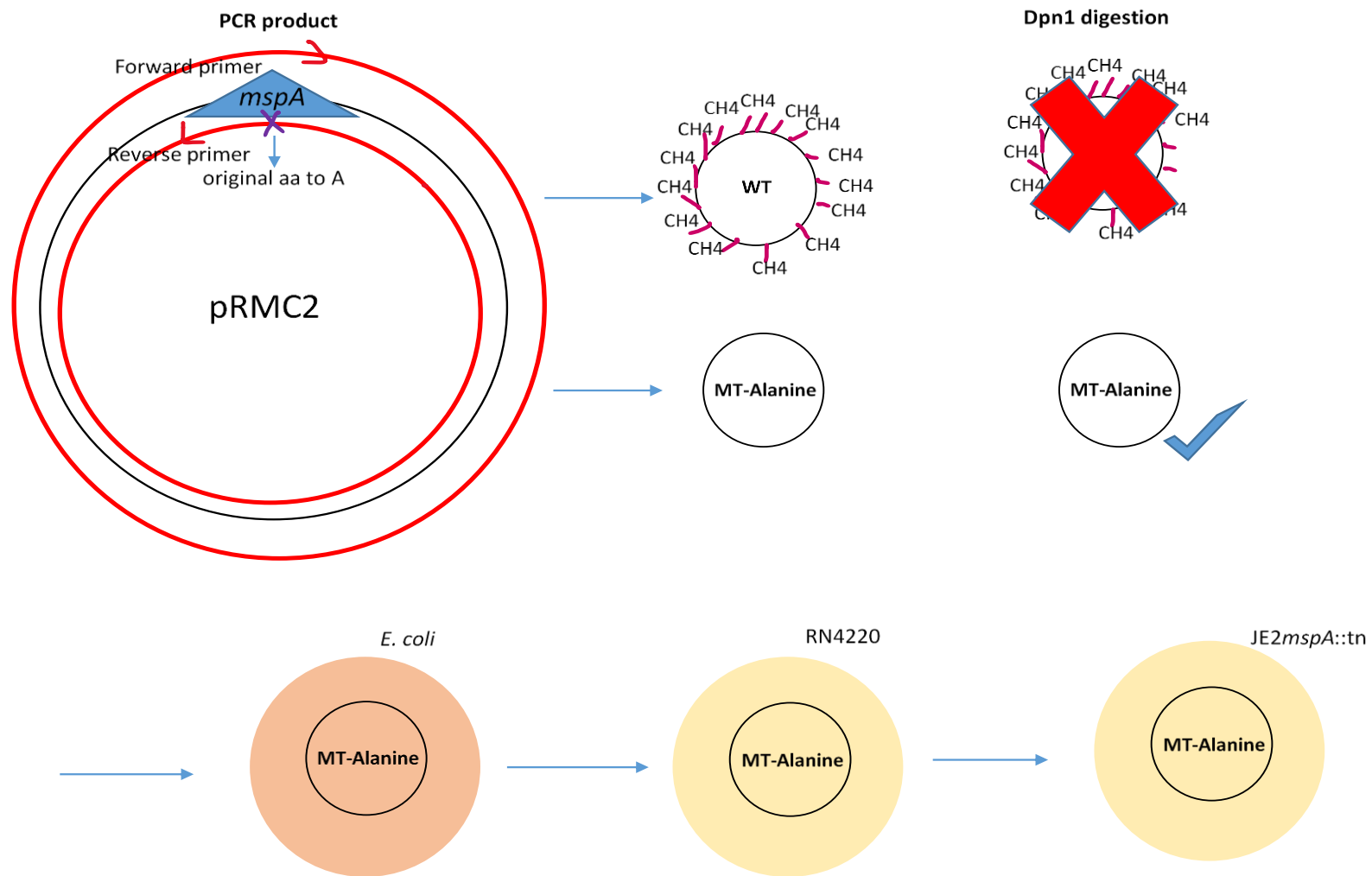
Fig. 2.4: Description of the method used to create the mutated variants with one aa changed to alanine.

Table 2.4: Primers used for domain removal and loop substitution.

Primer name	Primer sequencing
Domain 1-F	TTTATCTGGAAGTTGTATTTACTCGCATTTTG
Domain 1-R	ATACAACTTCCAGATAAAATTGCATATGATAACTTCC
Domain 2-F	ATTTACTCGCTTTCCAAAAGAGAATTGGTGG
Domain 2-R	TTTGGAAAGCGAGTAAATACAACTTCCATTTTAAAAATAC
Domain 3-F	CAAAAGAGAATAAAATGCTTAAAAAAGATCTAAAAGGC
Domain 3-R	AGCATTTTATTCTCTTTTGGAAAACTCATCGTCGTTAA
Domain 4-F	ATATAGAGCTCTTAGTTTACGCCTTTTAGATCTTTTTTAAG
Domain 4-R	ATATATAACGAGCTCGAATTCACTG
Loop 1-F	GGTGGTGGTGGTGGTGGTATTTTGAGAATTATTATGGGTGTG
Loop 1-R	ACCACCACCACCACCACCCATTTTAAAAATACTAATAAAACTAACAATTAG
Loop 3 s1F	GAAGTTGTATTTACTCGCATCTTGAATTTAATGTCATTATTTATCTTTG
Loop 3 s1R	GCGAGTAAATACAACTTCAAATCCTGTCACTTCGACATTAC

Finally, the transformed bacteria were screened on LB agar with ampicillin (100 µg/ml) and presence of the plasmid was verified by colony PCR using the pRMC2 primers described below.

Primer name	Primer sequence
pRMC2_seq_F	ATTCAGGCTGCGCAAC
pRMC2_seq_R	ATCTAATCTAGACATCATTAATTCCTC

The colony PCR started by heating at 95°C for 10 min, then, subjected to a further 35 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 2.5 min, with a final extension step at 72°C for 5 min. The transformed bacteria were grown overnight on LB broth with ampicillin (100 µg/ml). Then, plasmid was extracted and verified by colony PCR using pRMC2 primers. Next day, the correct plasmid was sent for sequencing (Eurofins, Germany) to check the right mutation or variant. Then, the plasmid was transformed in to *Staphylococcus aureus* strain RN4220 through electroporation in 0.2 cm cuvettes. Following electroporation, 700 µl TSB or BHI media were added, and bacteria were incubated for 1 h at 37ºC with shaking. The transformed bacteria were then plated on TSA media containing chloramphenicol (10 µg/ml).The recombinant plasmid was electroporated into *S. aureus* RN4220. Lysostaphin was added (100 µg/ml) to isolate the plasmid from *S. aureus* RN4220 as previously described. Plasmid was then passed to JE2*mspA::tn* though

electroporation and plated on TSA containing chloramphenicol (10 µg/ml) and erythromycin (5 µg/ml). Finally, presence of the plasmid was verified again by colony PCR using pRMC2 primers.

2.3.28. DNA agarose gel electrophoresis

The PCR products were run on 1-1.5% agarose gels. The gel was made using 1X TAE or 1X TBE and stained using SYBR Safe stain. When the samples were amplified using Green Master Mix, they were run without loading dye. If not, DNA loading dye was mixed with each sample before running the gel. The gel was run for around 60 min at 90 V. Ladder loaded on to the gel was either 1 Kb or 50 bp and bands were visualized on a UV transilluminator.

2.3.29. Other routine experiments used

All PCR purifications were done by using the GeneJET PCR purification kit. DNA was extracted from the gel by using the GeneJET gel extraction kit. All plasmid extractions were done by using the GeneJET Plasmid miniprep kit. All the DNA samples were stored at -20°C until use.

2.4. Data analysis

The data presented in this thesis represent the arithmetic mean of between at least three and up to to six independent biological experiments, and the error bars represent standard error of biological replicates. Statistics were performed with an unpaired t-test or one-way ANOVA for comparison of multiple groups, and Tukey for multiple comparison (statistical data are shown in the appendix section), where the significance at different levels was determined by using p values, e.g., * ≤ 0.05,** ≤ 0.01,*** ≤ 0.001, **** ≤ 0.0001. Means with the same significance letters are not significantly different. If two variables have different letters, they are significantly different.

2.5. Computer software used

Name of the software	Description
Protter	For protein visualization
I-Tasser	For 3D structure prediction
ExPASY	To detect the ORF
GraphPad prism	For statistical analysis
SnapGene	To design the primers and create the plasmid maps
Benchling	To check the DNA sequences
ImageJ	To measure the cell wall thickness

3. MspA, a newly identified small transmembrane stabilizing protein with pleiotropic effects on the pathogenicity of *Staphylococcus aureus*

3.1. Introduction

Staphylococcus aureus is a major human pathogen. It can cause a variety of infections, ranging from superficial skin infections, such as abscesses and impetigo, to severe infections, such as sepsis, bacteraemia, endocarditis and osteomyelitis (Lowy, 1998). Treatment is problematic due to the rise of antibiotic-resistant strains, and so new methods to prevent and treat infections are needed. To develop these methods, we need to better understand how this bacterium causes the disease. This can be categorized into three main strategies, where the bacteria can utilize one or two and sometimes all three strategies to cause the disease. These strategies are adhesion, i.e, the ability of bacteria to bind and enter host cells and tissues; toxicity, i.e, secretion of proteins that cause damage to human cells and tissues; and evasion, i.e, the ability of *S. aureus* to interfere with the protective ability of the human immune system (Foster et al., 2014; Seilie & Wardenburg, 2017). Despite our current understanding of the pathogenicity of *S. aureus*, we have not yet been successful in developing a vaccine to prevent infections, and even in well-resourced countries with good health-care infrastructure, the incidence of invasive disease is increasing (Clegg et al., 2021), which suggests that there is a lot more for us to learn.

To address these issues, Professor Massey's group have developed a new approach to studying pathogenicity, where they apply genome-wide association studies (GWAS) to collections of clinical *S. aureus* isolates to identify new genes and proteins that affect the ability of *S. aureus* to produce cytolytic toxins (toxicity). In one such study, the toxicity of each of 90 MRSA isolates was measured, and statistical tests on the genome sequence and toxicity data performed to identify loci that associated with changes in toxicity level of the isolates. While doing this, the group found several genes that positively correlated with toxicity (Laabei et al., 2014; Recker

et al., 2017). One of these genes, which is the focus of this thesis, was annotated as SATW20_23930 in the TW20 genome and predicted to encode a membrane-bound protein with 105 amino acids (aa), with four transmembrane domains and three loops (Fig 3.1). We named this gene *mspA* (membrane stabilizing protein A) based on our understanding of its activity and the focus of this chapter was to characterise its activity in *S. aureus* with a specific emphasis on how it affects toxin production.
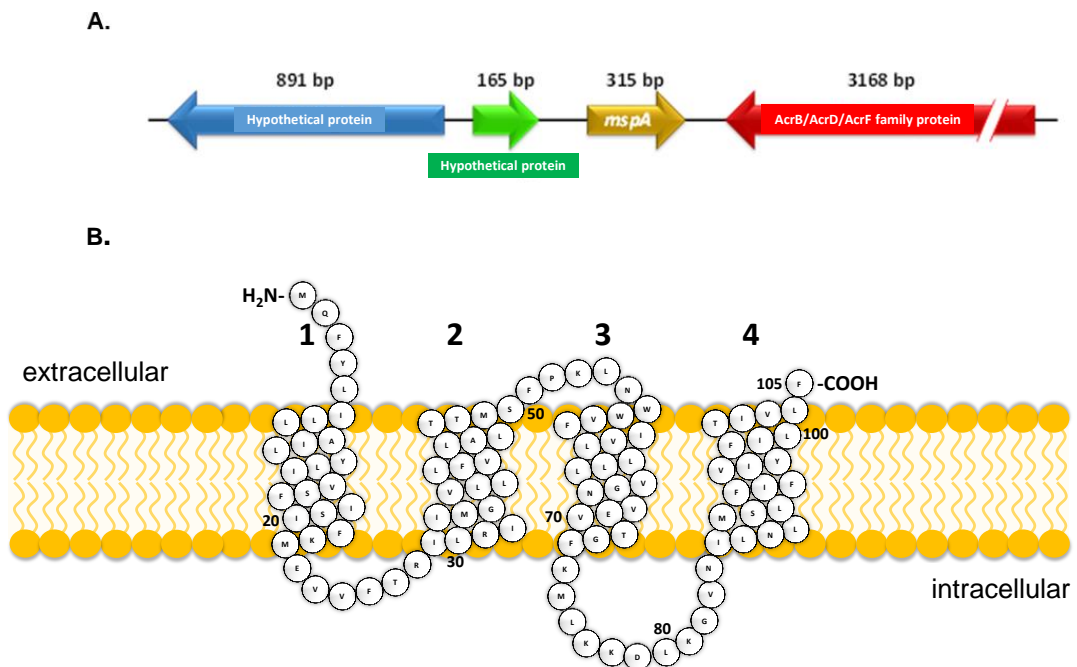
Fig 3.1: The *mspA* gene locus and predicted 2D structure of the protein. A. The *mspA* gene (315 bp) is situated between genes with the locus tags SAUSA300_2212 (165 bp) and SAUSA300_2213 (3168 bp) . B. The 2D structure of MspA protein (105 aa) with four transmembrane domains, three loops and no detectable signal sequence (Protter - interactive protein feature visualization and integration with experimental proteomic data (Omasits et al., 2014).

3.2. Aims of this chapter

1. Verify the contribution of the MspA protein to the toxicity of *Staphylococcus aureus.*

2. Characterise the mechanism by which MspA protein affects *Staphylococcus aureus* toxicity and investigate whether there is any relation between iron efflux/influx and MspA protein.

3. Examine the contribution of other proteins known to affect iron homeostasis: HrtA, HrtB and Fur.

4. Determine whether MspA protein is associated with staphyloxanthin production.

5. Investigate the possible roles of *mspA*-neighboring genes in two different genetic bacterial backgrounds, i.e, JE2 and SH1000.

3.3. Results

3.3.1. Confirmation of the correct open reading frame (ORF) of *mspA* gene

According to the genomic analysis previously made by (Holden et al., 2010) for the TW20 *S. aureus* strain (sequence type 239), the *mspA* gene was annotated as encoding a putative membrane protein (CBI50258.1, Expasyfetch). However, when we examined the same region in the genome of FPR3757 USA300 (JE2) strain, it was annotated as a non-coding intergenic region between SAUSA300_2212 and SAUSA300_2213 loci (Laabei et al., 2014). Given the different annotations across two sequenced strains, we sought to further characterize this region. We used ExPASy software to identify potential ORFs within this 748 bp region between SAUSA300_2212 and SAUSA300_2213 loci. The results in (Fig. 3.2) indicated that reading frame 2 in the 5`-3` orientation of the locus contains the longest ORF across all six frames. This identified ORF of frame 2 has several possible initiation codons (Fig 3.3). The correct ORF ought to start ~10 nt downstream of a Shine Dalgarno consensus sequence. Analysis of this sequence identified an ORF with a length of 315 bp encoding a protein with 105 aa that has a likely Shine Dalgarno consensus sequence ~10 nt upstream of its initiation codon. This suggested that the annotation of the TW20 gene is correct and that there is a gene encoding a 105 aa protein within this 748 bp region (Fig 3.4).
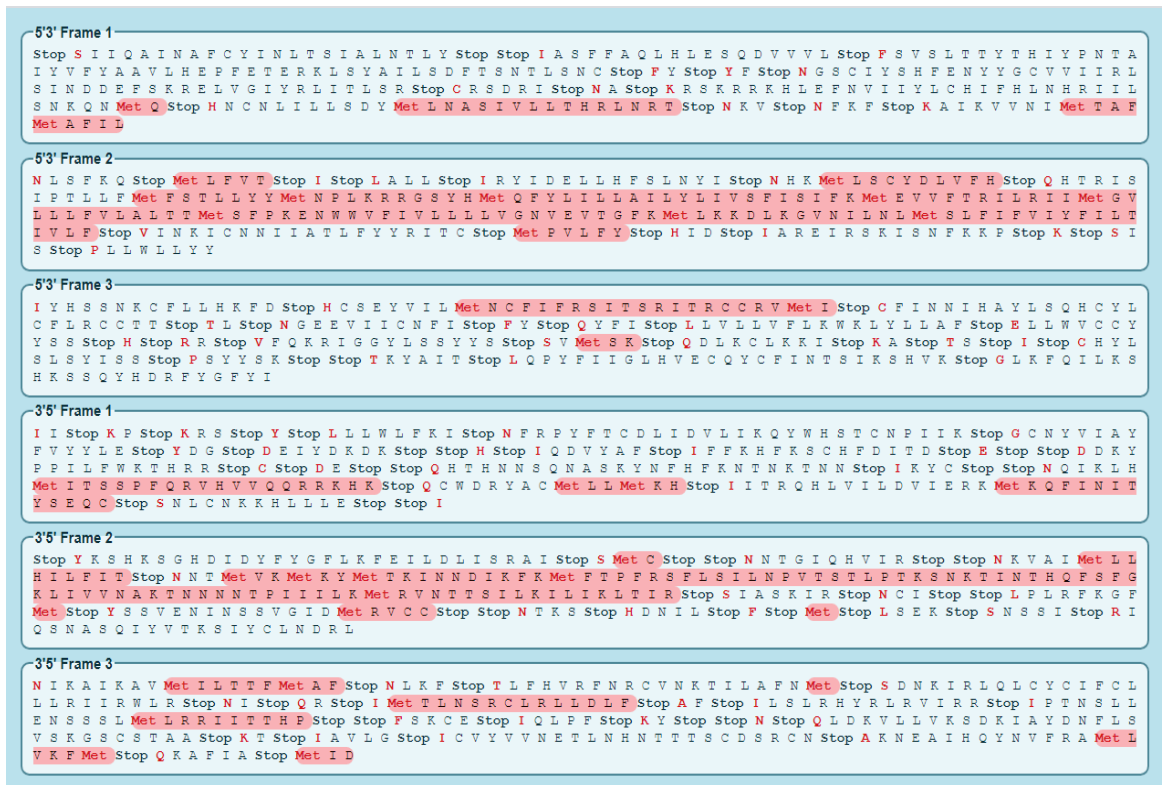
Fig 3.2: Identity of ORFs Predicted. ORFs predicted in the six frames of the intergenic region (748 bp) between SAUSA300_2212 and SAUSA300_2213 loci.
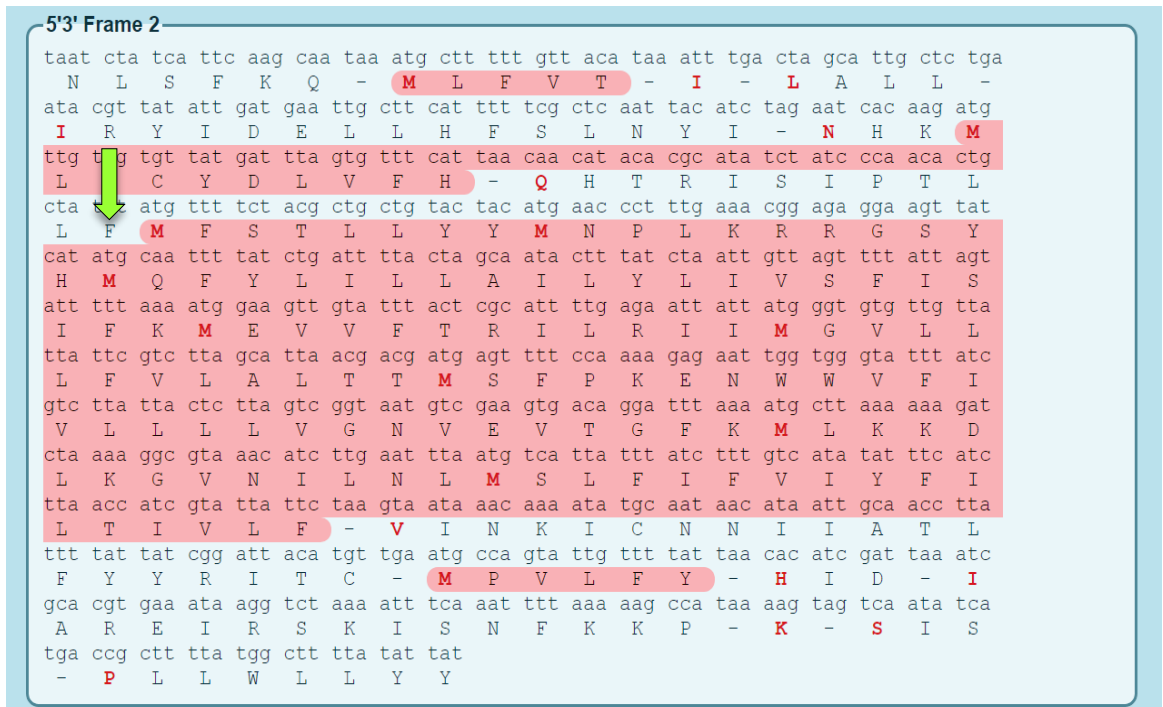
Fig 3.3: Identified ORF of frame 2. The assigned ORF in frame 2 with initiation codon (referred to by the green arrow) resulting in gene length of 315 bp.
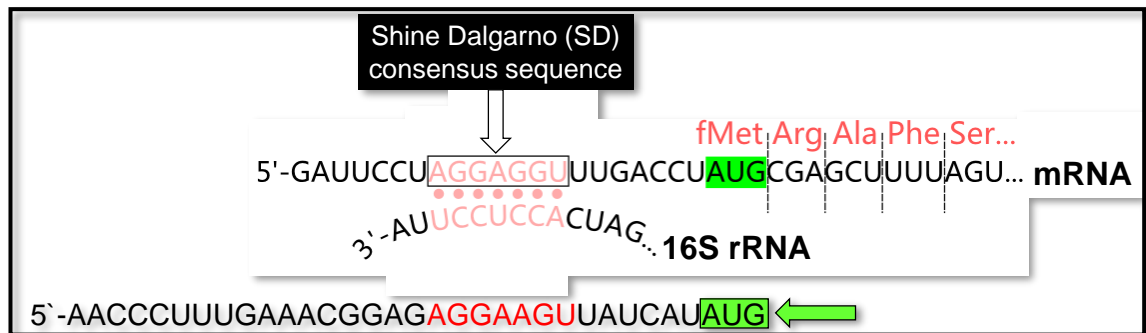
Fig 3.4: Sequence upstream of the initiation codon. Indicates that the one referred to by the green arrow is the correct one as a Shine Dalgarno sequence exists ~10 nt upstream this initiation codon.

3.3.2. Confirmation of transposon insertion within *mspA* gene

A mutant with a transposon insertion in the region believed to contain the *mspA* gene was provided to us by the group that constructed the Nebraska Transposon Mutant Library (NTML) (Bae et al., 2008). To confirm the correct insertion of the transposon (Tn) within the *mspA* gene in the mutant we used a primer within the Tn (Upstream 5` CTCGATTCTATTAACAAGGG 3`) and another within the *mspA* gene (MspRV: 5` GCGAGCTCGTTGCAATTATGTTATTGC 3`) in a PCR reaction. The resulting PCR product was confirmed as the expected 786 bp size by agarose gel electrophoresis (Fig 3.5), verifying that the Tn was in the expected site and inactivated the *mspA* gene.
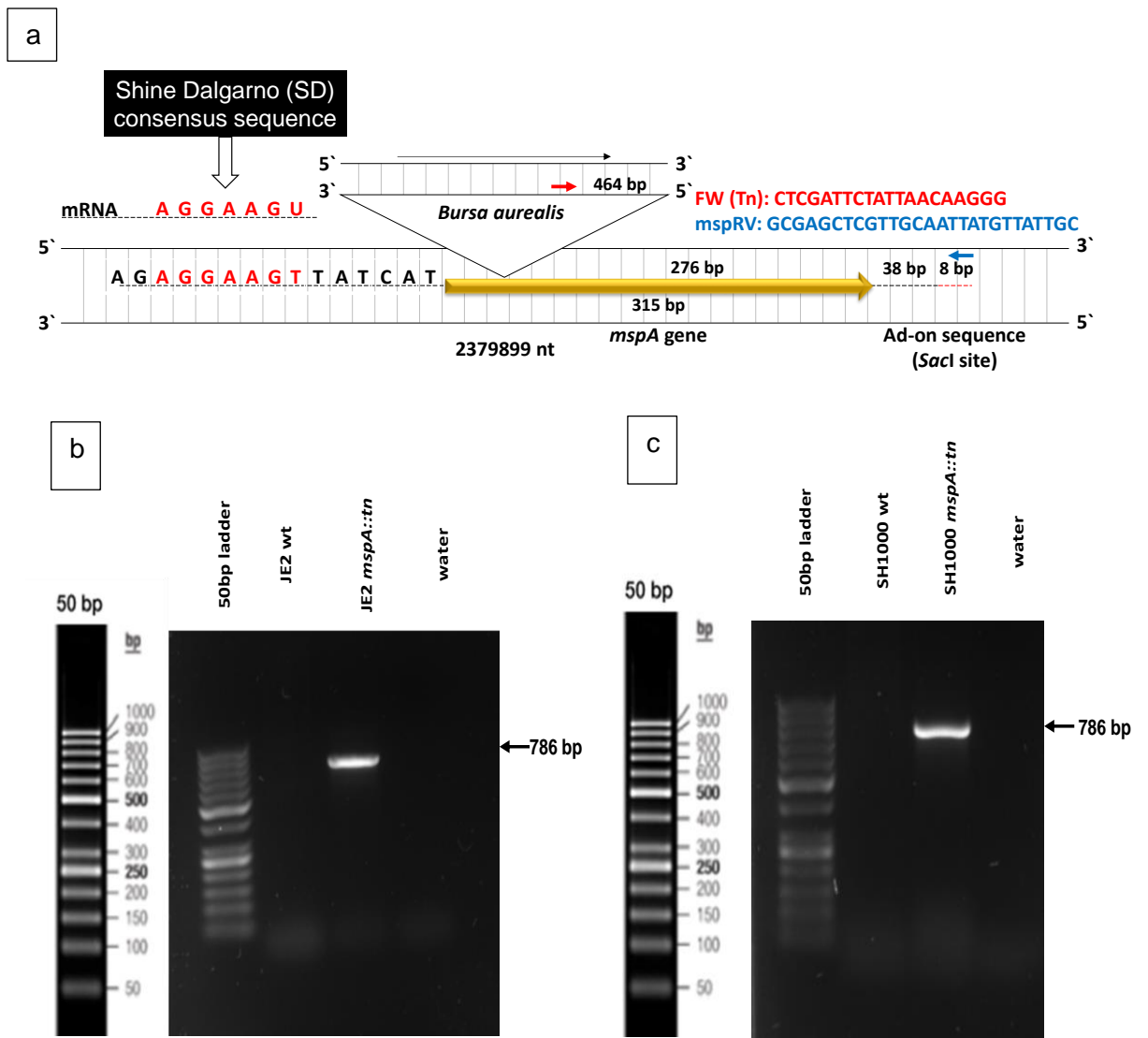
Fig 3.5: Transposon insertion in the *mspA* gene. (a) Diagram referring to the Tn (Bursa aurealis) insertion position in the *mspA* gene and the expected amplicon size to confirm insertion. Agarose gel electrophoresis for amplicon (786 bp) resulting from PCR with the forward Tn primer (Upstream 5` CTCGATTCTATTAACAAGGG 3`) and the reverse *mspA* primer (MspRV: 5` GCGAGCTCGTTGCAATTATGTTATTGC 3`) in (b) JE2 background or (c) SH1000 background.

3.3.3. Effect of *mspA* knockout on *Staphylococcus aureus* toxicity

To examine the contribution of MspA to the cytotoxicity of *S. aureus*, the supernatant of the JE2 wild type and *mspA* knockout mutant were incubated with THP-1 cells, and the lysis of these cells was quantified. The wild type JE2 strain was significantly more cytolytic than the *mspA* mutant strain (Fig. 3.6). To verify that the MspA protein has an effect on toxicity, the wild type gene was cloned into an expression vector such that its transcription was driven by an inducible tetracycline promoter of the pRMC2 plasmid (Corrigan & Foster, 2009), resulting in the p*mspA* plasmid (this cloning was performed by another member of the group and provided to me). I introduced this plasmid into the JE2 *mspA* mutant strain by electroporation and quantified whether this restored the toxicity of the mutant strain when complemented with the wild type gene. This allowed me to comprehensively examine the effect MspA has on the toxicity of *S. aureus* strain JE2 by directly comparing a set of isogenic mutants, including wild type JE2, the JE2 *mspA* mutant and the complement strain of JE2 *mspA* mutant containing the p*mspA* plasmid at varying expression levels of the *mspA* gene (gene expression was induced by adding tetracycline at increasing concentrations of 0, 100, 200 and 250 ng/ml). As the tetracycline concentrations increased, more THP-1 cells died due to the increased rate of *mspA* gene expression (Fig. 3.6). The means of JE2wt. followed by the complement strain JE2*mspA*::tn(p*mspA*) 250ng/ml tet. showed the highest significant value among on strains, while the knocked out JE2 *mspA*::tn and JE2*mspA*::tn(p*mspA*) 0ng/ml tet. showed the lowest. This work confirmed the effect of MspA on toxicity, as induction of *mspA* gene expression in the mutant complemented with the wild typeol gene resulted in a significant increase in the toxicity of the mutant.

To confirm that the effect of MspA on toxicity is not specific to the MRSA genetic background, I transduced the mutation into the genetically distinct methicillin sensitive *S. aureus* (MSSA) strain SH1000 (Horsburgh et al., 2002), which was then verified by PCR as described above (Fig. 3.5c). This mutant was also transformed with the p*mspA* plasmid. The toxicity of this second set of isogenic mutants was again measured by comparing their ability to lyse THP-1 cells (Fig. 3.7). The means of SH1000wt. followed by the complement strain SH1000*mspA*::tn(p*mspA*) 250ng/ml tet. showed the highest significant value among on strains, while the knocked out SH1000*mspA*::tn and SH1000*mspA*::tn(p*mspA*) 0ng/ml tet. showed the lowest. The JE2 strain was consistently more toxic resulting in higher THP-1 cell death compared

to SH1000. As such, while the JE2 supernatant was diluted in TSB to 10%, the SH1000 supernatant was only diluted to 30%. In both genetic backgrounds, inactivation of the *mspA* gene resulted in a significant reduction in the ability of the bacteria to lyse the human cells (p-value = 0.000856 for the JE2 background and 0.00439 for SH1000 background). These results indicated that both MRSA and MSSA *S. aureus* strains have a reduced ability to lyse THP-1 cells when *mspA* is inactivated. To confirm that this effect was a result of the inactivation of the *mspA* gene in the SH1000 MspA mutant, expression of *mspA* from p*mspA* was induced with tetracycline. As with the JE2 set of strains, as the tetracycline concentration increased, more THP-1 cells died due to the increased rate of *mspA* expression. This work demonstrated that in the absence of MspA protein, the ability of *S. aureus* to cause THP-1 cell death was diminished, but the near wild type phenotype was recovered when expression of the *mspA* gene, driven by the tetracycline inducible promoter was functionally complemented (Fig. 3.7).
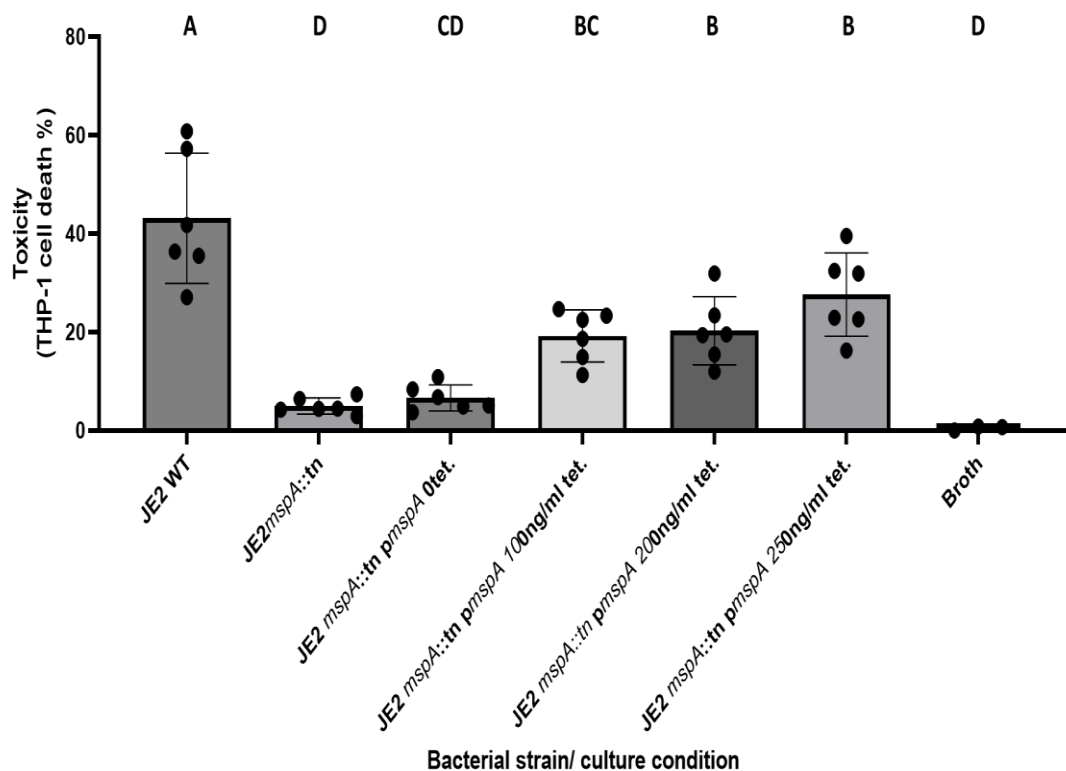
Fig. 3.6: Percentages of cell death (monocytic THP-1 cell line) reflecting effects of *mspA* gene on cytolytic toxin secretion in *S. aureus* strain JE2. This effect was complemented by inducing expression of the *mspA* gene inserted in an expression plasmid (p*mspA*). Gene expression was induced by adding tetracycline at increasing concentrations of 0, 100, 200 and 250 ng/ml. Data represent the arithmetic mean of six biological repeats with two technical repeats, and error bars the standard error of the mean. Statistics were performed with one-way ANOVA using Tukey for multiple comparison.
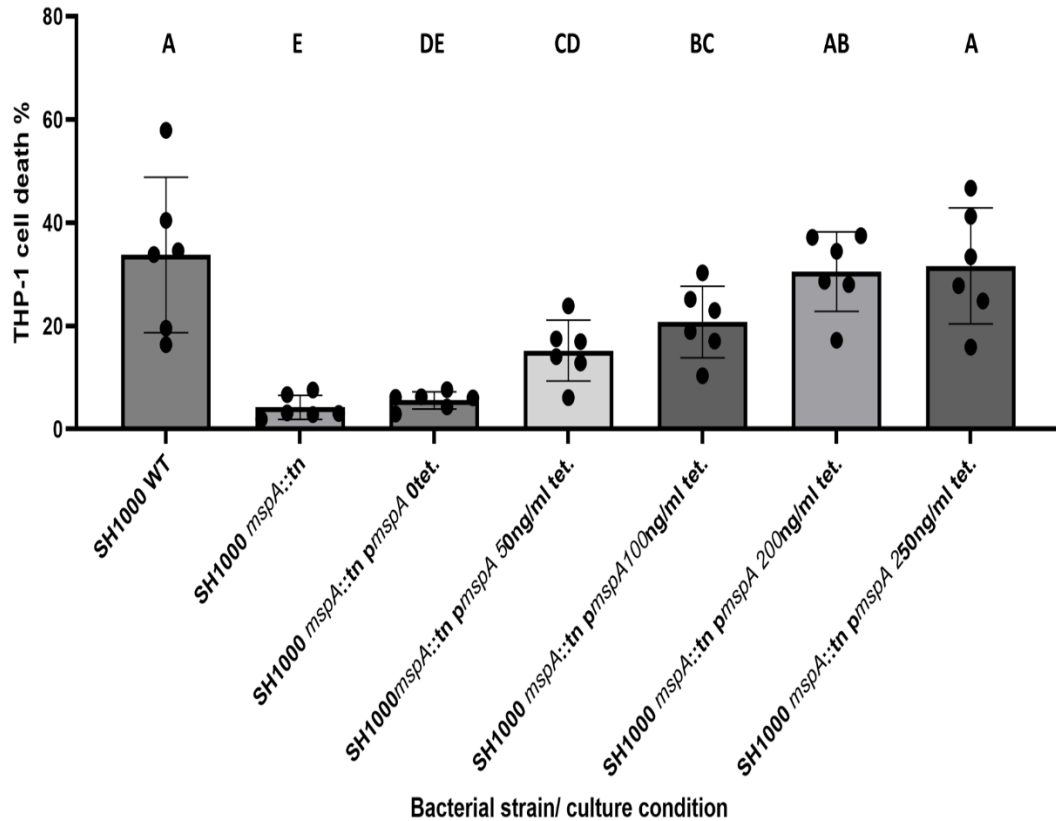
Fig. 3.7: Percentages of cell death (monocytic THP-1 cell line) reflecting effects of *mspA* gene on cytolytic toxin secretion in *S. aureus* strain SH1000. This effect was complemented by inducing expression of the *mspA* gene inserted in an expression plasmid (p*mspA*). Gene expression was induced by adding tetracycline at increasing concentrations of (0,50, 100, 200 & 250 ng/ml). Data represent the arithmetic mean of six independent experiments biological repeats with two technical repeats, and error bars the standard error of the mean. Statistics were performed with one-way ANOVA using Tukey for multiple comparison.

3.3.4. Effect of *Staphylococcus aureus mspA* knockout on PSMs production and Hla protein (alpha toxin) production

As the loss of MspA protein results in the reduction of the cytolytic activity of *S. aureus*, we sought to determine whether this loss resulted in a reduction of expression of any specific group of toxins. To quantify the relative expression of the phenol-soluble modulins (PSMs), they were extracted from bacterial supernatants by TCA precipitation, and the samples were loaded each in two volumes and were run on SDS-PAGE gels, which were then stained overnight using Blue Safe protein stain. Due to the small size of the PSMs. We loaded different volumes of supernatant because this is a challenging issue as overloading results in thicker and more robust bands, making it difficult to differentiate among bands, and the low volume makes it hard to distinguish among bands. So, we picked two volumes, 2.5 µl and 5 µl, for

loading (Fig 3.8). This provided a qualitative measure of the band's relative abundance. However, what was clear is that in both genetic backgrounds, the loss of MspA resulted in a significant decrease in PSM production, which explains the results of the THP-1 cell lysis data reported above. We quantified the effect when MspA is absent on the Hla toxin to confirm whether its activity had more general effect. The Hla protein (alpha toxin) was more abundant in the cell lysate of the mutant than in the wild type bacteria. To determine that, we performed Western blotting and identified a thicker band in the mutant in the whole-cell lysate compared with the wild type. Also, we could see a slight reduction in the supernatant of the mutant strain compared with wild type. This may have also contributed to the reduction in the toxicity of the *mspA* mutant (Fig 3.9).
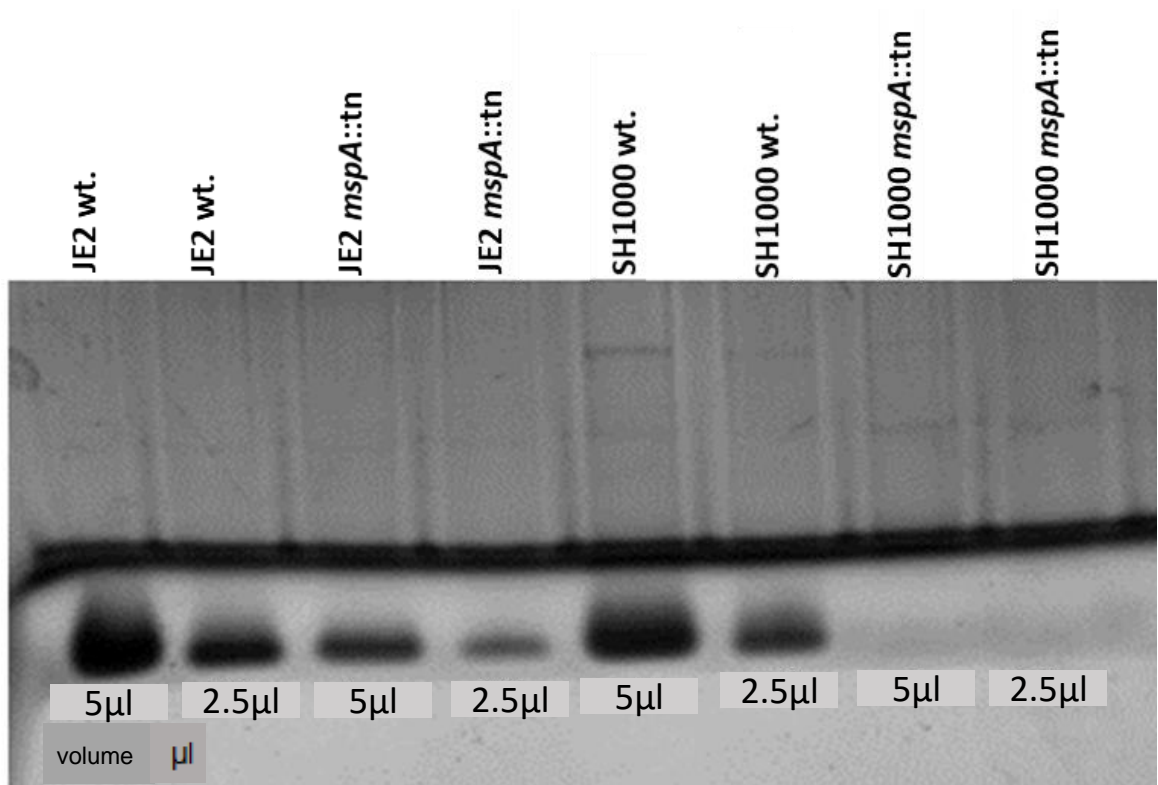
Fig 3.8: SDS-PAGE demonstrating that inactivation of *mspA* leads to reduced PSM production. Both SH1000*mspA::tn* and JE2*mspA::tn* produced lower amounts of the PSMs compared to their respective wild type strains, the samples were loaded each in two volumes (5 µl and 2.5 µl).
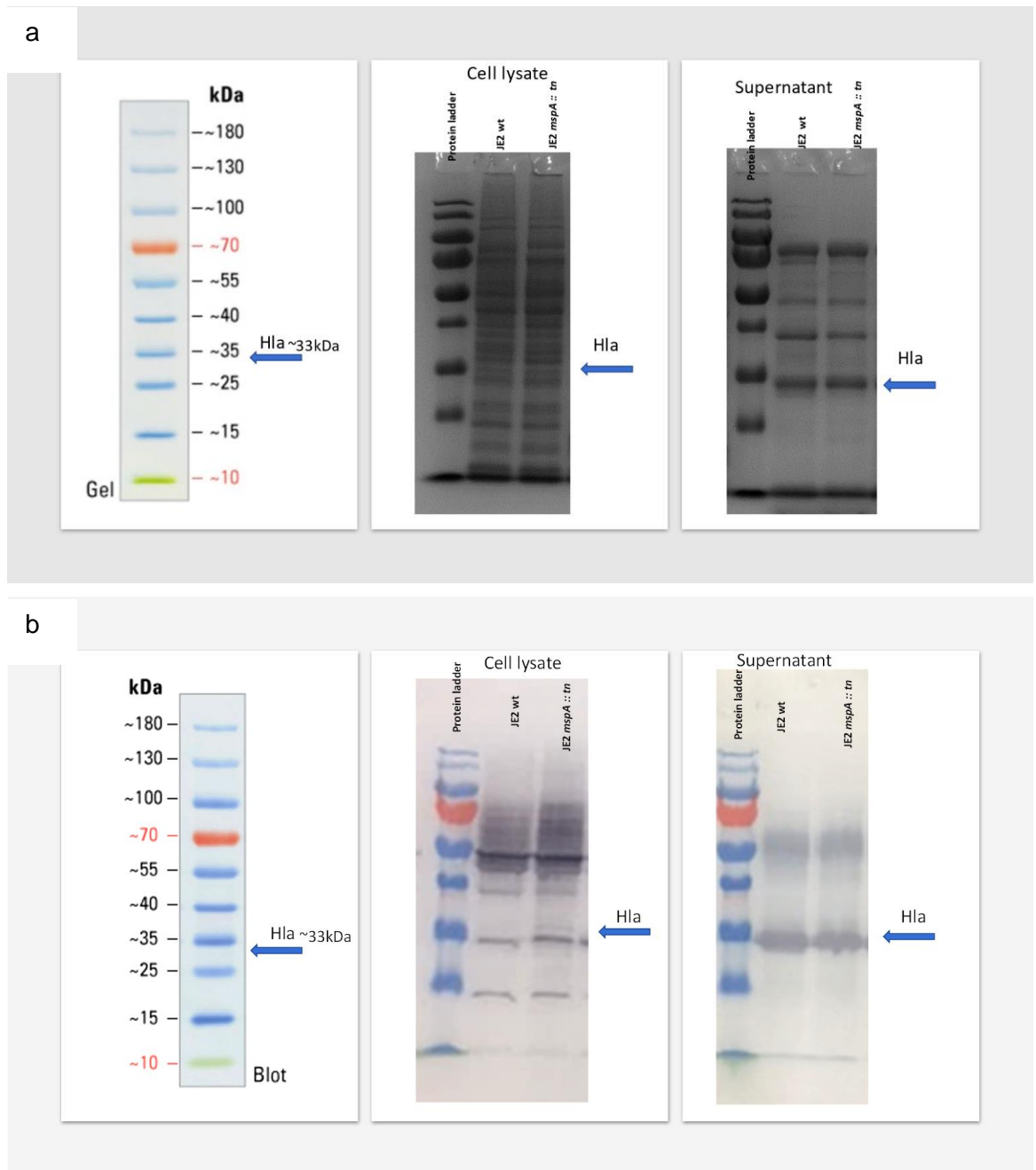
Fig 3.9: Hla protein extraction from JE2 wt and JE2*mspA::tn***.** Hla protein is more abundant in the whole cell lysate of *mspA* mutant compared with wild type. (a) SDS-PAGE gel to check the quality of the protein in the supernatant versus the whole cell lysate in the JE2 wt and JE2*mspA::tn* strains. (b) Nitrocellulose membrane, indicating that Hla is more abundant in the whole cell lysate of the *mspA* mutant compared to wild type and slightly less abundant in the supernatant.

3.3.5. The Agr system in the *mspA* mutant is less active than in the wild type

Inactivation of the *mspA* gene resulted in the loss of the ability of *S. aureus* to produce some cytolytic toxins. To determine whether the reduction in the secretion of toxins was mediated through suppression of the Agr quorum sensing system, we transformed the wild type and the mutant strain using electroporation with a plasmid that contained RNAIII promoter fused to GFP (green fluorescence protein). A single colony was incubated in TSB at 37°C for 18 h with shaking at 180 rpm. The samples were diluted to 1:1 in TSB into a black fluorescence 96-well plate and 200 µl was loaded to the plate, and the readings were taken at a range of excitation of 470 nm: emission of 515 nm to detect GFP using a CLARIOstrar plate reader. Fig 3.10 shows that Agr was expressed at a lower level in the *mspA* mutant compared with the JE2 wild type strain, suggesting it was less active, which would explain the toxicity results described above.
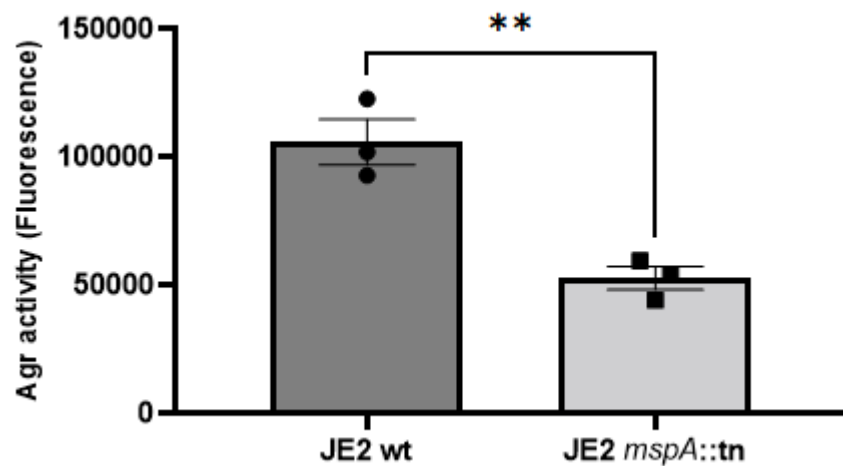
Fig 3.10: The downregulation of Agr when *mspA* is absent. Inactivation of *mspA* leads to less fluorescence which shows down regulation of the Agr system. Data represent the arithmetic mean of three independent experiments biological repeats with two technical repeats, and error bars referring to standard error of the mean Statistics were performed with t-test where the significance was determined by p values as the following: *≤ 0.05, **≤ 0.01, ***≤ 0.001.

3.3.6. MspA-dependent proteomic analysis

To understand how *MspA* might be affecting Agr activity, we wanted to determine the impact of MspA on the production of other *S. aureus* proteins. To do this a proteomic analysis of wild type (WT) *S. aureus* and *mspA* mutant was undertaken. We also wanted to identify any other regulatory changes that might explain how the loss of the *mspA* gene expression affected the cytolytic activity of the bacteria. We applied a proteomic approach of tandem mass tagging coupled to mass spectroscopy (TMT-MS) (Dayon, 2012) using whole cell lysates of the JE2 strain (WT) and its *mspA* mutant and used *S. aureus* USA300 proteome as a reference. We detected and quantified the abundance of 1149 proteins. Among these proteins, we found 63 differentially abundant proteins in the *mspA* mutant with 2 or more-fold difference (Table 3.1). This indicated that the loss of MspA had a pleiotropic effect on the *S. aureus* proteome.

Table 3.1: Differentially abundant proteins in whole cell lysate due to the absence of MspA. The small arrow refers to more/less abundant proteins in the cell lysate of the mutant compared to the wild type bacteria. Direction of the arrows, i.e, up/down refers to higher/lower abundance of a given protein in the cell lysate of the mutant bacteria compared to the wild type, respectively.

Accession #	Gene	Functional description	Fold change	Abundance	P value
Q2FVR0	*hrtB*	Hemin transport system permease protein HrtB	18.0	↑	0.0001
Q2G079	*nrdI*	Protein NrdI	5.8	↑	0.002
Q2G2P2		Globin domain protein	3.6	↑	0.001
Q2FUQ9		Cold shock protein	3.5	↑	0.027
Q2FX98		Uncharacterized protein	3.2	↑	0.003
Q2FZX4	*lipA*	Lipoyl synthase	2.7	↑	0.0001
Q2FVR1	*hrtA*	Hemin import ATP-binding protein HrtA	2.7	↑	0.006
Q2G0T9		Alpha amylase family protein	2.7	↑	0.003
Q2FXF4		Uncharacterized protein	2.7	↑	0.037
Q2FZA9	*arcC1*	Carbamate kinase 1	2.6	↑	0.0001
Q8KQR1	*isdC*	Iron-regulated surface determinant protein C	2.6	↑	0.002
P72360	*scdA*	Iron-sulfur cluster repair protein ScdA	2.6	↑	0.006
Q2G1N4		Periplasmic binding protein	2.5	↑	0.008
Q2G1X0	*hla*	Alpha-hemolysin	2.4	↑	0.008
Q2FWY2		Pyrazinamidase/nicotinamidase	2.4	↑	0.029
Q2FV74	*clpL*	ATP-dependent Clp protease ATP-binding subunit	2.4	↑	0.001
Q2G2R5		PTS system lactose-specific IIA component	2.3	↑	0.003
Q2FW75		ABC transporter periplasmic binding protein	2.3	↑	0.002
Q2FVE7	*cntA*	Peptide ABC transporter, peptide-binding protein	2.3	↑	0.004
Q2FWB7		Uncharacterized protein	2.2	↑	0.021

Accession	Gene	Protein	Fold	Direction	p-value
Q2FYK3	cfvC	Conserved virulence factor C	2.2	↑	0.004
Q2FXE1		Uncharacterized protein	2.2	↑	0.007
Q2FZB0	argF	Ornithine carbamoyltransferase	2.2	↑	0.0001
Q2FZJ9	qoxA	Probable quinol oxidase subunit 2	2.2	↑	0.006
Q2FVF9		Uncharacterized protein	2.1	↑	0.034
Q2FV17	fda	Fructose-bisphosphate aldolase class 1	2.1	↑	0.0001
Q2FZF0	isdB	Iron-regulated surface determinant protein B	2.1	↑	0.080
Q2G1Z3		Iron compound ABC transporter	2.1	↑	0.002
Q2FYN6		Uncharacterized hydrolase	2.0	↓	0.155
Q2G0L5	sdrC	Serine-aspartate repeat-containing protein C	2.0	↓	0.001
Q2G0W6		Uncharacterized protein	2.1	↓	0.006
Q2FUU5	lipA	Lipase 1	2.1	↓	0.002
Q2FWZ8	ftnA	Bacterial nonheme ferritin	2.1	↓	0.001
Q2G294		Acetyl-coenzyme A synthetase	2.1	↓	0.001
Q2FZI6	purH	Bifunctional purine biosynthesis protein PurH	2.2	↓	0.0001
Q2FVS2		Uncharacterized protein	2.2	↓	0.001
Q2G2P7	hutH	Histidine ammonia-lyase	2.2	↓	0.006
Q2FW51		Truncated major histocompatibility complex class II analog protein	2.2	↓	0.015
Q2FVQ4		L-Lactate permease	2.2	↓	0.006
Q2FV59	crtM	Dehydrosqualene synthase	2.2	↓	0.001
Q2FUX7	arcA	Arginine deiminase	2.3	↓	0.080
Q2FVE0	ahpD	Alkyl hydroperoxide reductase AhpD	2.3	↓	0.0001
Q2G1C7		Uncharacterized protein	2.3	↓	0.006
Q2FZR3		Oligopeptide ABC transporter	2.3	↓	0.004

Q2FZS2		Truncated major histocompatibility complex class II analog protein	2.4	↓	0.004
Q2G0W8		Uncharacterized protein	2.4	↓	0.008
Q2G2P5	*nikA*	Nickel-binding protein	2.4	↓	0.008
Q2FV34		Uncharacterized protein	2.5	↓	0.001
Q2G118	*parB*	Chromosome partitioning protein, ParB family	2.5	↓	0.005
Q2G1I8		Uncharacterized protein	2.5	↓	0.001
Q2FZS8	*clpB*	Chaperone protein ClpB	2.5	↓	0.001
Q2G087	*hisC*	Histidinol-phosphate aminotransferase	2.6	↓	0.047
Q2G1C9		Uncharacterized protein	2.6	↓	0.0001
Q2G0G1	*adh*	Alcohol dehydrogenase	2.8	↓	0.017
Q2FWN9		Uncharacterized leukocidin-like protein 2	2.9	↓	0.0001
Q2G1C8		Uncharacterized protein	3.0	↓	0.0001
Q2G1D0		Acetyl-coenzyme A acetyltransferase	4.4	↓	0.003
Q2G1K9		Aldehyde-alcohol dehydrogenase	4.6	↓	0.001
Q2FWP0		Uncharacterized leukocidin-like protein 1	4.9	↓	0.003
Q2FVJ5	*bioD*	ATP-dependent dethiobiotin synthetase BioD	6.3	↓	0.001
Q2G218	*ish1*	L-Lactate dehydrogenase 1	7.3	↓	0.001
Q2G091		ABC transporter	9.4	↓	0.0001
Q2FVJ7	*bioB*	Biotin synthase	9.7	↓	0.001

We selected several differentially abundant proteins for further analysis . They were the following:

- The CrtM protein (Q2FV59) that showed 2.2-fold reduction in abundance in the mutant strain. This protein is involved in the biosynthesis of the carotenoid pigment staphyloxanthin (Liu et al., 2005; Mishra et al., 2011).

Carotenoid provides a protective role against human innate immune attack, and acts alongside a protein called flotillin (FloA) in forming stable functional membrane microdomains (FMMs), which are important for membrane stability (Garcia et al., 2017). These results suggest that the stability of the bacterial membrane of the *mspA* mutants might be affected, which, in turn, might affect toxin production.

- Four proteins involved in iron homeostasis for *S. aureus* were more abundant in the *mspA* mutant. Two of them, HrtA (Q2FVR1) and HrtB (Q2FVR0), are involved in transport (efflux) of iron from the bacteria (Friedman et al., 2006), while the other two, IsdB (Q2FZF0) and IsdC (Q8KQR1), are involved in the acquisition (influx) of iron (Skaar & Schneewind, 2004). No differences in the abundance of Fur, a ferric uptake regulatory protein, or any other iron-heme related proteins were detected. Results of proteomic analysis for these four proteins indicated a possible impairment in the machinery of iron homeostasis inside the bacteria due to the knockout of the *mspA* gene.

- The Hla protein (alpha toxin) (Q2G1X0) was more abundant in the cell lysate of the mutant than in the wild type bacteria aligning with our finding by Western blotting that this protein was more abundant in whole cell lysate and less abundant in the bacterial supernatant.

3.3.7. Effect of *mspA* knockout on carotenoid biosynthesis

Staphyloxanthin is a carotenoid and is an important contributor to the ability of *Staphylococcus aureus* to protect itself from the human immune system. *S. aureus* can survive for a longer time and become widely distributed in hospital settings when the strain can produce staphyloxanthin (Beard-Pegler et al., 1988) (Xue et al., 2019). Strains producing staphyloxanthin are also more resistant to neutrophil killing (Liu et al., 2005). The CrtM protein helps the bacteria to form stable functional membrane domains, and it works alongside flotillin, encoded by the *floA* gene, which is involved in staphyloxanthin biosynthesis (Liu et al., 2005; Mishra et al., 2011; Garcia et al., 2017). Our proteomic data suggested that CrtM was less abundant in the *mspA* mutant compared to the wild type bacteria. To determine whether the mutant produced less staphloxanthin due to the low abundance of CrtM, we quantified the pigmentation in the wild type and *mspA* mutant as well as in *crtM* and *floA* mutants in the JE2 background. Complementing the proteomic data, the results indicated significant reductions in staphyloxanthin production in both *mspA* and *crtM* mutants, while no significant difference in staphyloxanthin production between the wild type and *floA* mutant (Fig 3.11). The means of JE2 *floA*::tn showed the highest significant value among on strains, while the JE2 *crtM*::tn showed the lowest.
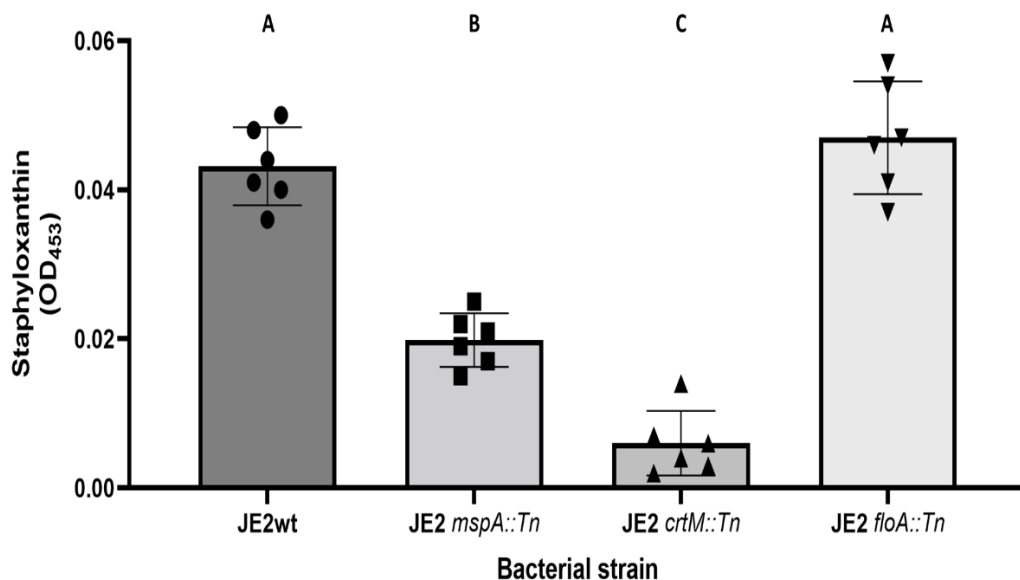
Fig 3.11: Staphyloxanthin production in *mspA, crtM* and *floA* mutants compared to the wild type in the JE2 background. Data represent the arithmetic mean of six independent experiments biological repeats with two technical repeats, and error bars the standard error of the mean. Statistics were performed with one-way ANOVA using Tukey for multiple comparison.

The integrity of the bacterial membrane bound components is important for the activity of all biological systems including the Agr system. With significantly less staphyloxanthin in the bacterial membrane of the *mspA* mutant, it is possible that this might directly affect the membrane and as a consequence plays a role in the effect we have observed on toxicity. To test this, we compared the toxicity of the *floA* and *crtM* mutants alongside the wild type JE2 and *mspA* mutant (Fig 3.12). The means of JE2 *crtM*::tn showed the highest significant value among on strains, while the JE2 *mspA*::tn showed the lowest. Toxicity was only affected in the *mspA* mutant suggesting that neither staphyloxanthin nor flotillin and FMMs are involved in the production of cytolytic toxins by *S. aureus*. This suggested that while MspA is involved in staphyloxanthin biosynthesis, this is unrelated to its effect on toxin production.
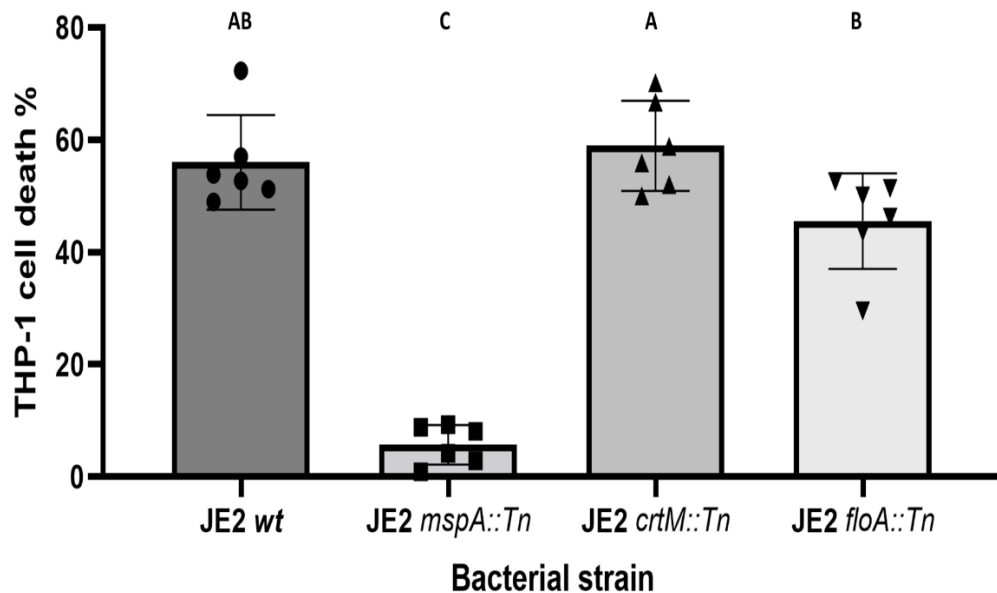
Fig 3.12: THP-1 cell death relating to toxin production/secretion in *mspA, crtM* and *floA* mutants compared to the wild type in the JE2 background. Data represent the arithmetic mean of six biological repeats with two technical repeats, and error bars the standard error of the mean. Statistics were performed with one-way ANOVA using Tukey for multiple comparison.

3.3.8. The sensitivity of *mspA* mutants to streptonigrin

With the proteomic analysis revealing that several proteins involved in either uptake or efflux of iron were differentially abundant, we hypothesised that the *mspA* mutant might be impaired in either iron sensing or uptake processes. To test this, we utilized the antibiotic streptonigrin, which can be used as an indirect measure for intracellular iron concentrations. The bactericidal action of the antibiotic streptonigrin is dependent upon intracellular iron (Dyer, Mckenna, & Woods, 1987).As such, resistance to streptonigrin can be used as a proxy for the level of intracellular iron within a bacterial cell. Streptonigrin causes RNA damage and breaks DNA strands in the presence of iron. The interaction between streptonigrin hydroquinone and O_2 in the presence of iron results in the generation of O_2 radicals, which causes the DNA and RNA damages (Dyer et al., 1987) (Bolzán & Bianchi, 2001). Forty µl streptonigrin at a concentration of 200 µg/ml was spotted onto filters that were transferred to wild type and *mspA*::tn growth plates. This experiment was performed for *mspA* mutants in both genetic backgrounds JE2 and SH1000. The plates were incubated to allow the bacteria to grow. The results indicated that the zone of clearance, as a result of the diffusion of the antibiotic, was larger for *mspA* mutants (Fig 3.13A), suggesting that the mutant contains higher levels of intracellular iron. In addition, we utilized a serial dilutions of streptonigrin in a 96-well plate and measured the OD_{600} after 8 hours growth of *mspA* mutant in the JE2 and SH1000 backgrounds as compared to the corresponding wild type strains and we used *fur* knockout mutant in JE2 background as a control (Fig 3.13B). For the JE2 strain at a streptonigrin concentration of 0.2344 µg/ml, there was a difference in the ability of the wild type and mutant to grow, where the *mspA* mutant was more sensitive, suggesting that the mutant had a higher intracellular concentration of iron. For the SH1000 strain at a streptonigrin concentration of 0.05866 µg/ml, there were differences between the wild type and *mspA* mutant in their ability to grow. This suggested that the *mspA* mutant in both backgrounds was more sensitive to streptonigrin and may, therefore, have higher intracellular iron relative to the corresponding wild type strains. That supports our hypothesis that iron homeostasis processes are impaired in the *mspA* mutant. Expectedly, the *fur* mutant showed a reduction in growth with the increasing concentration of streptonigrin.

a.
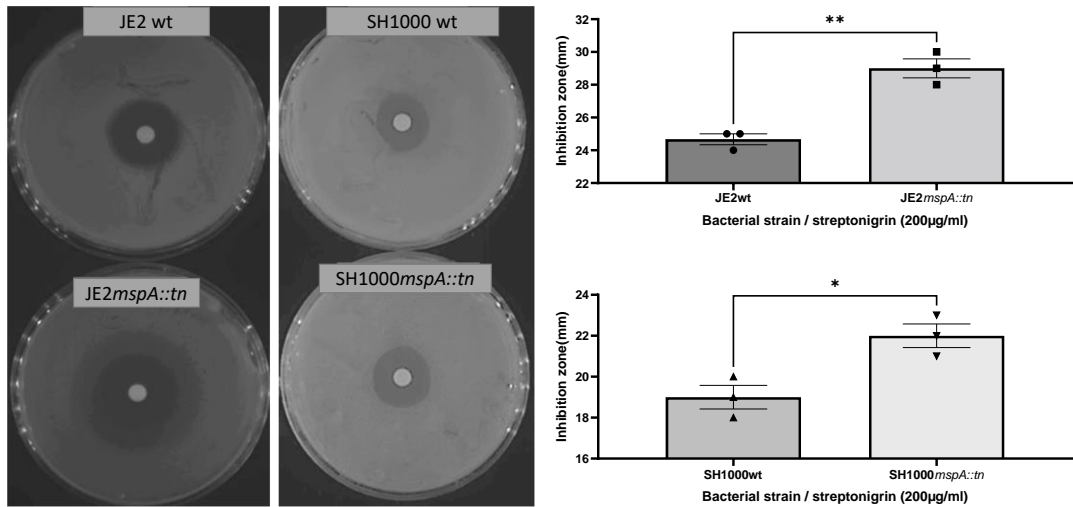
b.
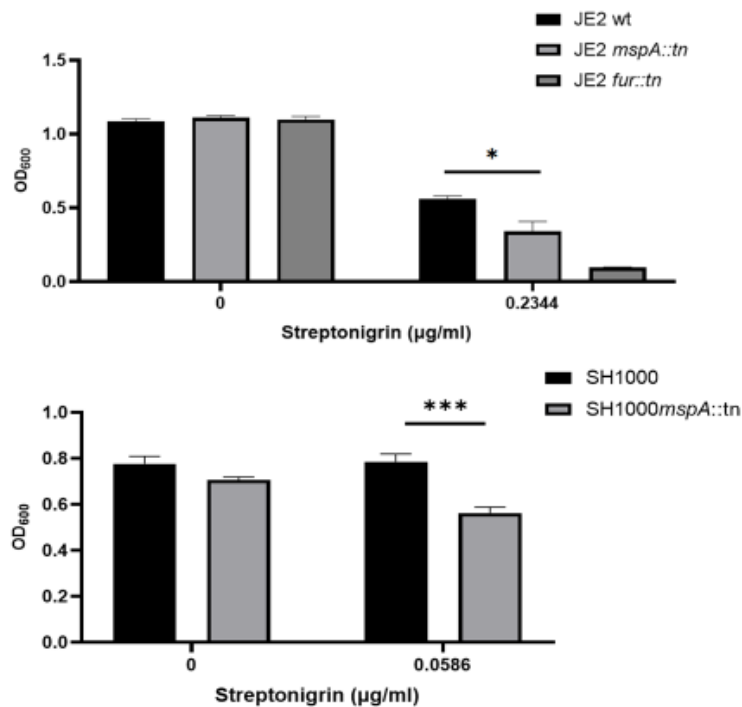
Fig 3.13: Comparison of sensitivity to streptonigrin between wild type and *mspA* mutant. (A) The zone of clearance was larger for the *mspA* mutant in the JE2 background as a result of the diffusion of the antibiotic. (B) To verify whether MspA contributes to iron uptake or sensing, we used streptonigrin at (0, 0.2344 µg/ml) for JE2 and (0, 0.0586µg/ml) for SH1000 background to exert its antibacterial activity. The *mspA* mutant in either JE2 or the SH1000 backgrounds was more sensitive to the antibiotic suggesting that the mutant had a higher abundance of intracellular iron. *fur* mutant showed a severe reduction of cell growth at the highest concentration. Data represent the arithmetic mean of three biological repeats with two technical repeats, and error bars represent standard error of the mean Statistics were performed with t-test where the significance was determined by p values as the following: *≤ 0.05, **≤ 0.01, ***≤ 0.001.

3.3.9. Effect of *mspA* inactivation on *Staphylococcus aureus* adaptation to iron

We constructed a heme adaption experiment to see if the activity of the Hrt system affects the *mspA* mutant, given that HrtA and HrtB proteins were more abundant. Bacteria can adapt to stressful environments, such as high iron, by altering the expression of proteins that allow them to manage the stress. For *S. aureus*, we can grow the bacteria overnight in low to moderate levels of iron and they can adapt the expression of their iron uptake (e.g.Isd) and efflux systems (e.g Hss and Hrt) to control how much iron is contained in the cell. Once adapted following overnight growth, the bacteria can survive and grow even if exposed to higher levels of iron much more rapidly, and this is the basis of our iron adaption assay. To determine the contribution of MspA in *S. aureus* to growth in a high iron environment, we grew wild types and *mspA* mutants of the two backgrounds overnight with and without hemin, and then recultured the bacteria with different hemin concentrations. We have chosen 0 and 40 µM to present here. When MspA was absent, neither bacterial strain could adjust well to media containing high iron concentration (Fig 3.14). The means on 0µM, JE2 wt. with no hemin overnight showed the highest significant value among on strains, while the knocked out JE2 *mspA*::tn with hemin overnight showed the lowest. For the means of 40µM, JE2 wt. with hemin overnight showed the highest significant value among on strains, while the knocked out JE2 *mspA*::tn with no hemin overnight showed the lowest. Fig 3.15 The means on 0µM, SH1000 wt. with no hemin overnight showed the highest significant value among on strains, while the knocked out SH1000 *mspA*::tn with hemin overnight showed the lowest. For the means of 40µM, SH1000 wt. with hemin overnight showed the highest significant value among on strains, while the SH1000wt. with no hemin overnight showed the lowest. In both the JE2 and SH1000 backgrounds, the *mspA* mutants were reduced in their ability to adjust to the presence of hemin compared to the wild-type strain. ANOVA test using Tukey was performed to compare the wt. vs. *mspA* mutant for the different conditions. This showed that, despite the increased abundance of Hrt proteins, the system's efflux function was reduced, which explains the increased intracellular iron concentrations reported.
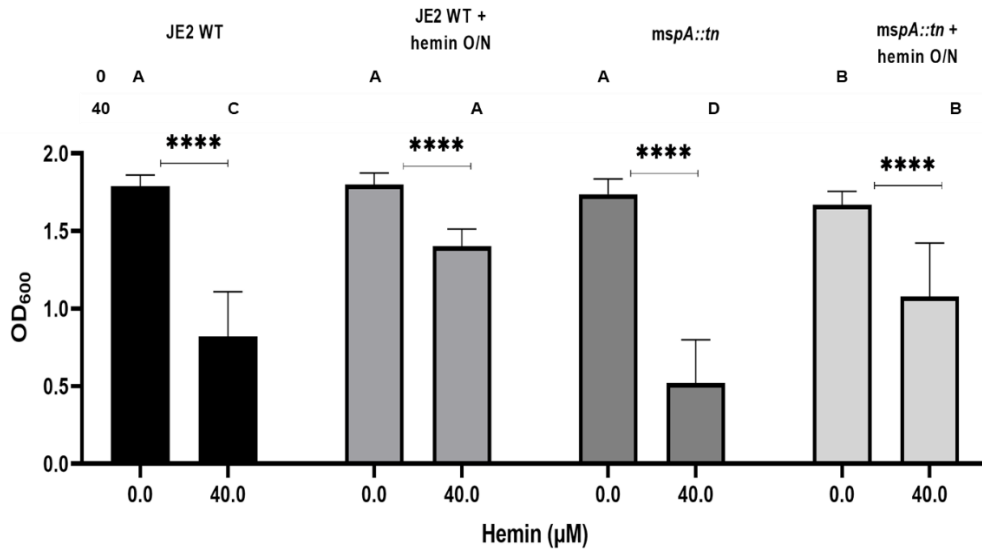
Fig 3.14: Comparison between *S. aureus* wild type JE2 strain and *mspA*::tn mutant for their contribution to the adaptation to high iron concentration. In this assay, bacteria were cultured overnight in TSB or hemin (10 µM), then reinoculated 1:1000 into TSB containing hemin 40 µM for 8 hours. Data represent the arithmetic mean of three independent experiments, and error bars represent standard error of the mean ANOVA test using Tukey was performed to compare the wt vs. *mspA* mutant for the different iron conditions. Another Statistics were performed with t-test where the significance was determined by p values as the following: *≤ 0.05, **≤ 0.01, ***≤ 0.001 and ****≤ 0.0001.
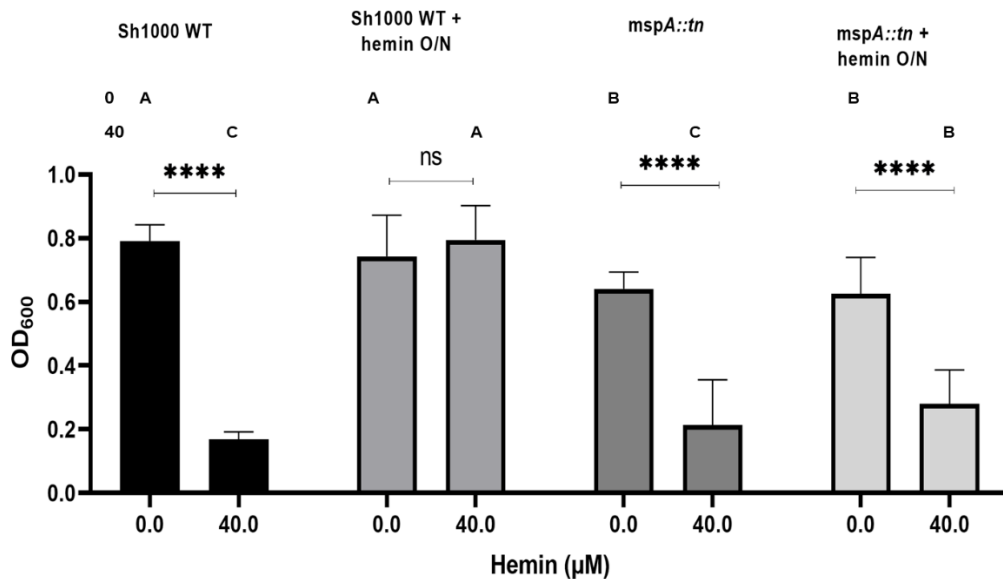
Fig 3.15: Comparison between *S. aureus* wild type SH1000 strain and *mspA*::tn mutant for their contribution to the adaptation to high iron concentration. In this assay, bacteria were cultured overnight in TSB or hemin (10 µM) then reinoculated 1:1000 into TSB containing hemin 40 µM for 8 hours. Data represent the arithmetic mean of three independent experiments, and error bars represent standard error of the mean ANOVA test using Tukey was performed to compare the wt vs. *mspA* mutant for the different iron conditions. Another Statistics were performed with t-test where the significance was determined by p values as the following: *≤ 0.05, **≤ 0.01, ***≤ 0.001 and ****≤ 0.0001.

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3.3.10. Effect of differential abundance of hemin on *Staphylococcus aureus* toxicity

Given the potentially toxic effect that iron has on bacteria, it was possible that its presence at a higher level in the bacterial cytoplasm was responsible for the reduction in cytolytic activity of the *mspA* mutant cells. To examine this, we sought to induce this condition by performing toxicity assays with the bacterial supernatant following growth in increasing levels of iron. The results demonstrated that by adding more hemin to the media, wild type *S. aureus* reduces ability to cause THP-1 cell death (Fig 3.16 and 3.17) The means of JE2wt and SH1000wt with 0 µM hemin showed the highest significant value , while 10µM showed the lowest. This suggest that the increased intracellular iron in the *mspA* mutants may participate in the observed reduction of toxicity.
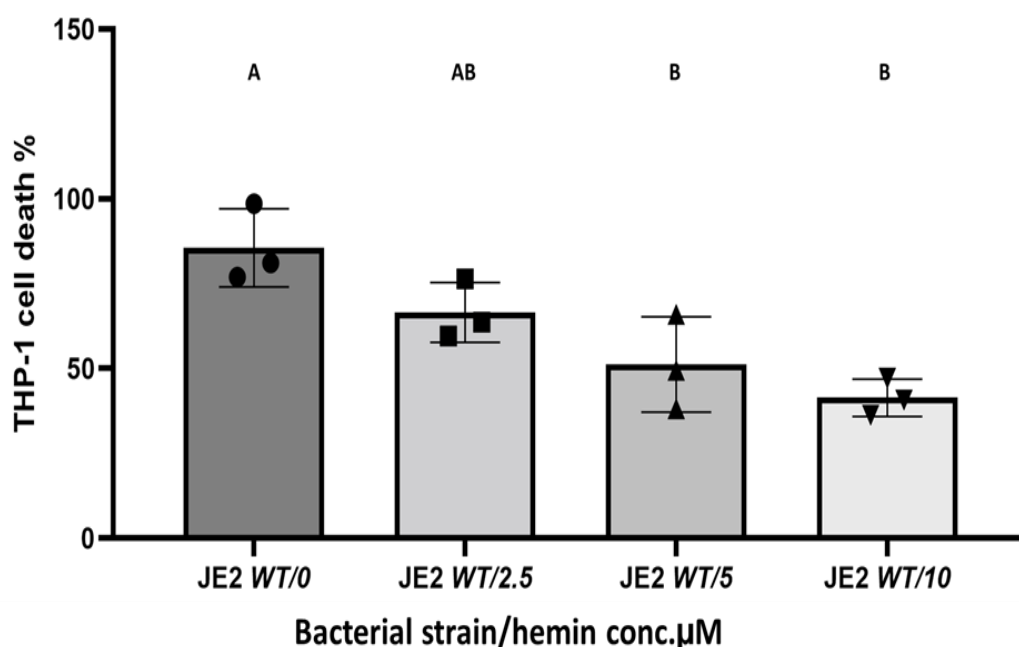
Fig 3.16: Effect of increased hemin on toxin production in wild type JE2 strain. . Data represent the arithmetic mean of three biological repeats with two technical repeats, and error bars the standard error of the mean. Statistics were performed with one-way ANOVA using Tukey for multiple comparison.
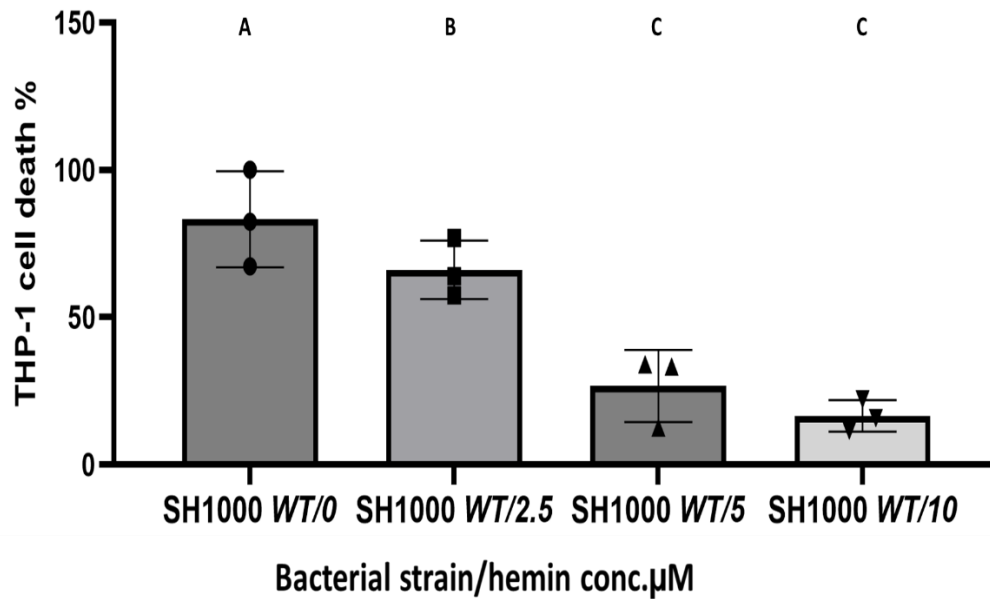
Fig 3.17: Effect of increased hemin on toxin production in wild type SH1000 strain. Data represent the arithmetic mean of three biological repeats with two technical repeats, and error bars the standard error of the mean. Statistics were performed with one-way ANOVA using Tukey for multiple comparison.

As the PSMs were the primary group of toxins downregulated in the *mspA* mutant we sought to examine whether these were also affected by hemin. To test this, the wild types JE2 and SH1000 strains were cultured overnight in various concentrations of hemin (0, 2.5, 5 & 10 μM/ml). The PSMs were precipitated from bacterial supernatants, and proteins were run on SDS-PAGE gels, which were stained overnight using Blue Safe protein stain. The results indicated a gradual decrease in PSM secretion associated with the increase in hemin concentration (Fig 3.18).
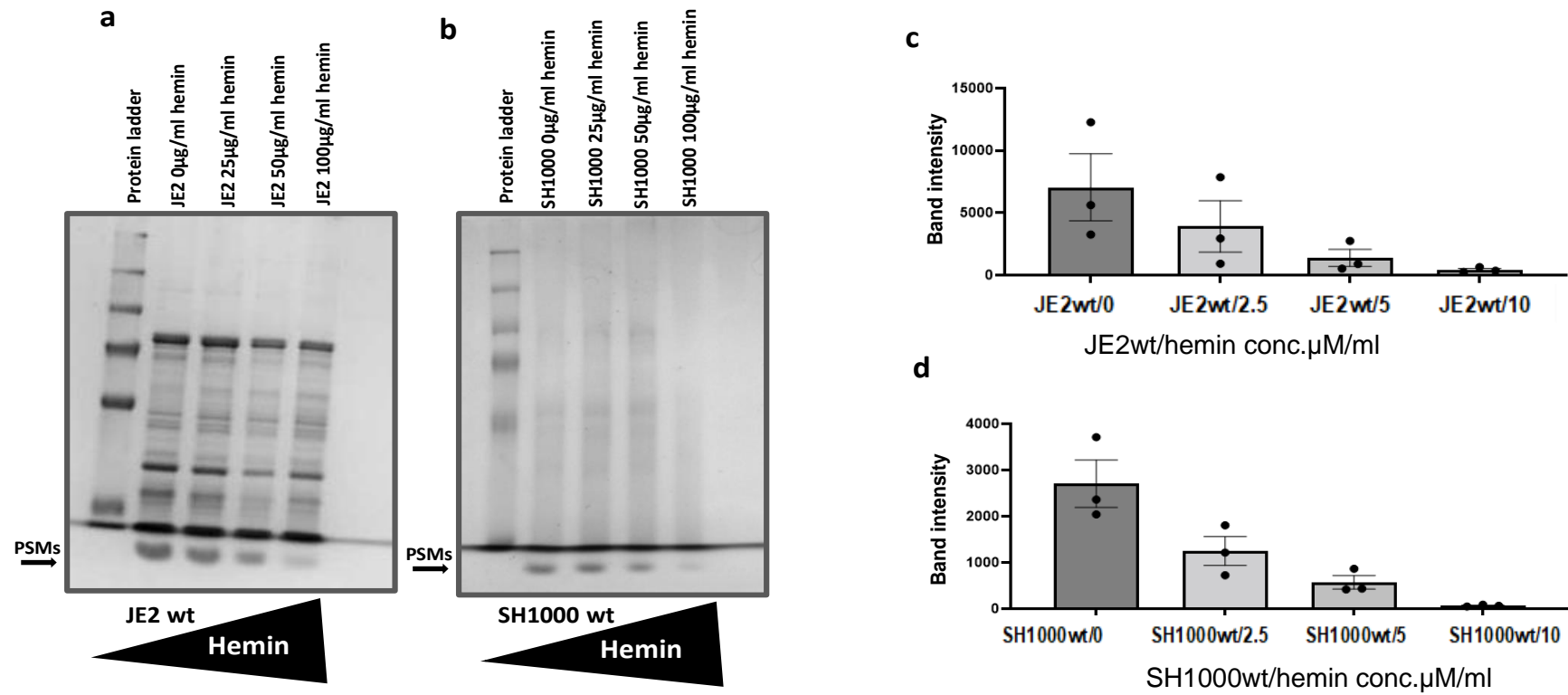
Fig 3.18: SDS-PAGE showing the effect of increased hemin on toxin production of the wild types JE2 and SH1000 strains. Bacteria were cultured overnight in various concentrations of hemin. Toxins (PSMs) were precipitated from bacterial supernatants in JE2 wild type (a) and SH1000 wild type (b). Bar chart showing the replicates of measuring the band intensity using ImageJ (c) in JE2 and (d) in SH1000 background.

3.3.11. Effect of other iron homeostasis genes on toxicity

3.3.11.1. The ability of different *Staphylococcus aureus* iron homeostasis mutants to lyse the THP-1 cell line

As we found that the inactivation of MspA affects iron homeostasis and this appeared to be contributing to the observed effects on toxicity, we sought to determine if other iron regulatory proteins were also involved. The ability of JE2 *hrtA::tn ,* JE2 *hrtB::tn* and JE2 *fur::tn* mutants in the JE2 background to cause THP-1 cell death was quantified as these genes are involved in iron homeostasis. Fig 3.19 shows that there was a statistically significant reduction in the ability of JE2 *hrtB::tn* to produce cytolytic toxins, but not the JE2 *hrtA::tn* and JE2 *fur::tn* mutants. And the means of JE2 *fur*::tn showed the highest significant value among on strains, while the JE2 *hrtB*::tn showed the lowest.
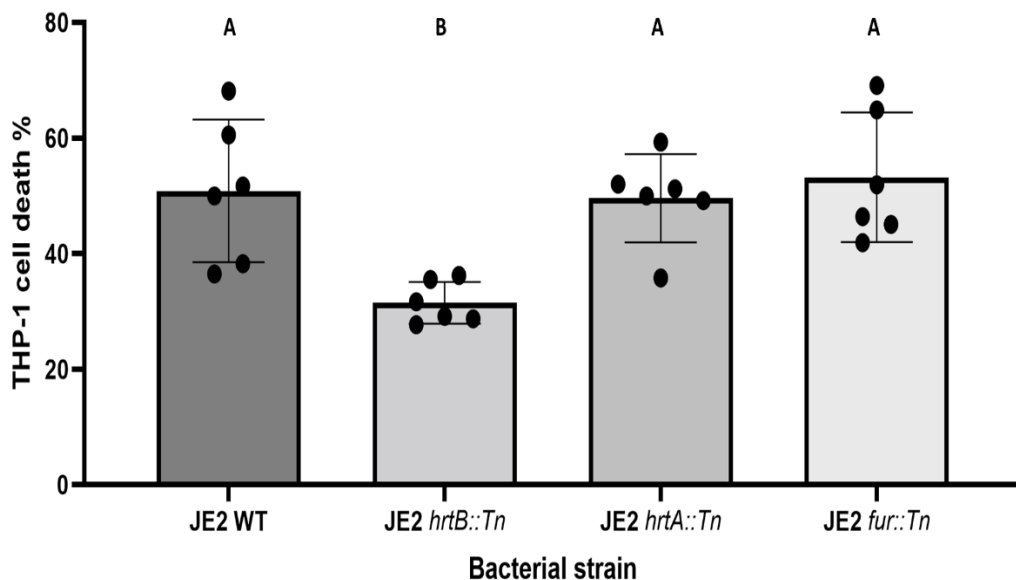
Fig 3.19: The ability of different iron homeostasis-related genes to lyse THP-1 cells when knocked out compared to the wild type. Inactivation of the *hrtB* gene slightly reduced the ability of the bacteria to produce cytolytic toxins and thus, to cause THP-1 cell death. No effects were observed when the other two genes, *hrtA* and *fur*, were knocked out compared to the wild type. Data represent the arithmetic mean of six biological repeats with two technical repeats, and error bars the standard error of the mean. Statistics were performed with one-way ANOVA using Tukey for multiple comparison.

3.3.11.2. Effect of different *Staphylococcus aureus* mutants with impaired iron homeostasis on PSMs production

To determine whether different genes involved in the control of intracellular iron affected PSMs production, we utilized the TCA precipitation method (described above) to isolate PSMs from bacterial supernatants. The samples were run on SDS-PAGE gels as described. The results suggested that PSM production from JE2 *hrtB::tn*, JE2 *hrtA::tn* and JE2 *fur::tn* were almost identical to the wild type, thus, these mutations did not affect PSMs production (Fig 3.20).
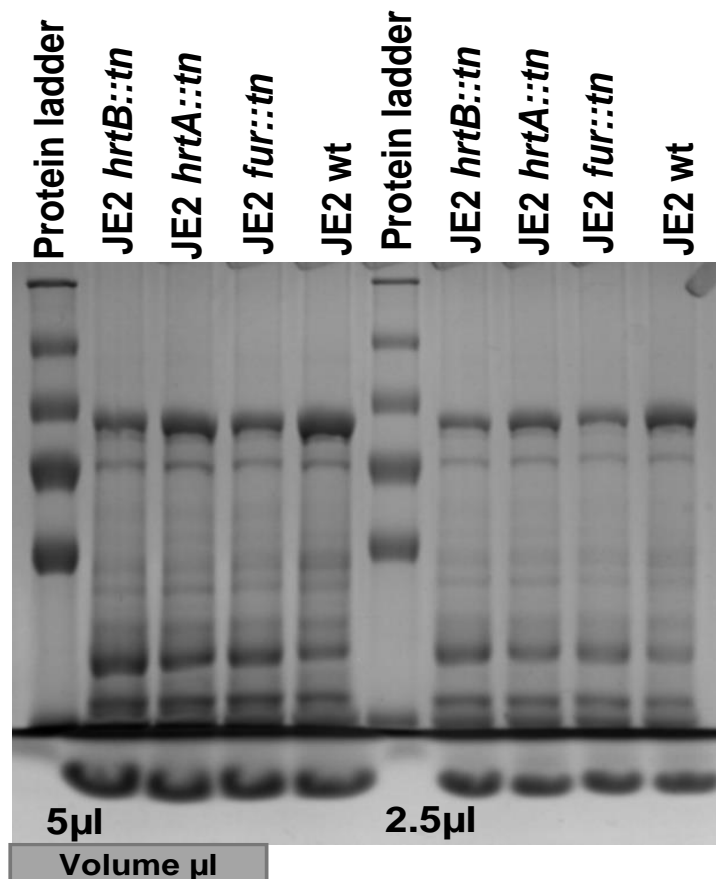
Fig 3.20: PSMs production of the mutant JE2 strains *hrtB, hrtA* and *fur* compared to the wild type. No effects of these mutations were seen on PSMs production, the samples were loaded each in two volumes (5 µl and 2.5 µl).

3.3.11.3. Detection of the sensitivity of JE2 *hrtB::tn* and JE2 *hrtA::tn* mutants to streptonigrin

As the HrtAB system is responsible for iron efflux, we sought to determine whether the JE2 *hrtB::tn* and JE2 *hrtA::tn* mutants had high intracellular iron levels, and so we performed the streptonigrin assay (described above). The result was a surprise as we did not see a difference. This was an unexpected result as we expected that HrtA and HrtB mutants would have a problem with iron efflux uptake, but they did not show that in this result (Fig 3.21).
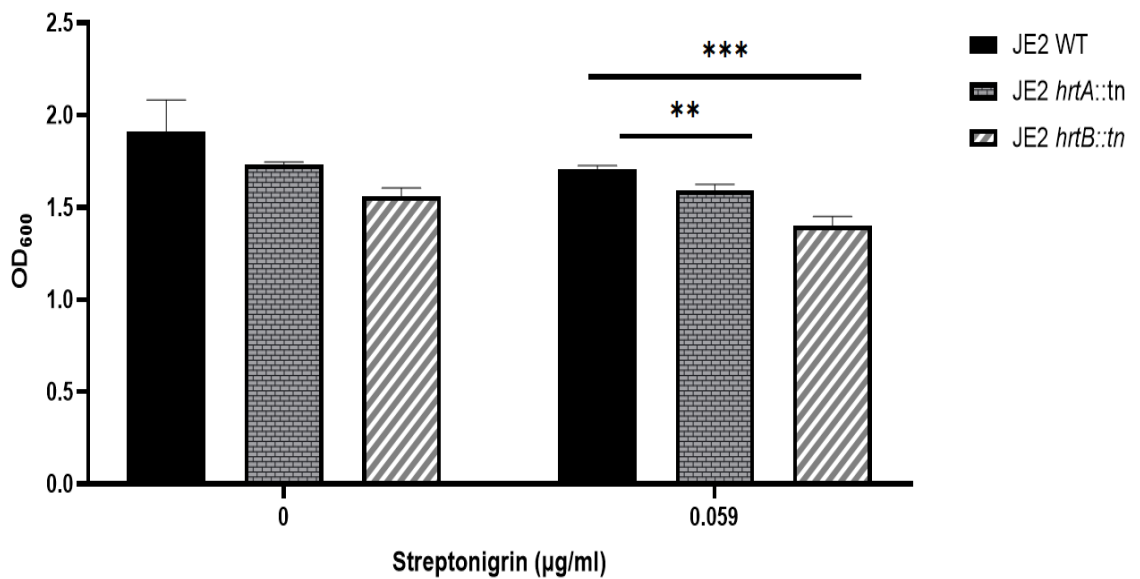
Figu 3.21: Comparison of sensitivity of wild type to streptonigrin compared to *hrt* mutants. *hrtA* and *hrtB* genes are essential in regulating and transporting iron from the cell. The results showed that these mutants are sensitive to streptonigrin, which suggests that mutants have more intracellular iron. *hrtB* mutant is more sensitive to streptonigrin than *hrtA*, so the former has a higher abundance of iron. Data represent the arithmetic mean of three biological repeats with two technical repeats, and error bars represent standard error of the mean Statistics were performed with t-test where the significance was determined by p values as the following: *≤ 0.05, **≤ 0.01, ***≤ 0.001.

3.3.11.4. JE2 *hrtB::tn* and JE2 *hrtA::tn* mutants lack adaptation to high iron level

To determine the effects of lacking *hrtB* and *hrtA* gene expression on bacterial growth in iron-rich media, we performed a hemin adaptation assay (described above), found that when *hrtA* and *hrtB* gene expression absent, the bacteria adapt to rich iron media, which was the opposite of what we had seen for the *mspA::tn* strain (Fig 3.22). The means on 0µM, JE2 *hrtA*::tn with no hemin overnight showed the highest significant value among on strains, while the JE2 wt. with no hemin overnight showed the lowest. For the means of 40µM, JE2 wt. with hemin overnight showed the highest significant value among on strains, while the knocked out JE2 *hrtA*::tn with no hemin overnight showed the lowest. We concluded that *hrtA* and *hrtB* do not contribute to bacteria adaptation in iron rich media.
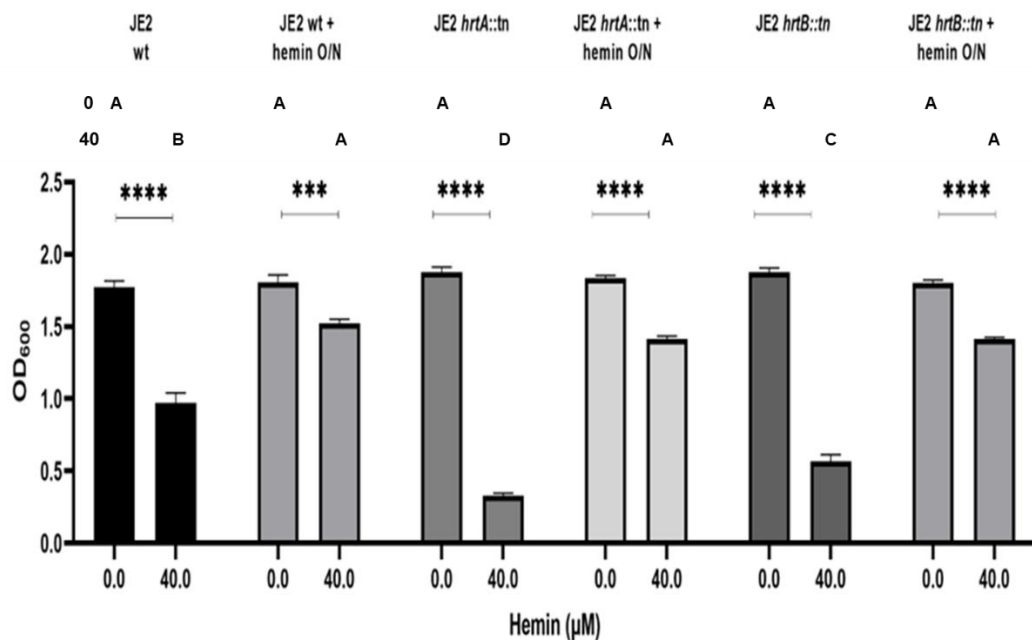
Fig 3.22*: hrtA* and *hrtB* mutants retain *S. aureus* adaptation to high iron concentration. In this assay, bacteria were cultured overnight in TSB or hemin (10 µM) then reinoculated 1:1000 into TSB containing hemin 40 µM for 8 hours. Data represent the arithmetic mean of three independent experiments, and error bars represent standard error of the mean ANOVA test using Tukey was performed to compare the wt vs. *hrtA and hrtB* mutants for the 0 and 40µM iron conditions. Another Statistics were performed with t-test where the significance was determined by p values as the following: *≤ 0.05, **≤ 0.01, ***≤ 0.001 and ****≤ 0.0001.

3.3.12. *Staphylococcus aureus mspA*-neighbouring genes

As the proteins encoded by co-located genes often work together, we examined whether *mspA*-neighbouring genes also affected toxicity. We moved the transposon from mutants in genes either side of *mspA* from the JE2 background (Fig 3.23) to the SH1000 background to compare knockout mutants of these three genes in the two different genetic backgrounds.

3.3.12.1. Moving the transposons of neighbouring genes to SH1000 background

Phage lysate (ϕ11) was used to move the transposons within SAUSA300_2211 (encoding hypothetical protein), SAUSA300_2212 (encoding hypothetical protein) and SAUSA300_2213 (encoding AcrB/AcrD/AcrF family protein) loci from the JE2 background into the wild type SH1000 background. DNA contained within the phage lysate of the three mutants was transduced into SH1000 wild type via ϕ11 as previously described. Successful transductants should contain an inserted transposon with a gene for erythromycin resistance, which we used to select for transformants. Oxacillin sensitivity was also confirmed to ensure that none of the original mutants within the JE2 background survived in the phage lysate process (Fig 3.23a/b). The correct construction of the new mutants that were both erythromycin-resistant and oxacillin sensitive was further confirmed by PCR. A schematic representation of the insertions within the three genes and expected amplicon sizes (316, 627 & 1058 bp, respectively) during PCR with a primer within the transposon and the other within the genebeing mutated is shown in (Fig 3.24). The results of PCR confirmed successful transduction from the JE2 background to the SH1000 background (Fig 3.23c).

a.

SH1000 *NE42::tn*
SAUSA300_2212

SH1000 *NE627::tn*
SAUSA300_2211

SH1000 *NE866::tn*
SAUSA300_2213

b.

SH1000 *NE42::tn*
SAUSA300_2212

SH1000 *NE627::tn*
SAUSA300_2211

SH1000 *NE866::tn*
SAUSA300_2213

c.

SH1000 *NE42::tn*
SAUSA300_2212

SH1000 *NE627::tn*
SAUSA300_2211

SH1000 *NE866::tn*
SAUSA300_2213
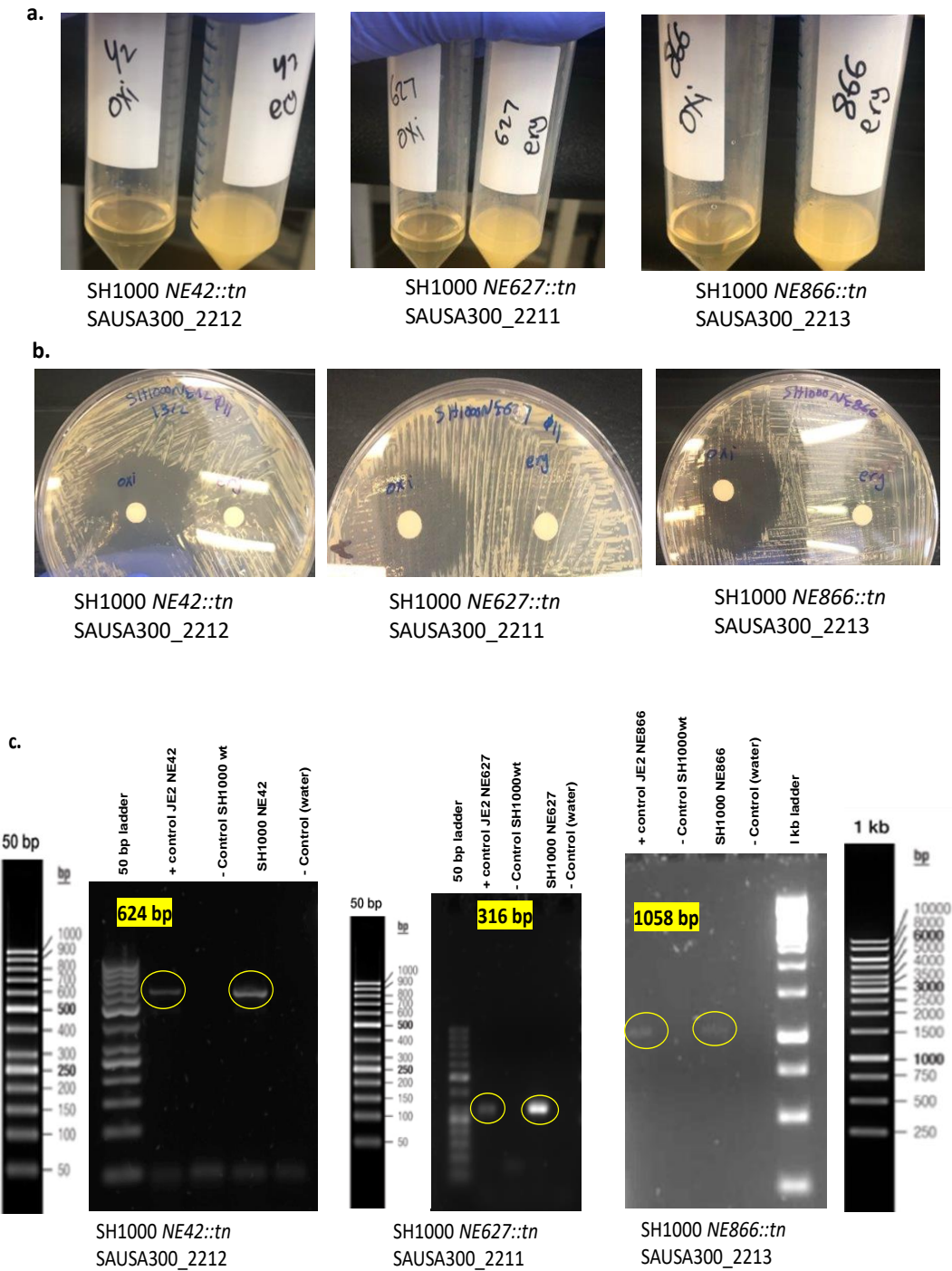
Fig 3.23: Verification of transposon movement via φ11 from the JE2 to SH1000 backgrounds for SAUSA300_2211 (NE627) mutant, SAUSA300_2212 (NE627) mutant and SAUSA300_2211 (NE627) mutant. (a and b) The strains were grown on two media (a) broth and (b) agar, one containing oxacillin and the other containing erythromycin (5 µg/ml). The results show that the mutants are sensitive to oxacillin and resistant to erythromycin. (c): Agarose gel electrophoresis of the PCR product from the SH1000 background transposon mutant showing an amplicon of the expected size (labelled in yellow in each gel). Mutants in the JE2 background were used as a positive control, while SH1000 wild type was used as a negative control.
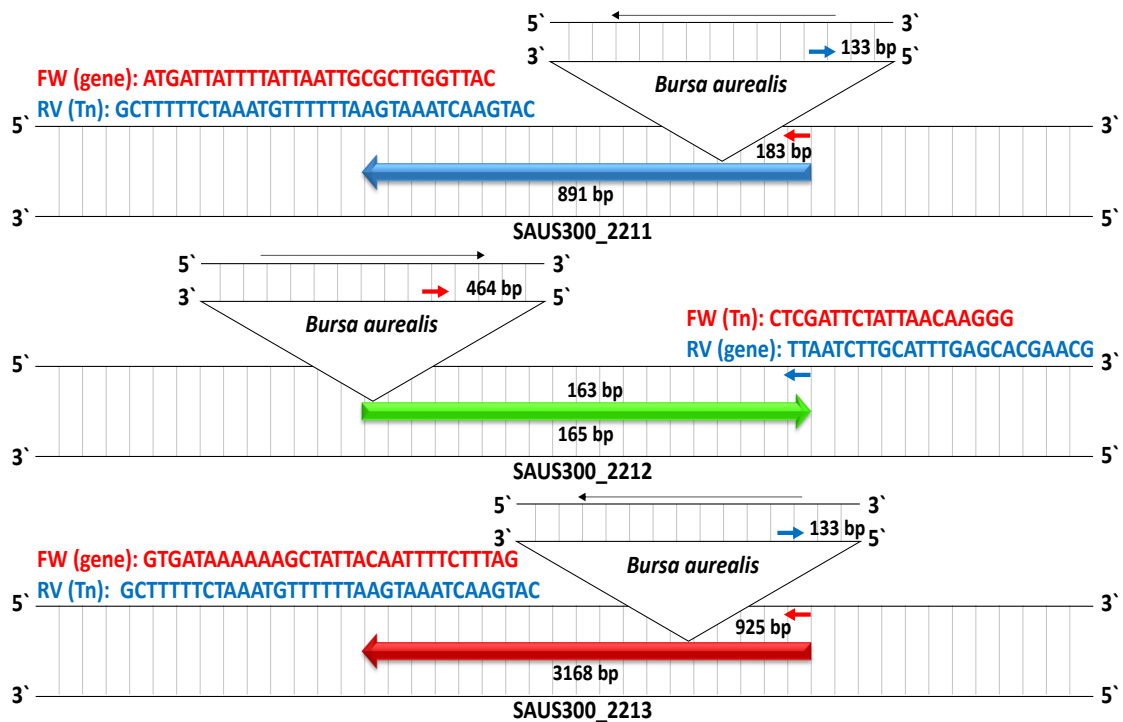
Fig 3.24: Schematic representation of the transposon insertion (*Bursa aurealis*, 3237 bp) within each of the three loci SAUSA300_2211, SAUSA300_2212 and SAUSA300_2213. Expected amplicon sizes (316, 627 and 1058 bp, respectively) during PCR with one primer within the transposon and the other primer within one of the genes being mutated are indicated.

3.3.12.2. *Staphylococcus aureus mspA*, but not neighbouring genes, contributes to toxin secretion

To determine whether the genes to either side of the *mspA* gene were involved in toxin production, we measured the ability of bacterial supernatants of the transposon mutants in the two genetic backgrounds (JE2 & SH1000) to cause THP-1 cell death. We found that the bacteria lost the ability to secrete cytolytic toxins and cause THP-1 cell death when *mspA* was inactivated but not when the expression of the neighbouring genes was absent (Fig 3.25 and 3.26) . In fig 3.25 the means showed the highest significant value in the JE2wt. strain , while JE2*mspA*::tn showed the lowest. The means of Fig 3.26 showed in SH1000 NE866 the highest significant value, while SH1000*mspA*::tn showed the lowest.
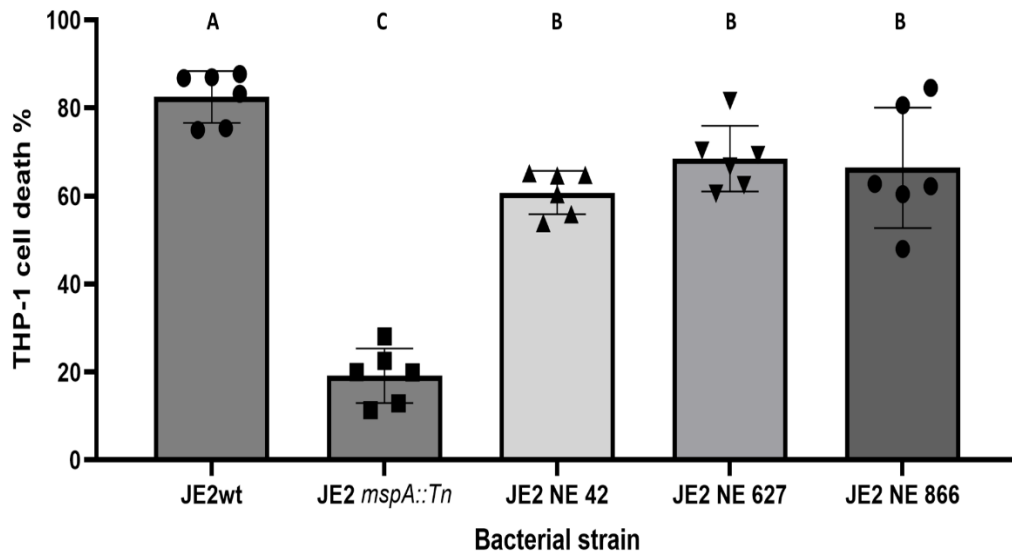
Fig 3.25: JE2 *mspA*::tn, but not neighbouring genes contributes to toxicity. Inactivation of the *mspA* gene in the JE2 background leads to reduced ability to cause THP-1 cell death due to loss of toxin secretion but this effect is not apparent when *mspA*-neighbouring genes are knocked out. . Data represent the arithmetic mean of six biological repeats with two technical repeats, and error bars represent the standard error of the mean. Statistics were performed with one-way ANOVA using Tukey for multiple comparison.
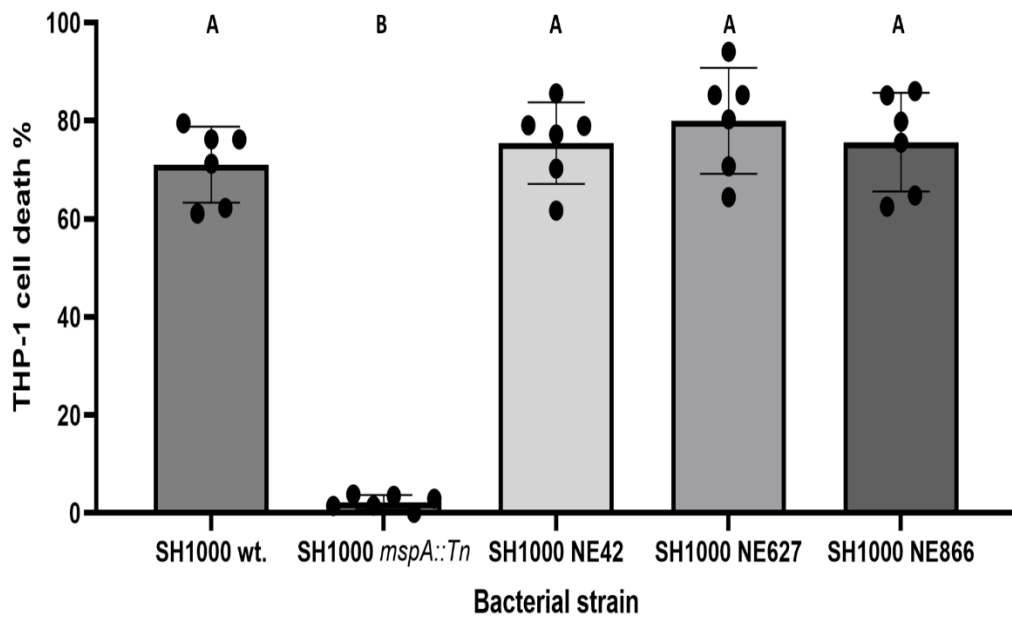
Fig 3.26: SH1000 *mspA*::tn, but not neighbouring genes contributes to toxicity. Inactivation of *mspA* gene in SH1000 background leads to reduced ability to cause THP-1 cell death due to loss of toxin secretion but this effect is not apparent when *mspA*-neighbouring genes are knocked out. . Data represent the arithmetic mean of six biological repeats with two technical repeats, and error bars represent the standard error of the mean. Statistics were performed with one-way ANOVA using Tukey for multiple comparison

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3.3.12.3. *Staphylococcus aureus mspA*, but not neighbouring genes, affects PSM production

To determine the effects of *mspA* and its neighbouring genes on PSM production, we extracted total proteins including low-molecular-weight PSMs from bacterial supernatant as described above by the trichloroacetic acid (TCA) precipitation method and analysed them by SDS-PAGE. We found that when the *mspA* gene was inactivated, this led to decreased PSMs production in both genetic backgrounds, but not when *mspA*-neighbouring genes were knocked out (Fig 27a & b). The effect of MspA was less clear in the JE2 background compared to that in the SH1000 background.
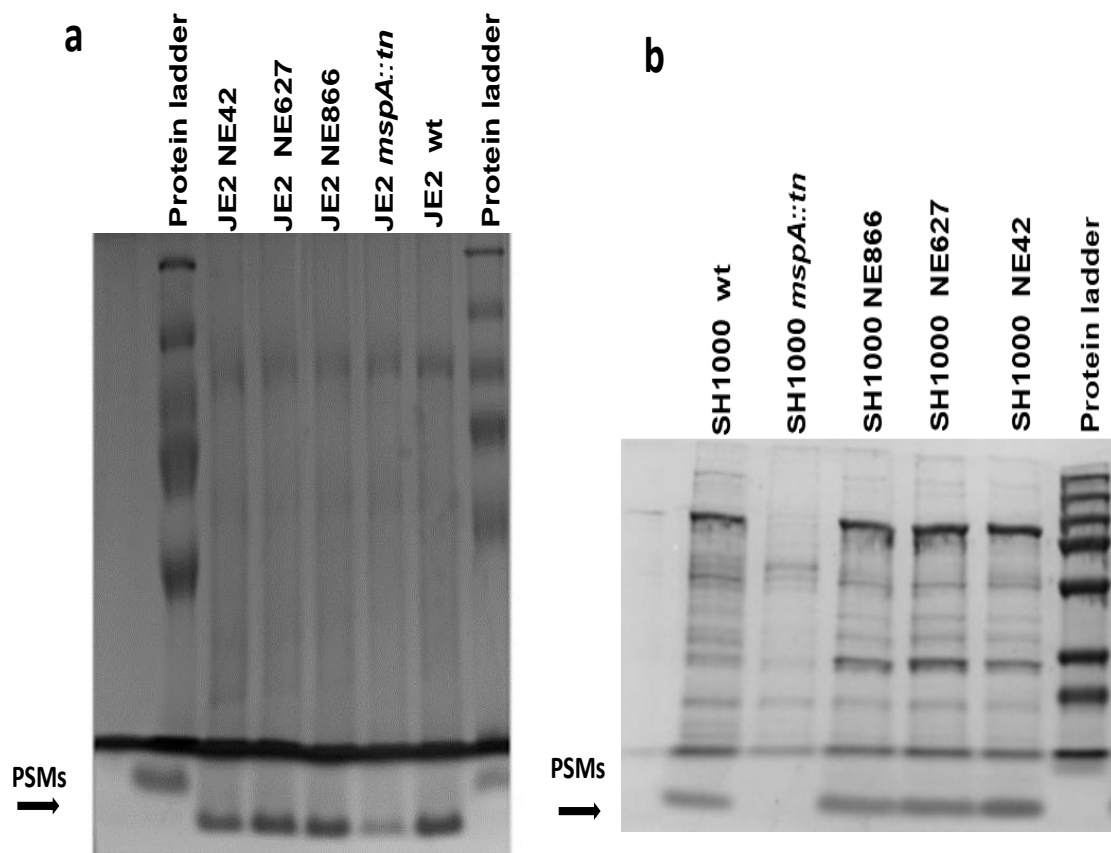
Fig 3.27 *mspA*, but not neighbouring genes, affects PSM production. Inactivation of *mspA* leads to reduced PSMs production in both backgrounds of (b) SH1000 and (a) JE2. These data indicate that *mspA*-neighbouring genes do not contribute to PSMs production.

3.3.12.4 The sensitivity of *mspA* and neighbouring genes to streptonigrin

To determine whether intracellular iron levels varied in knockout mutants of the *mspA*-neighbouring genes compared to the *mspA* mutant, we performed streptonigrin resistance assays (described above). The results confirmed that the *mspA* mutant in both genetic backgrounds was more sensitive to streptonigrin, however the mutants of the *mspA* neighbouring genes were as resistant as the wild type strains in both the JE2 and SH1000 background (Fig 3.28 & 3.29). This suggested that the genes neighbouring *mspA* are not involved in iron homeostasis.
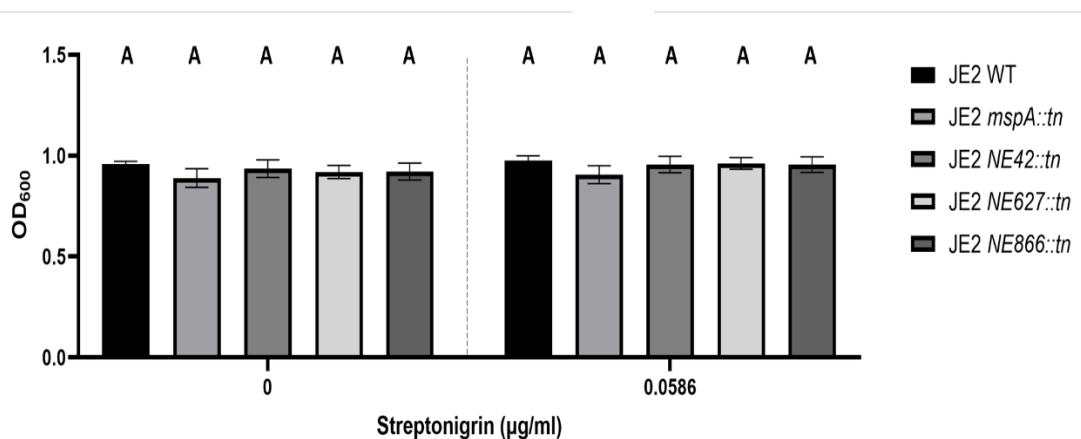
Fig 3.28: The sensitivity of the JE2 *mspA* mutant to streptonigrin compared to mutants of its neighbouring genes. *mspA* mutant is more sensitive to streptonigrin suggesting it has more intracellular iron. . Data represent the arithmetic mean of six biological repeats with two technical repeats, and error bars reprents the standard error of the mean. Statistics were performed with one-way ANOVA using Tukey for multiple comparison
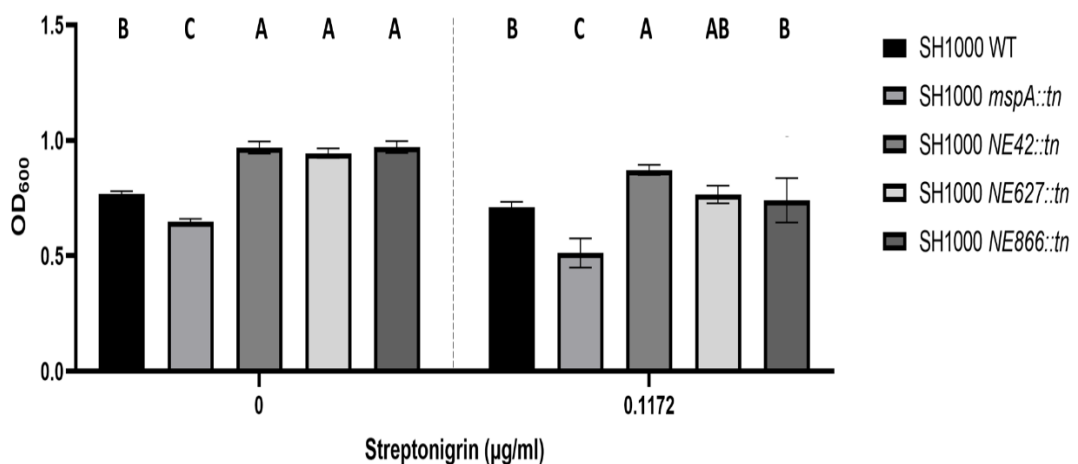
Fig 3.29: The sensitivity of the SH1000 *mspA* mutant to streptonigrin compared to mutants of its neighbouring genes. *mspA* mutant is more sensitive to streptonigrin suggesting it has more intracellular iron. Data represent the arithmetic mean of six biological repeats with two technical repeats, and error bars represents the standard error of the mean. Statistics were performed with one-way ANOVA using Tukey for multiple comparison

3.3.12.5 The *mspA*-neighbouring genes do not facilitate adaptation to iron

Analysis of the contribution of the *mspA* mutation and its neighbouring genes to hemin adaptation indicated that *mspA*, but not the up and downstream genes, help the cell to adapt well in media containing high iron (Figs 3.30 & 3.31). Fig 3.30 showed the means on 0μM and 40μM were showed the same as the highest was JE2 NE866::tn with hemin overnight among on strains, while the JE2*mspA*::tn with no hemin overnight showed the lowest. In the fig 3.31 with 0μM with hemin overnight the results identified that the mean of SH1000 NE866::tn showed the highest significance value and SH1000 *mspA*::tn was the lowest among the strains, and 40μM SH1000 NE866::tn with hemin overnight showed the highest significant value and SH1000*mspA*::tn with no hemin overnight showed the lowest.
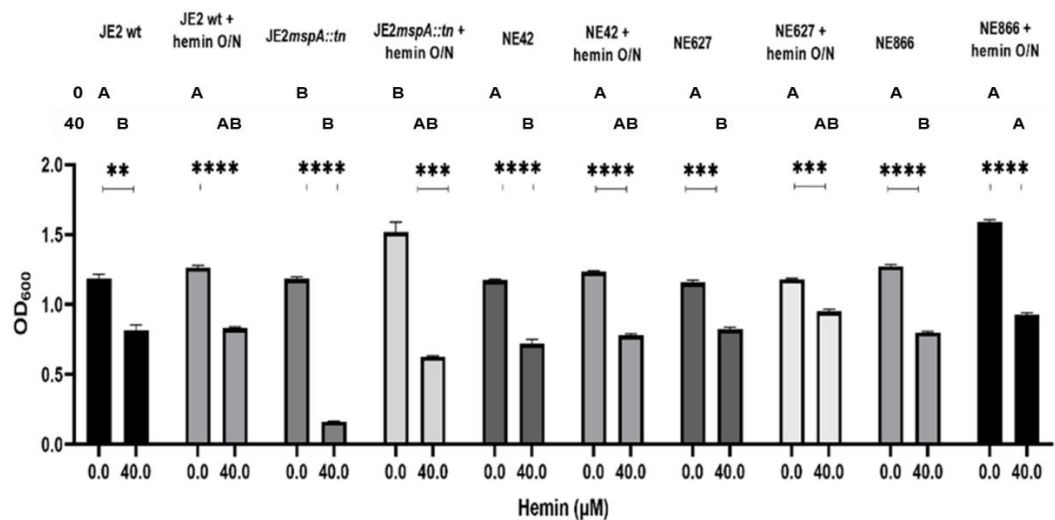
Fig 3.30: MspA in *S. aureus* JE2 background, but not its neighbouring genes, contribute to adaptation to high iron concentrations. In the assay, bacteria were pre-exposed to hemin, then sub-cultured in 40 μM hemin. Data represent the arithmetic mean of three independent experiments, and error bars represent standard error of the mean ANOVA test using Tukey was performed to compare the wt vs. *mspA* and neighbouring genes for the 0 and 40μM iron conditions. Another Statistics were performed with t-test where the significance was determined by p values as the following: *≤ 0.05, **≤ 0.01, ***≤ 0.001 and ****≤ 0.0001.
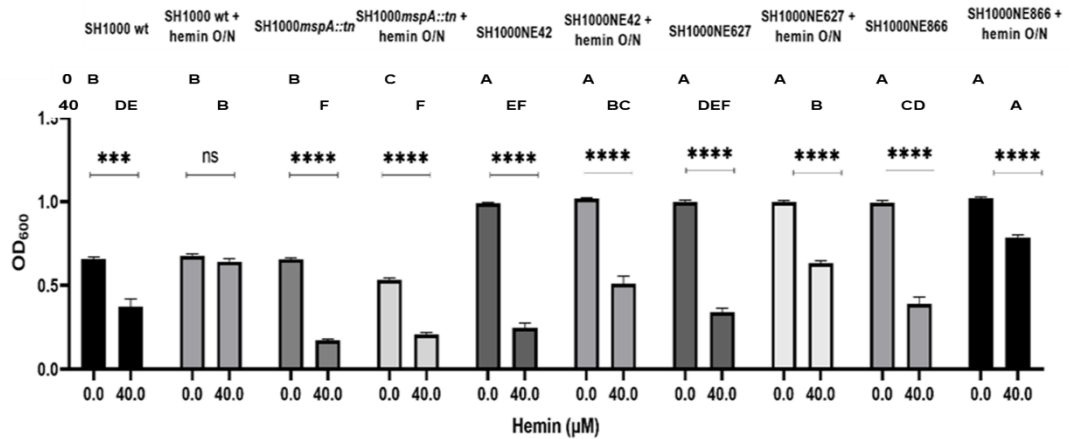
Fig. 3.31: MspA in *S. aureus* SH1000 background, but not its neighbouring genes, contribute to adaptation to high iron concentrations. In the assay, bacteria were pre-exposed to hemin, then sub-cultured in 40 μM hemin. . Data represent the arithmetic mean of three independent experiments, and error bars represent standard error of the mean ANOVA test using Tukey was performed to compare the wt vs. *mspA* and neighbouring genes for the 0 and 40μM iron conditions. Another Statistics were performed with t-test where the significance was determined by p values as the following: *≤ 0.05, **≤ 0.01, ***≤ 0.001 and ****≤ 0.0001.

3.4. Discussion

Staphylococcus aureus is considered a highly adaptable organism that causes diseases in both healthy and immune-compromised individuals (Lowy, 1998)(Gordon & Lowy, 2008). Development of new classes of antibiotics is a critical, given the high rate of *S. aureus* evolving resistance. Therefore, we need to improve our understanding of this pathogen in order to develop more efficient alternatives for therapeutic applications.

The aim of the work presented here relates to characterizing how the novel membrane-bound protein, MspA, affects toxin production with a view to understanding its role in *S. aureus* pathogenicity. MspA protein has a relatively small size of 105 amino acids, with only 28 amino acid residues predicted to sit outside the bacterial membrane, 11 outward and 17 inward of the bacterial membrane (Fig 3.1). Loss of this protein has pleiotropic effects for which we have created a pictorial summary (Fig 3.32). Such a loss affects offensive and defensive capabilities of the bacteria, which together render the bacteria incapable of causing infection to in humans.

A functional genomics approach has been utilized for the identification of the pleiotropic effects of this protein, which was tested for its contribution to *S. aureus* cytolytic toxin as well as iron production/ secretion. In the first instance, the earlier

finding for the role of MspA in toxin production was verified by comparing the cytolytic activity of two sets of isogenic mutants in different genetic backgrounds (i.e., JE2: MRSA & SH1000: MSSA). In both cases, inactivation of the *mspA* gene resulted in the reduction of cytolytic activity of the bacteria, and this effect was complemented by overexpressing the gene from an inducible expression plasmid introduced by genetic transformation (Fig 3.6 & 3.7).

To understand why the mutants were less cytolytic, we compared the level of expression of the PSMs family of toxins in the wild type and *mspA* mutant. The *mspA* mutant resulted in the production of reduced PSMs (Fig 3.8). This test could have been done at the level of transcription, but we thought this might not be indicative as posttranscriptional regulation might have influence on the final gene products. Besides, we needed to compare these toxin proteins with those generated from proteomic analysis, thus, we should ensure that their molecular weights are similar. From proteomic analysis, Hla protein (alpha toxin) was more abundant in cell lysate of the *mspA* mutant than in the wild type bacteria (Fig. 3.9). Duggan et al. (2020) showed a reduction in the differential abundance of alpha toxin. In other words, there was a significant drop in toxicity due to the drop in PSMs production. We hypothesized that inactivation of *mspA* was mediated by the reduced expression of the major regulator of toxin expression, the accessory gene regulatory (Agr) quorum-sensing system (Duggan et al., 2020). Our results of qRT-PCR of *Agr* gene indicated that expression of toxin genes might be retarded when *mspA* gene is knocked out. However, a further experimentation might be required to explain whether the drop in toxicity is due to the reduced expression of gene encoding members of PSMs, or due reduced expression of gene governing toxin secretion, ex., the repressor *pmtR* gene and downstream exporter *pmtABCD* genes that enable PSM secretion. Also, we suggest that the drop in toxicity might be due to the lack of toxin secretion as a consequence of the loss of MspA and subsequent decrease of staphyloxanthin production. Xue et al. (2019) showed the importance of staphyloxanthin in *S. aureus* virulence and neutrophil resistance. Reduced staphyloxanthin was shown to increase membrane permeability and decrease membrane stability (Fig 3.32), which might explain why the Hrt and Agr systems are inactive in the *mspA* mutant in our recent research (Duggan et al., 2020).

Inactivation of *mspA* led to downregulation of the Agr system (Fig 3.10). The accessory gene regulatory (*agr*) system is known to mediate quorum sensing or the regulation of toxin expression and secretion (Ky & Otto, 2015) (Queck et al., 2008). The changes in membrane structure lessens the offensive capabilities of the bacteria,

in terms of toxicity, and the defensive capabilities, in terms of survival inside macrophages or blocking the attack of the innate human immune system (i.e. neutrophil, blood and macrophage). Under the latter condition, human defensin (hNP-1) can cause pore formation and fluidity of the bacterial membrane as well as protein leakage (Duggan et al., 2020) (Fig 3.32). Produced by neutrophils and other immune cells, hNP-1 kills bacteria through pore formation driven by electrostatic attractions between these cationic peptides and the negatively charged bacterial membrane (Kagan et al., 1990; Duggan et al., 2020).

Table 3.1 refers to the effects of *MspA* on protein production and metabolic pathways. We found that the most differentially regulated proteins, when the *mspA* gene was inactivated, were the hemin transport system protein HrtB (important for the transportation of the substrate across the membrane) compared to the wild type. The levels of two other iron-related proteins also appeared in the proteomic data. They were iron-regulated surfaces determinant protein HrtA (an iron-sulfur cluster repair protein and iron compound ABC transporter) and bacterial non-heme ferritin.

As iron can be toxic to all cell types if the level becomes too high, bacteria have evolved systems to adapt to this by reducing the expression of the genes encoding proteins that import iron into the cells (e.g., the Isd proteins). These proteins were significantly increased in the *mspA* mutant resulting in a higher rate of iron influx. This action could result in intracellular iron toxicity. To examine whether MspA is involved in the ability of the bacteria to adapt to high levels of iron, we grew the bacteria overnight with and without hemin and then recultured them in increasing iron concentrations to see how quickly they will adapt. The results indicated that prior preadaptation of the bacteria to hemin (i.e. growth overnight) allowed the bacteria to adapt to increasing iron concentrations more rapidly.
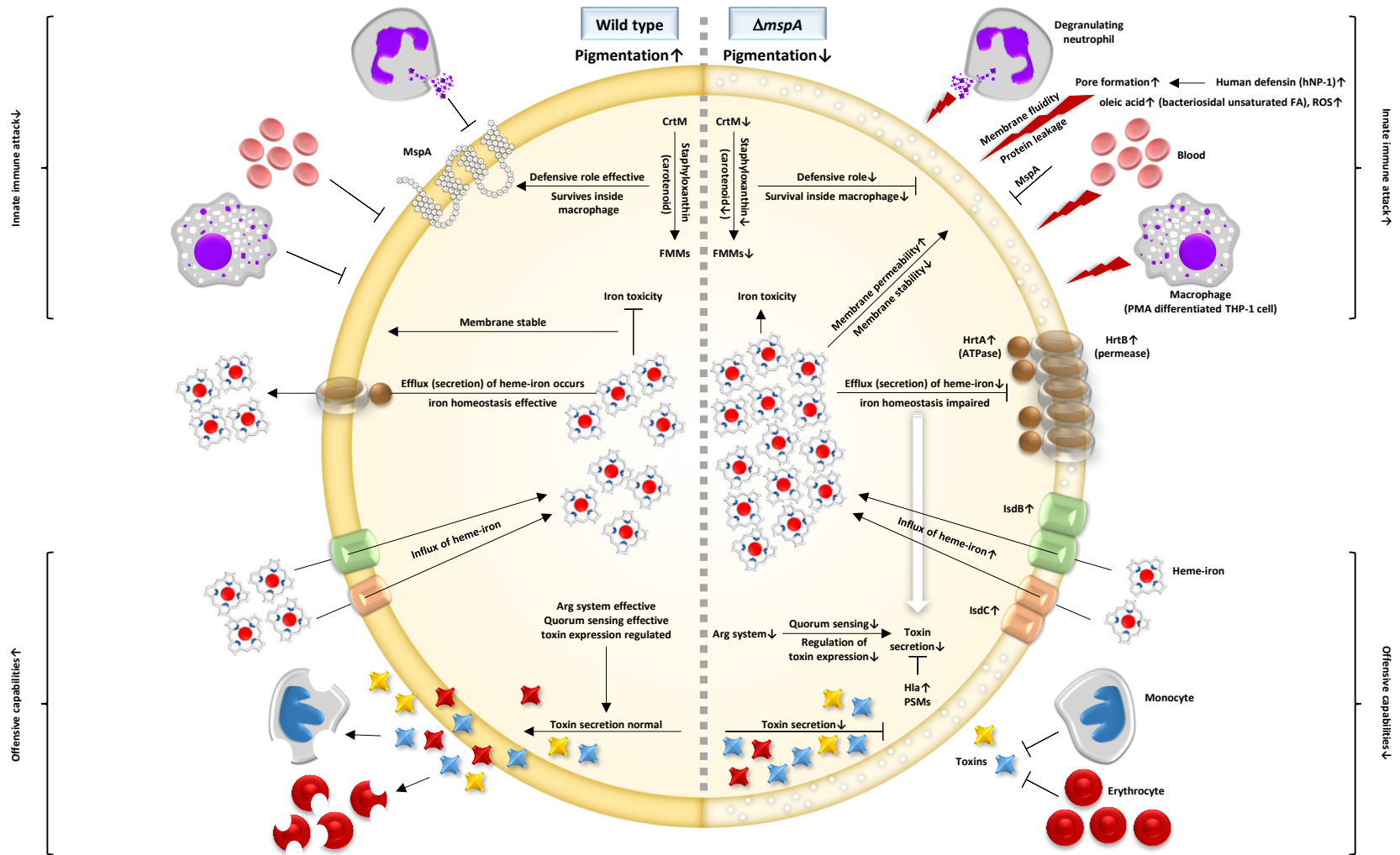
Fig. 3.32: Summary of the pleiotropic effects of knocking out *mspA* gene on the offensive and defensive capabilities of *S. aureus*. The Agr system does not become activated, which subsequently affects the production of cytolytic toxins, and might contribute to the reduced toxin secretion and immune susceptibility.

However, when compared to the wild type strains, the *mspA* mutant was significantly impaired in iron homeostasis (Fig 3.14 & 3.15). This suggests that *mspA* gene is positively involved in the adaptation of *S. aureus* to high iron environments. Given the potential toxicity issues associated with high levels of iron in the cytoplasm, we hypothesized that *mspA* gene contributes to iron homeostasis, on the one hand, and to toxin secretion, on the other hand. As indicated in Fig 3.16 and 3.17, we hypothesize that *mspA* could mediate the expression of the *agr* gene and we verified this using a reporter fusion system to support this hypothesis as we demonstrated via qPCR that *RNAIII* gene was downregulated in the *mspA* knockout mutant as we previously indicated (Duggan et al., 2020). This gene is proposed to regulate expression of many *S. aureus* genes encoding exoproteins and cell wall associated proteins as well as the system of Agr quorum sensing that promotes secretion of toxins. Thus, it is likely that reduced expression of *RNAIII* gene in the mutant will impair membrane structure and affect the machinery of toxin efflux, rather than toxin production.

As our proteomic data suggested that iron acquisition or utilisation was affected in the *mspA* mutant, we hypothesized that there might be differences in the intracellular abundance of iron in the bacterial cytoplasm. To test this hypothesis, we have used an antibiotic called streptonigrin, which acts as an indirect measure of intracellular iron (Fig 3.13). The *mspA* mutant appeared to be more susceptible to this antibiotic and this confirms that the *mspA* mutant has more intracellular iron.

To further confirm the effect of iron on toxicity, we examined the contribution of other proteins known to affect iron homeostasis, i.e., *hrtA, hrtB* and *fur* (Figs 3.19 & 3.20). The heme regulated transporter (HrtAB) is an efflux pump where HrtA is the ATP binding protein powering the pump and HrtB is the transmembrane efflux protein that takes intracellular heme and pumps it outside of the cell (Torres et al., 2007). The ferric uptake regulator (Fur), is a transcriptional regulatory protein that allows the bacteria to respond and adapt to environmental iron concentrations (Lojek et al., 2018). When we measured toxicity of these mutants, only *hrtB* mutant showed a high reduction in toxicity. For the *hrtA* mutant, we found no reduction in toxicity. We suggest that some other ATP binding proteins might substitute the activity of this protein. As HrtB is the major structural component of the efflux system, these data confirm that higher levels of iron negatively contribute to toxicity. As the *fur* mutant showed a level of toxicity similar to that of the wild type strain, we suggest that Fur regulates both the uptake and efflux of iron and its absence, in an environment with non-toxic levels of iron, results in no effects on toxicity.

When we examined the sensitivity of both *hrtA*, and *hrtB* mutants to streptonigrin, we expected that in the absence of the HrtAB system, the bacteria would become more

sensitive to streptonigrin. The result was unexpected, and this surprising result may be either because the Hrt system does not work properly in the JE2 background or JE2 has another iron efflux system (Fig 3.21). In the *mspA* mutant, the case is different as changes in membrane structure might result in blocking the HrtA,B system as well as any other iron transport systems due to blocking of iron secretion. Over and above, proteomic analysis indicated that the levels of Isd proteins (for iron efflux) in the *mspA* mutant were also increased. Thus, a large amount of iron is expected to enter the bacteria, which in turn maximizes the problem of iron toxicity and iron homeostasis impairment. The latter actions can be other reasons for the reduction in toxin secretion.

We also investigated the possible roles of *mspA*-neighbouring genes in two different genetic backgrounds. Although the effect of the *mspA* gene was consistent, there was no effect for the loss of expression of *mspA*-neighbouring genes on the ability of the bacteria to cause THP-1 cell death (Figs 3.25 & 3.26), on intracellular iron levels (Fig 3.28 & 3.29), or on the ability of the bacteria to adapt to hemin (Figs 3.30 & 3.31). This suggests that the effect of the *mspA* gene on toxicity and iron homeostasis is independent of the genes situated next to it at both sides on the bacterial chromosome.

The loss of *mspA* gene expression, which results in reduced toxicity, appears to be interfering with iron homeostasis, as higher intracellular iron levels in the bacteria shut the machinery of toxin secretion off. Furthermore, we have detected one more important factor for the reduced toxicity. The *mspA* mutant was noticeably less golden in colour than the wild type strain. These results reflect the lower level of carotenoid or staphyloxanthin (Fig 3.11). Proteomic data supports this phenomenon as CrtM, for staphyloxanthin production, was significantly reduced in the *mspA* mutant (Table 3.1). Staphyloxanthin provides the bacteria with protection against human innate immune attack (Liu et al., 2005). CrtM is believed to bind the scaffold protein flotillin (FloA) in order to form stable functional membrane microdomains (FMMs) (Garcı et al., 2017). FMMs are important for the protection of bacteria against attack by thr human innate immune system. Although levels of FloA were not affected in the *mspA* knockout mutant, we think FMMs will not properly due to the reduction CrtM .

Although we did not completely explain how this little protein has such a broad impact, our data can clearly suggest that an increase in heme levels in the cytoplasm of the *mspA* mutant is one of the results of MspA loss. As we continue to look into the molecular components of this protein's function, we came up with two working ideas. The first is that MspA works individually and interacts directly with *S. aureus'* iron homeostasis process(es). According to our proteomic findings, the mutant has more protein components of the heme efflux system Hrt. While increasing protein abundance is

generally associated with increased activity, mutant bacteria cannot adapt to iron-rich environments. Second, MspA may physically bind to these proteins, allowing heme to exit the cytoplasm. Alternatively, MspA might perform a direct structural role in bacterial membrane stabilisation, preventing proteins that require a certain amount of membrane stability from functioning correctly. Proteins may not be displayed or become accessible inside the membrane due to this instability, which might explain why the Hrt and Agr systems are inactive.

Overall results indicate that heme-regulated transporter (HrtAB) (Torres et al., 2007) increased (18-fold) at the protein level when *mspA* gene is knocked out, while iron level inside the cell increased, alleviating the possibility that expression of genes governing iron transport is impaired. As the proteomic analysis indicated that the levels of Isd proteins for iron efflux were also increased in the mutant might indicate that iron influx avenue is more active than the efflux avenue due to the possible impairment in the ferric uptake regulator (Fur) machinery that allows the bacteria to respond and adapt to environmental iron concentrations (Lojek et al., 2018). As toxin secretion was also impaired in the *mspA* knockout mutant, there is a change that a common mechanism in both toxin and iron secretion might be on charge.

4. *Staphylococcus aureus* is more resistant to β-lactam antibiotics in the absence of the MspA protein

4.1. Introduction

As a major human pathogen, *Staphylococcus aureus* is a global health concern due to its propensity to acquire antibiotic resistance (Lowy, 1998). Of particular note is methicillin resistant *S. aureus* (MRSA), due to the morbidity, mortality and financial implications associated with such an infection type (Gordon & Lowy, 2008). The increasing number of methicillin-resistant strains has made it difficult to treat severe infections (Deleo et al., 2010; Graveland et al., 2011). MRSA are resistant to β-lactam antibiotics, where this family of antibiotics covalently bind the penicillin-binding proteins (PBPs), to interfere with their peptidoglycan crosslinking activity, thus, inhibiting the growth of *S. aureus* leading to cell death. These four PBPs are high molecular weight (HMW) proteins called PBP1, PBP2, PBP3, and PBP4 that are important in providing the integrity of the bacterial cell wall structure as they harbor one domain that participates in the transpeptidation (crosslinking) of peptidoglycan on the external surface of the cytoplasmic membrane, and another domain that is involved in the transglycosylation process to extend the glycan chain (Stapleton & Taylor, 2002) (Fig. 4.1).
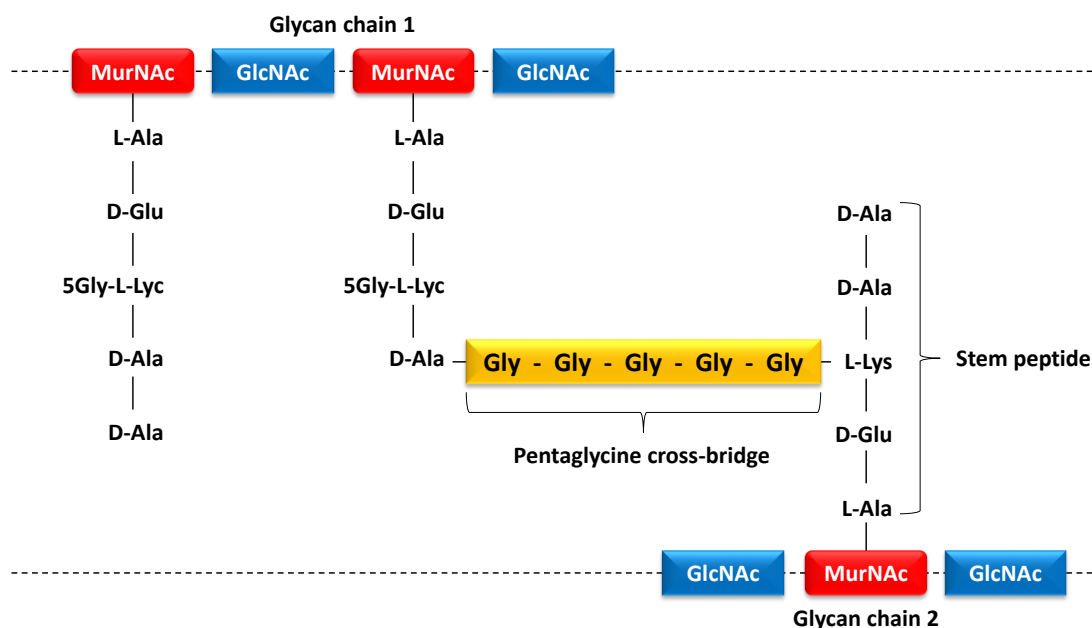
Fig. 4.1: A schematic representation of the cross-linking of two glycan chains in peptidoglycan of *S. aureus*. MurNAc, *N*-acetylmuramic acid; GlcNAc, *N*-acetylglucosamine. Taken from Stapleton and Taylor (2002).

The action of β-lactams results in the mechanical weakening of the cell wall, the release of some of the cytoplasmic contents and cell death (Giesbrecht et al., 1998). An early report suggested that a β-lactamase, an enzyme that degrades the β-lactam ring of these antibiotics, participates in the occurrence of β-lactam resistance in *S. aureus* (Montanari et al., 1996). However, for MRSA strains, resistance is not based on β-lactamase production, rather, it occurs due to the expression of a fifth penicillin-binding protein, namely PBP2a that is encoded by the *mecA* gene (Hartman et al., 1984; Chambers et al., 1994; Lu et al., 1999; Pinho et al., 2001; Stapleton & Taylor, 2002). The *mecA* gene is a part of a long and mobile stretch of DNA, namely the SCC*mec* element (Fig. 1.13), 40-60 kb), comprising a *mecA-mecR-mecI* coding region (Fig. 1.12). The PBP2a protein confers resistance to the action of methicillin due to the reduced affinity of its transpeptidation domain for β-lactam antibiotics. Thus, PBP2a can partly take over the transpeptidation reaction of the host PBPs upon exposure to β-lactam antibiotics to maintain cell growth in the absence of active versions of the other PBPs (Stapleton & Taylor, 2002).

Prior to the emergence of MRSA, some *S. aureus* strains were found to have the *mecA* gene, although it was expressed at a low level such that these strains were still susceptible to β-lactam antibiotics (Stapleton & Taylor, 2002). However, subsequent mutations in the promoter/operator region of the regulatory complex of *mecA* and its adjacent *mecR-mecI* genes were naturally evolved and rapidly spread due to the overuse

of the antibiotics, resulting in the emergence of MRSA strains with constitutive expression of the *mecA* gene and thus, higher methicillin resistance. There are also two types of resistance different MRSA strains can express to methicillin: homogenous or heterogenous. The first term refers to a bacterial population in which all cells are highly resistant to β-lactam antibiotics (Tomasz et al., 1991) (Sieradzki & Tomasz, 1998), an example of which is *S. aureus* strain DU4916 (Gustafson & Wilkinson, 1989). The second term refers to a population of *S. aureus* where the majority of cells exhibit a low level of antibiotic resistance, while a minority of cells are highly resistant, an example of which is strain DU4916- K7 (Gustafson & Wilkinson, 1989). Thus, the level of resistance of the population would reflect the level of heterogeneity in a given population of MRSA strain, e.g. JE2 in USA300 background. The concept of heterogeneity in more recent reports is often referred to as heteroresistance (El-Halfawy & Valvano, 2015), which also refers to a population of mixed resistant and susceptible bacteria to a given antibiotic. El-Halfawy and Valvano (2015) have developed a method called population analysis profiling (PAP) that is used as the gold standard to determine heteroresistance in bacterial populations. The method detects bacteria that exhibit a range of susceptibilities to a given antibiotic, i.e. methicillin. Detecting the level of heterogeneity via the PAP method is important for detecting the exact response of bacteria to antibiotics when a given gene, like *mspA*, is knocked out.

In recent work aiming to characterize the process involved in producing cytolytic toxins by *S. aureus,* the MspA protein was identified, where it appears to stabilize the bacterial membrane (Duggan et al., 2020). We have identified novel loci mediating the ability of *S. aureus* to both secrete cytolytic toxins and form biofilm (Laabei et al., 2015; Recker et al., 2017; Duggan et al., 2020). The loss of MspA has pleiotropic effects on the bacteria such that they do not produce cytolytic toxins, the Agr system does not become activated, their iron homeostasis is affected, they produce less staphyloxanthin and are more susceptible to many membrane damaging agents such as the human defensin hNP1 and common detergents such as SDS (Duggan et al., 2020). Given the range of effects the loss of MspA has on the bacteria, and the importance of understanding how antibiotic resistance is conferred, in this study, we examined whether MspA also contributes to the susceptibility of the bacteria to the β-lactam family of antibiotics. We found the *mspA* mutant to be more resistant to cell wall attack by β-lactam antibiotics. We demonstrate that this is due to a dysregulation of the transcription of the PBP genes, such that there are higher levels of these proteins produced, conferring increased resistance. We hypothesize that this is a compensation action within the cell envelope. The cell walls seems to become

stronger to allow the bacteria to survive the loss in stability of the bacterial membrane in the absence of the MspA protein.

4.2. Results

4.2.1. β-lactam resistance is increased when the *mspA* gene is inactivated.

Given the enhanced susceptibility of the *mspA* mutant of *S. aureus* to many cell membrane attacking agents, we sought to determine whether the mutant was also more susceptible to cell wall biosynthesis-attacking antibiotics. Using a disk diffusion assay, we compared the susceptibility of the wild type of strain JE2 and its isogenic *mspA* mutant to three β-lactam antibiotics, i.e, oxacillin, amoxycillin with clavulanic acid and ampicillin. Contrary to our expectations, we found that the *mspA* mutant was more resistant to all three β-lactam antibiotics (Fig. 4.2a).

To verify the role of MpsA in the susceptibility level of *S. aureus* to β-lactam antibiotics, we repeated the disk diffusion assays focusing on oxacillin, only, as it is the most clinically relevant antibiotic for *S. aureus*. In this expreriment, we included an *mspA* mutant strain complemented with the *mspA* gene in the pRMC2 plasmid (namely p*mspA*) under the control of an inducible promoter (Corrigan & Foster, 2009). This plasmid restored the natural susceptibility level to oxacillin in the complemented bacteria (Fig. 4.2b).

a
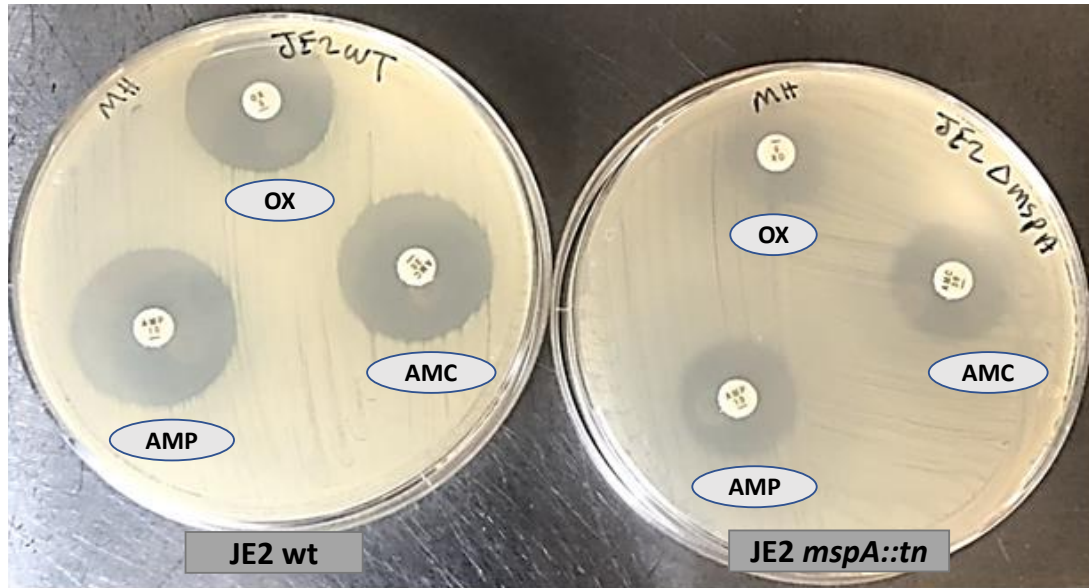
Antibiotics/Bacterial strains	oxacillin OX 5µg/ml	amoxycillin/clavulanic acid AMC 30µg/ml	ampicillin AMP 10µg/ml
JE2 wt	25mm	25mm	26mm
JE2 mspA⁻	12mm	19mm	17mm

b

Fig. 4.2: **In the absence of the MspA protein the resistance of *S. aureus* to β-lactam antibiotics is increased.** (a) The resistance of the MRSA strain JE2 to the antibiotics oxacillin, amoxycillin with clavulanic acid and ampicillin was examined by disk diffusion, where the *mspA* mutant was more resistant. (b) Resistance to oxacillin was restored to wild type levels when the *mspA* mutant was complemented with the *mspA* gene of p*mspA*.

4.2.2. Oxacillin disk diffusion test in an MSSA background

To measure the inhibition zone around the disk of the wild type and *mspA* mutant in the SH1000 background, we grew the bacteria on Muller Hinton agar with a 5µg/ml oxacillin disk. The zone of inhibition for both the wild type and *mspA* mutant was almost the same, suggesting that *mspA* does not affect oxacillin resistance in the SH1000 background (Fig. 4.3).
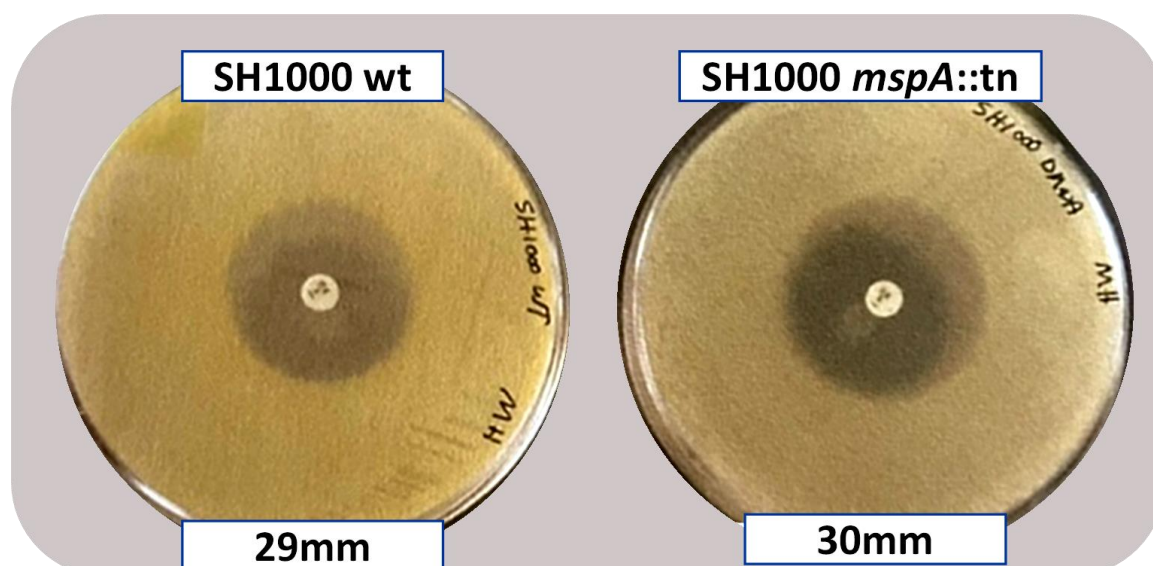
Fig. 4.3: Oxacillin disk diffusion test in SH1000 background. There is no significant difference between SH1000 wt and SH1000*mspA::tn* using the disk diffusion assay around the oxacillin disk.

4.2.3. Loss of the MspA protein is associated with homoresistance to oxacillin in the JE2 background.

As the absence of MspA resulted in an increase in resistance to oxacillin in the JE2 background (Fig. 4.1), we sought to determine the MIC for the wild type and mutant strain, and whether the increased resistance was a result of increased hetero- or homo-resistance levels in the MspA mutant population. Using a PAP-AUC assay, we were able to compare the heteroresistance levels of the wild type of strain JE2 and its isogenic *mspA* mutant. We have used overnight cultures of the two genotypes in a wide range of oxacillin concentrations and bacterial growth levels were quantified at each of these concentrations. The results indicated that the two genotypes harbored low levels of heteroresistance (i.e. that they are homo-resistant to oxacillin) as bacterial growth was maintained up to high concentrations of the antibiotic, and then dropped to zero. Had the population been heteroresistant, we would have expected to see two distinct drop-off points before the MIC was reached. These would represent the concentrations at which first the low level resistant, and then the high-level resistant subpopulations were inhibited. The results

showed a sharp growth reduction of the wild type strain at 4 μg/ml antibiotic concentration, while at 8 μg /ml for the *mspA* mutant strain (Fig. 4.4). These results confirmed that the *mspA* mutant is able to resist a higher concentration of the antibiotic compared with its respective wild type strain and that at a population level, the resistance of both genotypes is homogeneous.
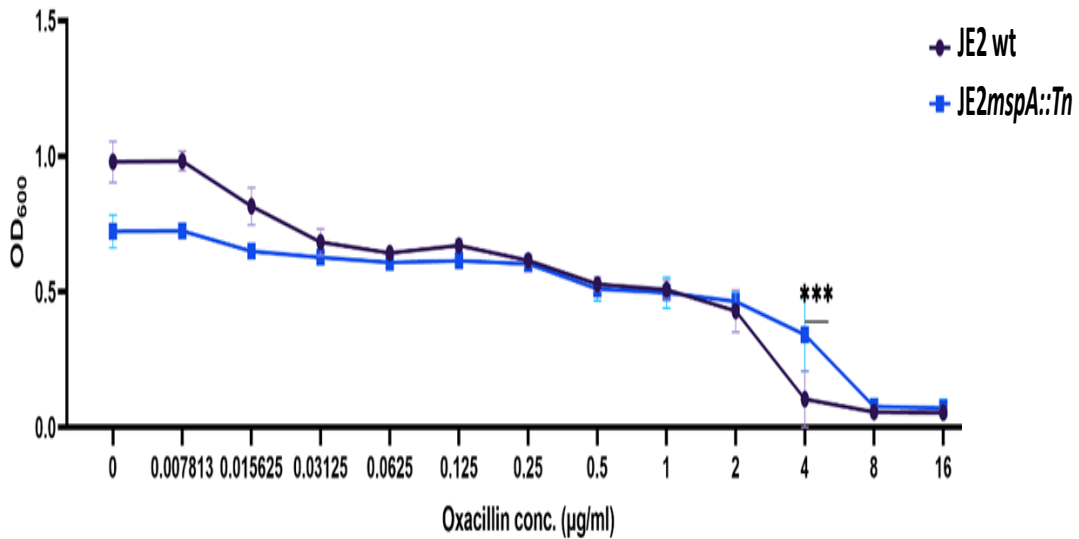
Fig. 4.4: PAP-AUC analysis describing the low level of heteroresistance in the JE2 wild type strain and its JE2 *mspA::tn* mutant. Data represent the arithmetic mean of three biological repeats with two technical repeats, and error bars represent standard error of the mean.Statistics were performed with t-test where the significance was determined by p values as the following: *≤ 0.05, **≤ 0.01, ***≤ 0.001.

4.2.4. The *mspA* mutant does not produce any β-lactamase enzyme

To examine whether the higher level of resistance of the *mspA* mutant to β-lactam antibiotics was due to the production of the β-lactamase enzyme, we performed a nitrocefin assay. Nitrocefin is a cephalosporin chromogenic detector used to detect the presence of the β-lactamase enzyme. The results in Fig. 4.5 shows that for the JE2 wild type and knockout mutant strains, as well as the PBS (used as the negative control), the colour did not change from yellow to red, suggesting that they do not produce a β-lactamase enzyme. Whereas, the colour changed to red for an *E. coli* strain that was producing β-lactamase enzyme (used as the positive control).
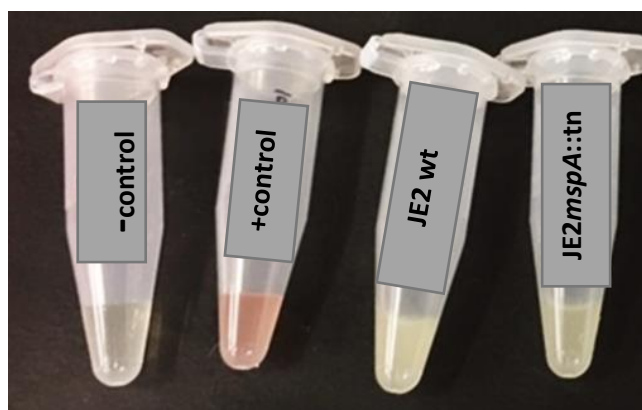
Fig. 4.5: The MspA mutant does not produce any detectable levels of β-lactamase enzyme. Nitrocefin assay for the JE2 wild type strain and its respective *mspA* mutant strain (JE2 *mspA::Tn*). An *E. coli* strain was used as a positive control, while PBS was used as a negative control.

4.2.5. Inactivation of the *mspA* gene results in a significant reduction in the expression of PBP genes

The penicillin binding protein (PBP) enzymes that are involved in the biosynthesis of peptidoglycan are the target of the β-lactam family of antibiotics (Livak & Schmittgen, 2001), and so to understand the molecular basis of the increased resistance to oxacillin in the *mspA* knockout mutant, we quantified the levels of transcription of the five genes involved in peptidoglycan biosynthesis, i.e, *pbp1, pbp2, pbp3, pbp4* and *pbp2a*. Gene expression was quantified by qRT-PCR, where the data were normalized by comparison to the level of expression of the housekeeping gene *gyrB*. The expression of each *pbp* gene in the *mspA* mutant is presented here relative to the expression of the same gene in the wild type JE2 strain (Fig 4.6). We found that *pbp1* and *pbp4* were expressed at significantly lower levels in the *mspA* mutant compared to their levels in the wild type JE2 strains, whereas *pbp2* and *pbp3* were expressed at significantly higher levels. There was no significant difference between the wild type and *mspA* mutant strains in the level of *pbp2a* expression (Fig. 4.6).

JE2 *mspA*::tn
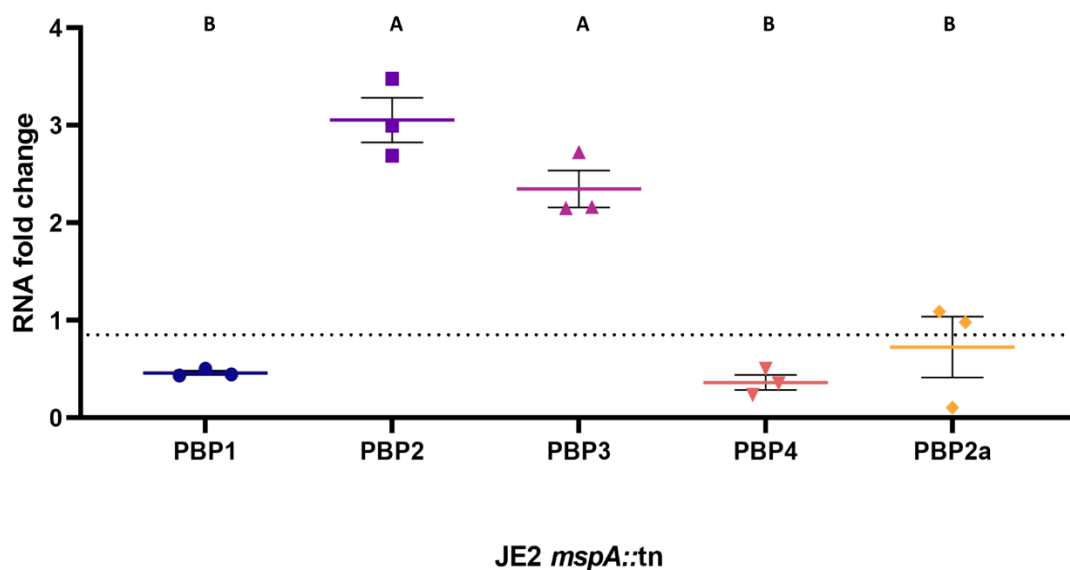
Fig 4.6: Expression levels of genes encoding the principal five peptidoglycan biosynthesis enzymes in MRSA as quantified by qRT-PCR. The expression of each *pbp* gene in the *mspA* mutant is presented here relative to the expression of the same gene in the wild type JE2 strain. Data represent the arithmetic mean of three biological repeats with two technical repeats , and error bars represent standard error of the mean Statistics were performed with one-way ANOVA using Tukey for multiple comparison.

4.2.6. Inactivation of *mspA* increased bocillin binding

To examine whether this dysregulation of the peptidoglycan biosynthetic genes affects the total abundance of penicillin binding proteins (PBPs) in the bacterial membrane, we incubated both the wild type and *mspA* knockout mutant in bocillin, which is a fluorescently-labelled penicillin that labels and allows the semi-quantification of PBPs (Zhao et al., 1999). The results indicated that the *mspA* mutant significantly bound more bocillin, thus, possessing more abundant PBPs than in the wild type strain and this effect was restored to the wild type levels in the complemented mutant strain (Fig. 4.7). These data suggested that the increase in abundance of the PBPs, but not PBP2a as it does not bind to bocillin (Kim et al., 2012), could titrate out the oxacillin, rendering the bacteria more resistant to oxacillin. Or, there could be some other oxacillin resistance effects of the dysregulation of the transcription of the *pbp* genes that results in an increase in PBP enzyme presence in the membrane.
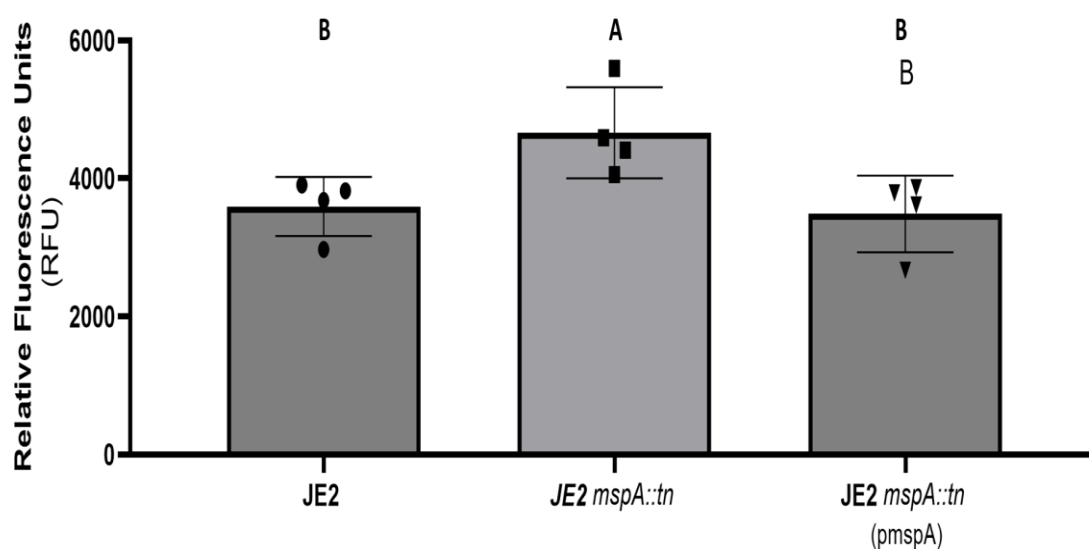
Fig. 4.7: In the absence of MspA the bacteria bind higher levels of bocillin. Bocillin binding test for the JE2 wild type strain, its respective *mspA* mutant strain (JE2 *mspA::Tn*) and the complemented strain harboring p*mspA*. Data represent the arithmetic mean of four biological repeats with two technical repeats, and error bars represent standard error of the mean. Statistics were performed with one-way ANOVA using Tukey for multiple comparison.

4.2.7. The loss of the MspA protein decreases autolytic activity

To examine whether there were any notable differences in peptidoglycan structure or integrity, we compared the autolytic activity of the wild type and mutant strains. When cells are lysed within a population, this is considered as a direct action of their lytic enzymes in a process known as autolysis. This acts as an indication of the sensitivity of peptidoglycan within the cell wall to the bacterial autolytic enzymes (Komatsuzawa et al., 1994) (Fig. 4.8). To test this activity, we induced autolysis by adding Triton X-100 to the cells and measuring the growth and autolytic rate during 6 hours. We found the *mspA* knockout mutant to be less autolytic compared to the wild type strain, although autolysis was more affected compared to that for a transposon mutant of the major autolysin gene of *S. aureus*, *atlA* (Oshida et al., 1995) (Fig. 4.8). The effect of the loss of MspA protein on autolysis was complemented by expressing the gene in trans. This suggested that there may be structural changes to the peptidoglycan of the MspA mutant that renders it less susceptible to autolytic enzymes, as well as β-lactam antibiotics. Altered structural change of cell wall was not discussed by Oshida and colleagues (1995) to possibly correlate the autolytic activity and β-lactam resistance when autolysin gene was knocked out.
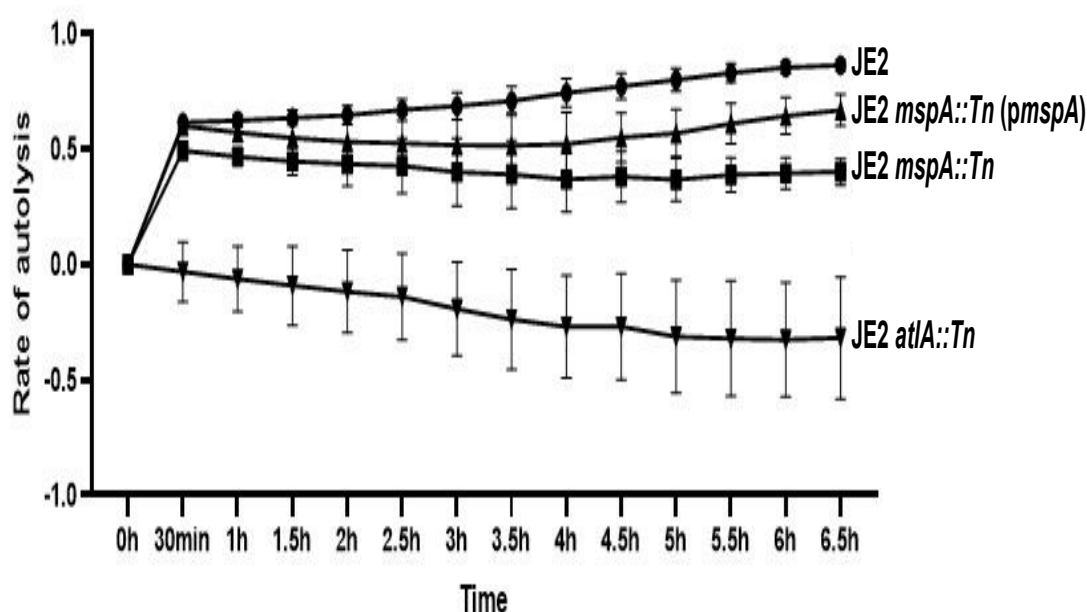
Fig. 4.8: Autolytic activity upon exposure to Triton X-100 for the JE2 wild type strain, its respective *mspA* mutant strain (JE2 *mspA::Tn*) and the complemented strain harbouring p*mspA*. A mutant strain with the major autolysin (*atlA*) of *S. aureus* knocked out was used as a control. Data represent the arithmetic mean of three biological repeats with two technical repeats, and error bars represent standard error of the mean.

4.2.8. Loss of MspA protein results in a change of cell wall thickness

Based on the speculation that peptidoglycan may be structured differently in the cell wall of the *mspA* mutant, we examined structural changes to the bacterial cell wall under the transmission electron microscopy (TEM) in both the JE2 (Fig. 4.9) and SH1000 backgrounds (Fig. 4.10). Analysis of the TEM images revealed that both the wild type and mutant strains looked intact but that the *mspA* mutant had a marginally thicker cell wall. To quantify this, we measured the distance between the membrane and the outside of the cells at four sides of the cell across 23 TEM cell images. We found that the cell wall of the *mspA* knockout mutant was significantly thicker (~30%) than that of the wild type strain in the JE2 background (Fig. 4.9), and there was a smaller and less significant difference in the SH1000 background (Fig. 4.10).

a

JE2wt JE2*mspA::tn*

b
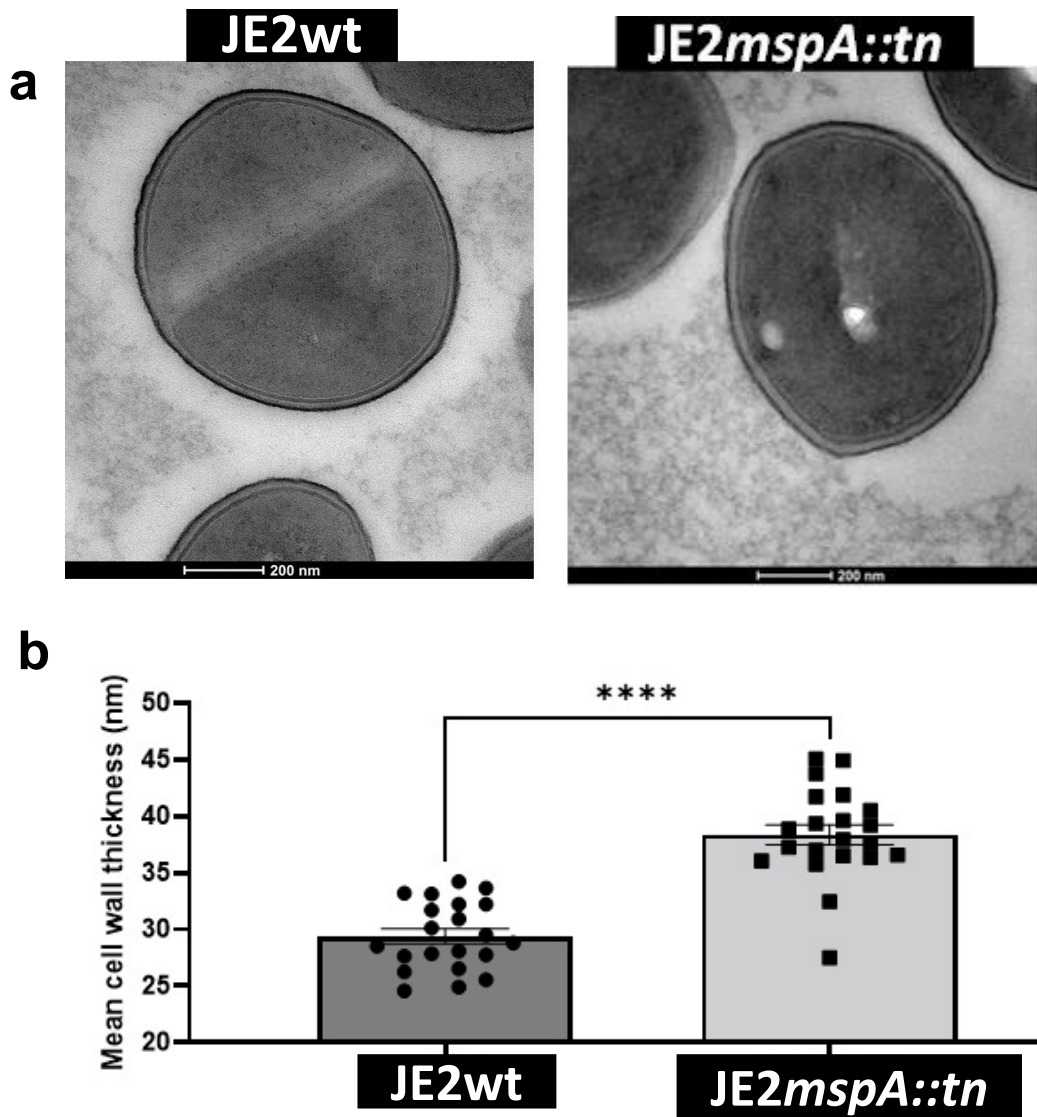
Fig. 4.9a and b: TEM of JE2 and JE2*mspA::tn* strains, showing that the loss of MspA protein has led to the increase of cell wall thickness. Statistics were performed with t-test where the significance was determined as a P value: ****≤0.0001.

a

SH1000 WT   SH1000 *mspA::tn*
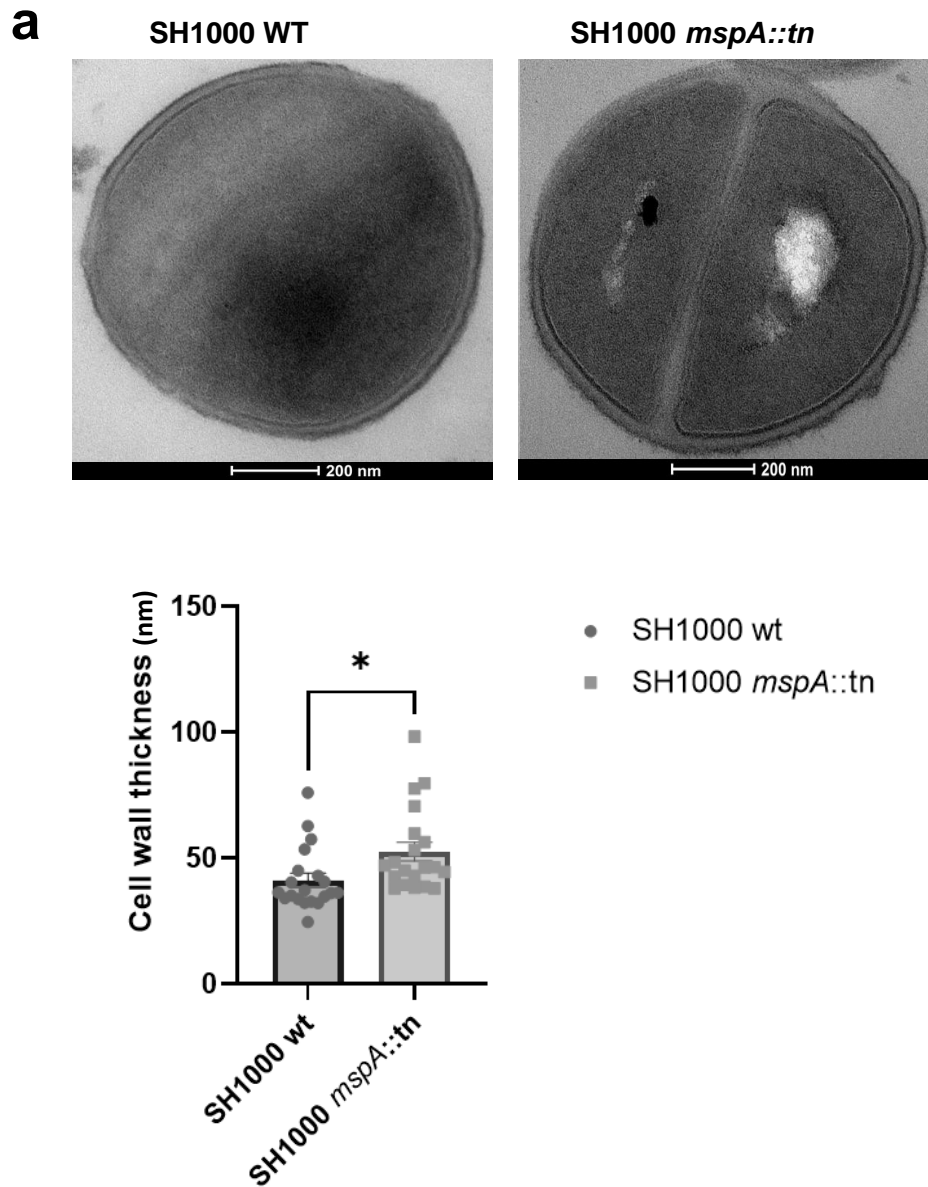
Fig. 4.10: TEM of SH1000 and SH1000*mspA::tn* **strains showing that the loss of MspA has led to the increase of cell wall thickness.** Data represent the arithmetic mean of at least three biological repeats, and error bars represent standard error of the mean. Statistics were performed with t-test where the significance was determined as a P value: *≤0.05.

4.2.9. Verification of subinhibitory concentrations of oxacillin for the wild type and *mspA* mutant.

As oxacillin resistance is typically only expressed in response to oxacillin, we sought to examine whether more obvious structural changes would occur to the *S. aureus* cell wall under subinhibitory concentrations of oxacillin. From (Fig. 4.4) the concentration of 0.5 µg/ml of oxacillin was selected as a suitable dose that would not kill either the wild type or *mspA* mutant. To verify this for the bacteria over a full growth curve we repeated this to determine the growth rate of the *mspA* mutant during overnight culture in the presence of oxacillin compared to that of the wild type strain. The growth curve was performed with and without exposure to oxacillin at concentrations of 0 and 0.5 µg/ml. The results indicated that both the wild type and mutant grew stably over 12 hours in the presence of oxacillin at 0.5 µg/ml (Fig. 4.11).
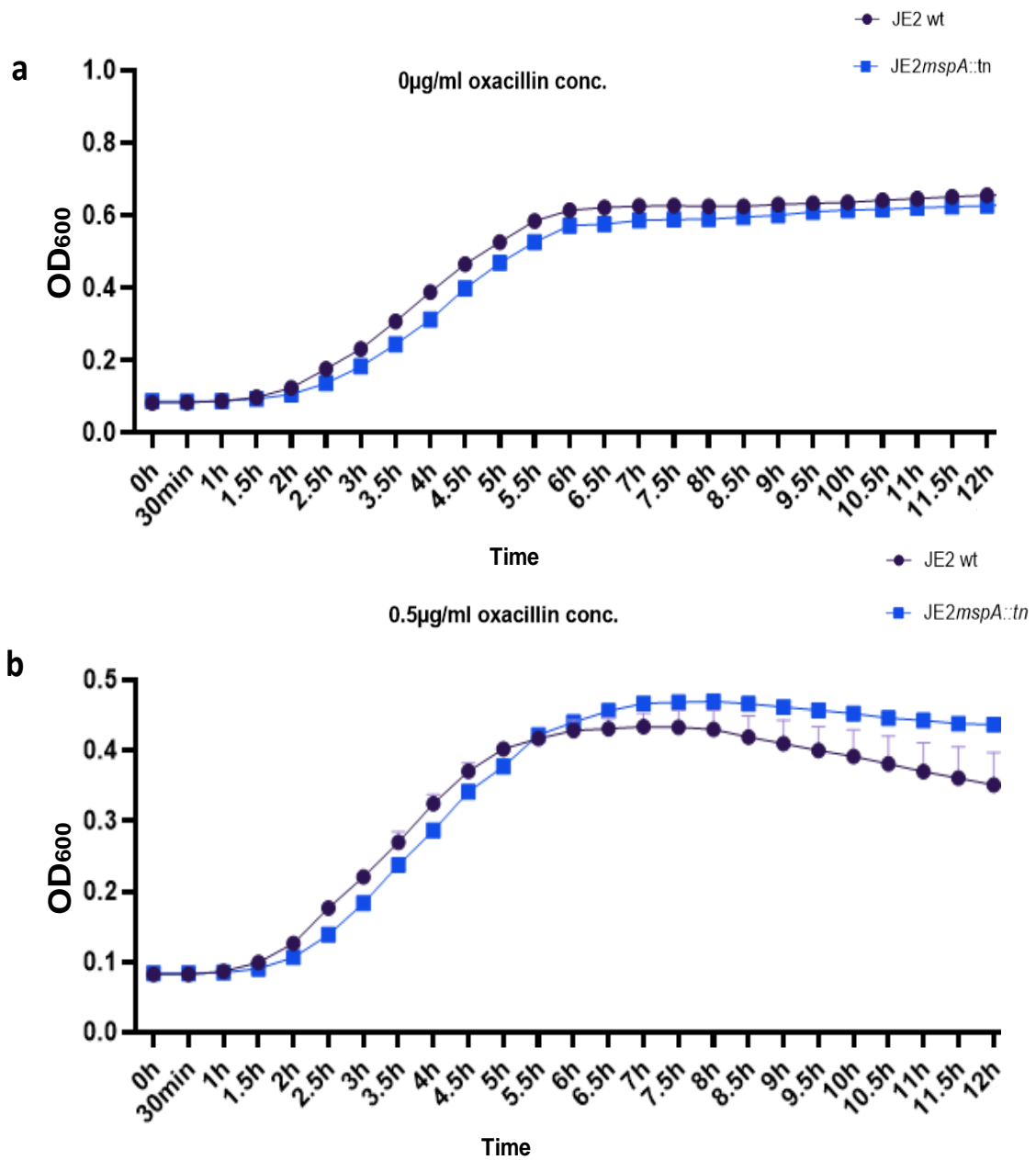
Fig. 4.11: Growth curve analysis of JE2 and JE2*mspA::tn* **before and after oxacillin exposure.** JE2 and JE2 *mspA::tn* (a) without and with treatment with 0.5 µg/ml oxacillin. The mutant strain had grown more than the wild type in the presence of oxacillin at this concentration. Data represent the arithmetic mean of three biological repeats with two technical repeats, and error bars represent standard error of themean.

4.2.10. Cell wall integrity maintained upon loss of the MspA protein after exposure to oxacillin

Having established a subinhibitory concentration of oxacillin for use, we performed TEM on the wild type and *mspA* mutant following overnight growth in 0.5 µg/ml of oxacillin. The results indicated that the cell wall of the wild type strain was damaged when exposed to 0.5 µg/ml oxacillin. However, in the mutant strain, there was no noticeable change in the cell wall structure when exposed to the same concentration of oxacillin. This suggests that in the absence of MspA, the mutant produces a more robust peptidoglycan cell wall.

WT   ***mspA* knockout mutant**
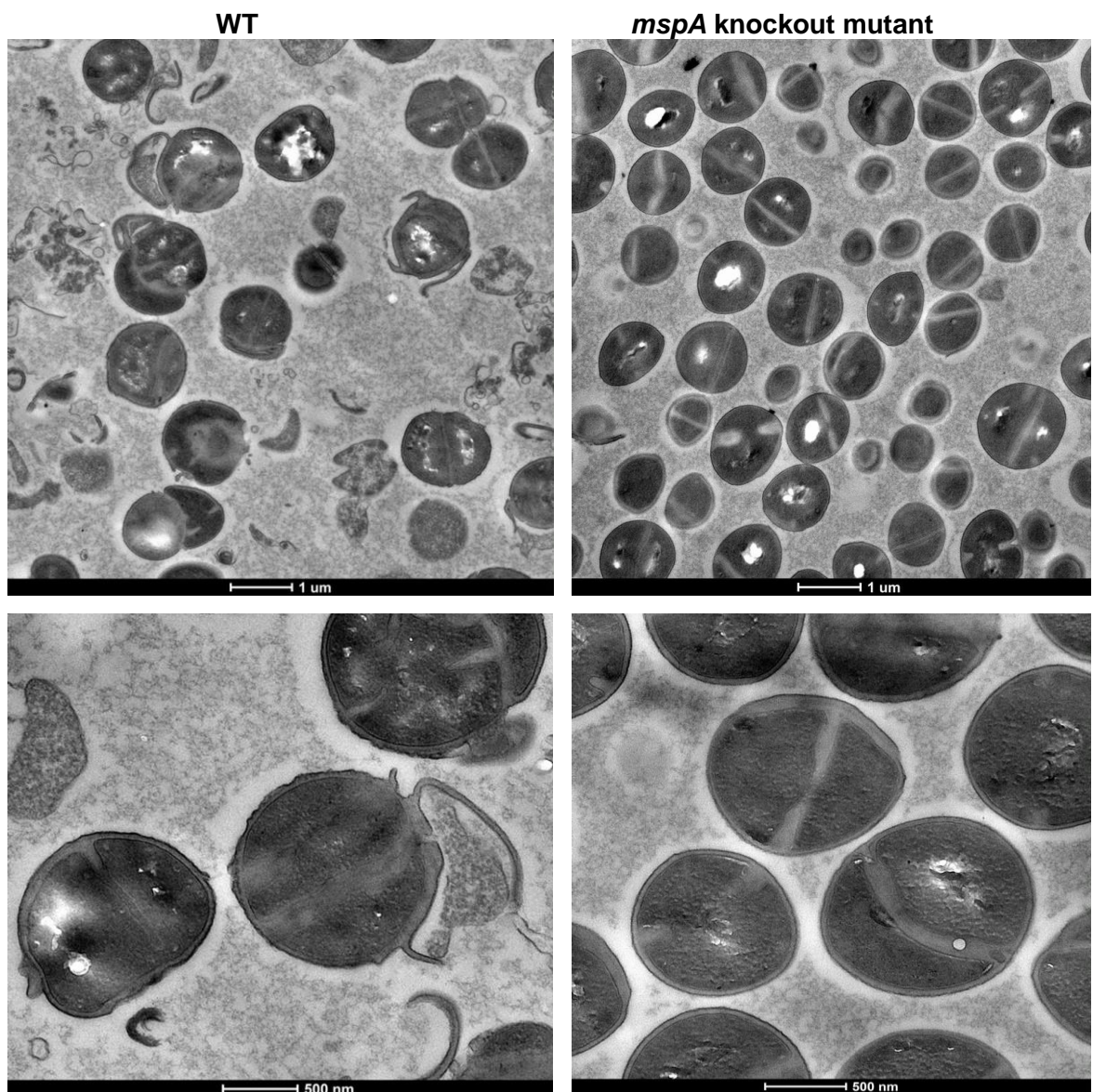
Fig. 4.12: TEM images of JE2 wild type (WT) and JE2*mspA::tn* strains after exposure to 0.5 µg/ml oxacillin. The images showed that the loss of MspA protein served to maintain the cell wall integrity in the knockout mutant when exposed to 0.5 µg/ml oxacillin. However, the cell wall in the JE2 wild type was entirely damaged by the drug at this concentration.
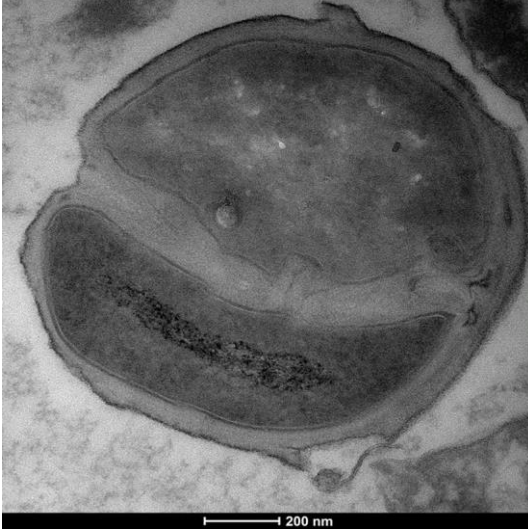
WT	*mspA* knockout mutant
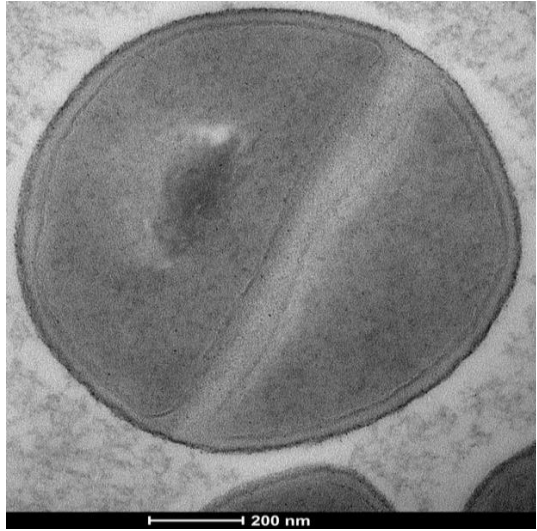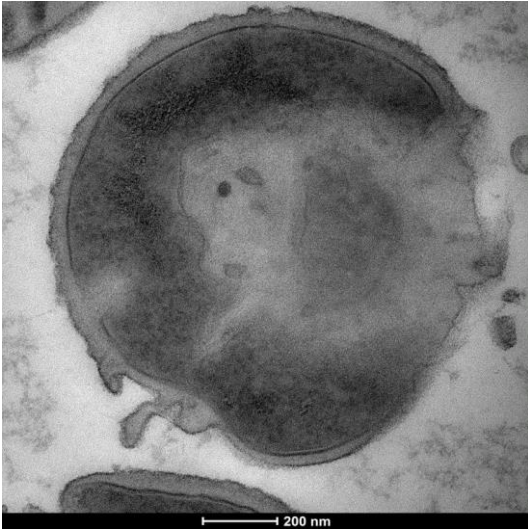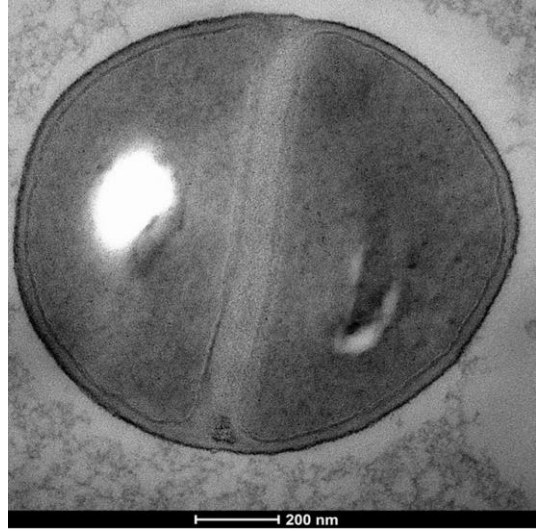
Fig. 4.12: Continued

4.3. Discussion

As we continue to decipher how the MspA protein affects so many metabolic and associated pathogenicity systems in *S. aureus*, here we add another activity to its repertoire where we demonstrate that it also contributes to this pathogen's antibiotic resistance profile. When MspA is absent, the strain becomes more resistant to β-lactam compared to the wild type strain, and the resistance was proven not to be mainly due to β-lactamase enzyme, but rather due to the increased amount of PBPs in the cell membrane. Then, we compared the autolytic activity in the wild type and mutant strain as an indicator of the sensitivity of peptidoglycan within the cell wall to the bacterial autolytic enzymes. The results indicated that the *mspA* knockout mutant is less autolytic compared to the wild type strain. We further found that the *mspA* mutant cell wall is stronger (thicker) compared to the wild type. The results further indicated occurrence of no damage in the bacterial cell when it was exposed to oxacillin treatment for the *mspA* mutant, while the cell wall of the wild type strain was damaged.

Only a few genes have been shown to increase β-lactam resistance to date (Fujimura & Murakami, 1997; Singh et al., 2019). For instance, inactivation of *clpP* and *clpX* genes increased resistance to β-lactam (Baek et al., 2014). This data aligns with ours where *mspA* knockout mutant showed less autolytic activity and higher β-lactam resistance probably due to the change in cell wall structure, similar to what has been shown by Baek and colleagues (2014) for the mutated *clpPX* genes. Interestingly, increased autolytic activity was also reported to correlate with increased β-lactam resistance (Gustafson et al., 1992; Corrigan et al., 2011; Dengler et al., 2013). Our findings are contrary to this, suggesting that this process hs levels of complexity to it that we are as yet unawre of. The *tarS* gene is vital to maintain a wild type level of β-lactam resistance (Brown et al., 2012; Sobhanifar et al., 2016). D-alanyl-lipoteichoic acid (*dlt*) putative operon also was shown to increase resistance to β-lactam (Nakao et al., 2000; Coupri et al., 2021). Most remarkably, disruption of the *gdpP* gene decreased sensitivity to penicillin by as much as 32 fold, and the *gdpP* mutant strain also showed a decrease in cell size and an increase in cross-linked peptidoglycan (Corrigan et al., 2011; Sommer et al., 2021). However, our data instead suggest a role for the differential expression of the *pbp* genes in the increased resistance of the *mspA* mutant. Here, we demonstrate that the expression levels of *pbp1* and *pbp4* genes were significantly reduced, while those of *pbp2* and *pbp3* genes were significantly increased when the *mspA* gene was knocked out (Fig. 4.5). This dysregulation of expression may explain the observed increase in resistance for the following reasons:

- PBP1 is a transpeptidase involved in transglycolosation. In previous work, it was reported that PBP1 dysfunction can be compensated for by decreasing the expression of autolytic genes (Dumitrescu et al., 2011; Poon et al., 2022). It is hypothesized that alterations in the peptidoglycan composition due to PBP1 dysfunction can be compensated for by the high production of PBP2 and PBP3 (Georgopapadakou et al., 1986). The latter phenomenon might even lead to the occurrence of higher levels of β-lactam resistance. Sumita et al. (1990) also stated that PBP2 and PBP3 are important for cell growth.

- PBP2 is involved in peptidoglycan transpeptidation to support the strong role of PBP2 in transglycosylation. Pinho et al. (2001) indicated that inactivation of the transglycosylase domain of this protein resulted in the excess recovery of shorter glycan chains, and hence, a marked decrease in methicillin resistance.

- PBP3 is involved in a peptidoglycan transpeptidase role and confers β-lactam resistance as was also confirmed by Sieradzki and Tomasz (1997).

- PBP4 is a monofunctional nonessential transpeptidase, which is reported to function in concert with PBP2 and PBP2a in MRSA to build highly cross-linked peptidoglycan (Wyke et al., 1981; Sieradzki et al., 1999; Lęski & Tomasz, 2005; Da Costa et al., 2018 Fergestad et al., 2020). PBP4 was reported to be required for cross-linking peptidoglycan in the wild type strain USA300 (Memmi et al., 2008).

Considering these activities, we speculate that overexpressing *pbp2* and *pbp3* genes in *mspA* mutant cells might compensate for the decrease of *pbp1* and *pbp4* gene expression. The extra PBPs in the mutant strain might also prevent the oxacillin from killing the bacteria. Under this condition, we needed more oxacillin to cause cell death. Further experimentation is required to detect whether the increase of PBPs in the membrane of the mutant strain occurs exclusively for PBP2, or for PBP3, or both. However this would require the generation of specific antibodies, as none are commercially available.

An alternative explanation is that PBP2a is playing a role here. In support of this, we did not observe any effect on oxacillin resistance in the SH1000 background, which as it is MSSA does not contain the *pbp2a* (*mecA*) gene. While the transcriptional analysis did not reveal any differences in expression of the *pbp2a* gene between the wild type and *mspA* mutant, this does not mean that there is not a difference in the level of PBP2a proteins between these strains. This hypothesis is supported by findings for the *clpP* and *clpX* mutants, which are also more resistant to β-lactams due to increased levels of PBP2a, which was also observed to result in a thicker cell wall by TEM (Baek et al., 2014).

As PBP2a does not bind bocillin, we would need to use a specific anti-PBP2a antibody to quantify this in the future.

In this chapter, we have identified and characterised the effect the MspA protein has on the β-lactam resistance of *S. aureus*. This adds to the list of effects MspA has on the activity of other membrane bound proteins. With regards to whether the effect on abundance of PBPs is due to a direct impact of the activity of the MspA protein, or due to an indirect, compensatory response of the bacteria due to the MspA-induced destabilization of the bacterial membrane is currently unknown. But what is clear is that understanding the activity of MspA will provide great insights into the biology of this major human pathogen.

5. Identification of the amino acids, domains and loops within the MspA protein that are responsible for toxicity, staphyloxanthin/ caretenoid production, and resistance to oxacillin

5.1. Introduction

As described earlier, the MspA protein consists of 105 amino acids comprising four transmembrane domains and three loops (Fig 5.1). To identify regions and amino acids critical to the activity of MspA, site-directed mutagenesis (SDM) was utilized, which is a procedure that introduces specific or targeted changes in the coding DNA of the gene of interest to study how these changes affect the activity of the protein. We have used SDM for two reasons. First, to detect the important regions of the MspA protein in order to understand the biology behind this protein. Second, once we find the important regions of this protein, they can be a target for future therapeutic approaches.
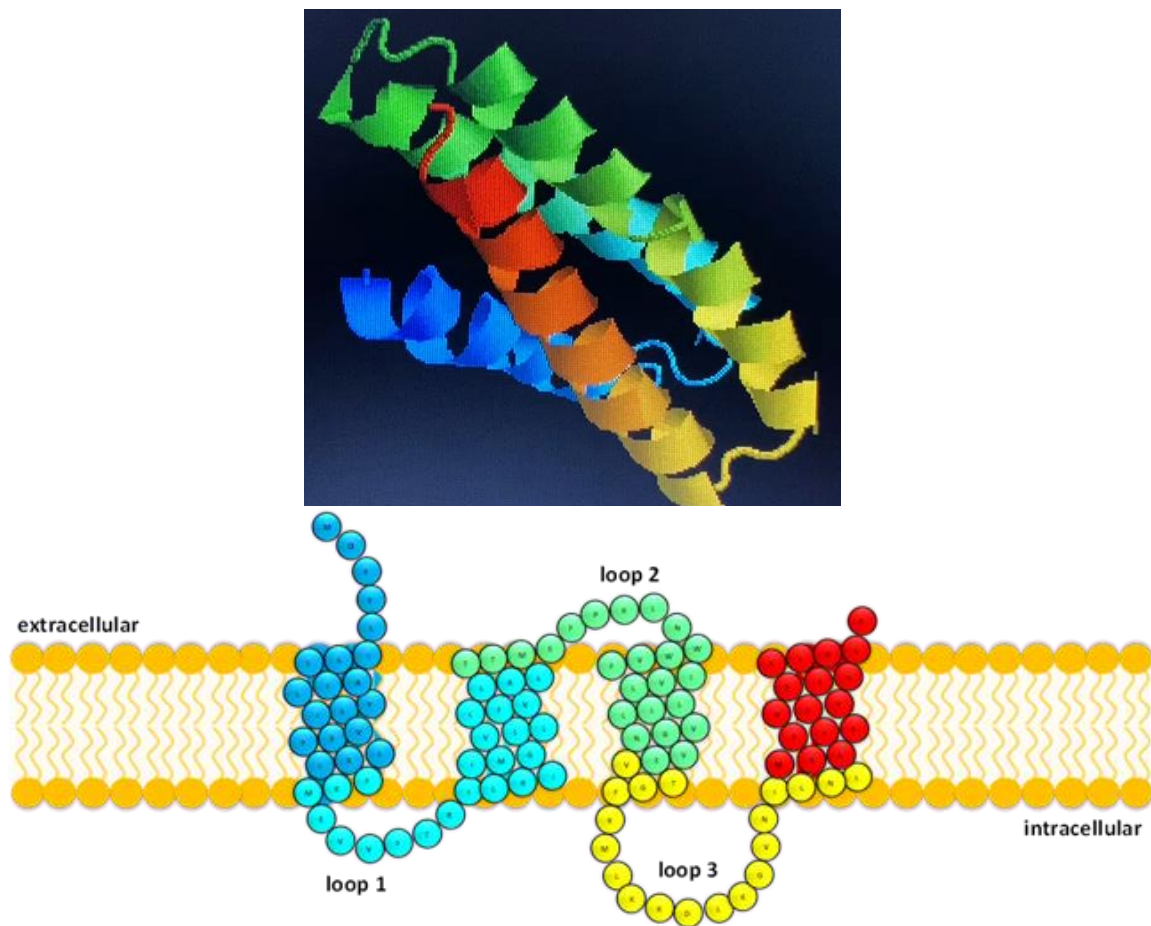
Fig. 5.1: The 3D structure of the MspA protein (105 aa) (I-TASSER- protein structure and function predictions, https://zhanggroup.org/I-TASSER/). The dark blue represents domain one, the light blue represents domain two, the green colour represents domain 3, and red colour represents domain 4. Loop 1 has a light blue colour, loop 2 has a green colour and loop 3 has a yellow colour.

In this chapter, two approaches of SDM to study the consequences of changing MspA protein structure/sequence were undertaken. For the first approach, we first made an amino acid sequence alignment of the MspA protein of *S. aureus* alongside other bacteria to detect conserved amino acids (AAs) in each of the different domains and loops (Fig. 5.2a & b). The alignment identified 26 conserved amino acids (Fig. 5.2c),and we substituted each of these with an alanine. Although alanine is four-fold degenerate (i.e, encoded by four different codons), we chose only one out of the four alanine codons, i.e, GCA, as *S. aureus* was shown to be biased to this codon and rarely uses the GCU, GCC or GCG codons. Alanine was used as it is a nonpolar, hydrophobic, aliphatic α-amino acid to replace the 26 conserved amino acids that were mostly hydrophobic , especially in the domain residues. Previous reports have used SDM to identify residues involved in ligand recognition by replacing residues in transmembrane domains individually with alanine as it is usually used to exchange polar amino acids; a procedure called alanine-scanning mutagenesis (Kim et al., 1995; Ishii et al., 1997). During this SDM approach, we changed

only one amino acid, at a time. In addition to the conserved amino acids, one amino acid L62, which was the amino acid idenfied in the original GWAS study that linked MspA to toxicity, was also substituted with alanine.

In the second approach, we removed the four domains, one at a time, and made substitutions of two loops, one at a time. Loop 1 was substituted for poly-glycine as glycine is the simplest amino acid in structure and has acid-base properties (Hammes et al., 1973). Loop 2 was not changed as no amino acid within this structure was identified as affecting the MspA related phenotypes. Finally loop 3 was substituted for loop 1. The importance of this approach lies in the theory that domains or loops that participate in the three studied MspA activities in this chapter, i.e, toxicity, staphyloxanthin production and the resistance to oxacillin, should harbour individual amino acids within their structures that have role(s) in these three activities. Fig. 5.3 shows the predicted protein structures after removing each domain or substituting the loops. All the steps that we applied to remove the entire domain or substitute the loops will be explained further. We hypothesised that substituting the conserved amino acids with alanine would affect MspA protein function. Thus, we would have the chance to detect the most important domain(s) and/or loop(s) as well as amino acid(s) acting as an important region(s) of this protein.

A

```
pseudointermedius   --------------------MYLVVAYISIFKMRIMMPKLLRVIMGLLLIIVVATSLVY   39
Streptococcus       ---------------------------------VVFTRILRIIMGVLLLFVLALTTMS   25
aureus              -----------MQFYLILLAILYLIVSFISIFKMEVVFTRILRIIMGVLLLFVLALTTMS   49
Vibrio              -----------------------------MEVVFTRILRIIMGVLLLFVLALTTMS   27
argenteus           -----------MQFYLILLAILYLIVSFISIFKMEVVFTRILRIIMGVLLLFVLALTTMS   49
pettenkoferi        -----------MQLYLILLPILYLIVAYISIFKMKTIFTTILRIIMALLLLFVVALTTVS   49
cohnii              -----------MQLYLIFLPVLYLIVSYXSIFKMNTIITRILRIIMSLLLLFVVAITTLS   49
lugdunensis         MIGSGLERKSKMQLYLILLPILYLIVSYISIYKMPTIFTRILRIIMGILLLFVIAITTLQ   60
warneri             -----------MKLYLILLPLLYLIVSYISIFKMNSIFTRILRIIMAVLLLFVVAITTMQ   49
haemolyticus        -----------MKLYLLLLPVLYLIVSYISIFKMHSIFIRILRIIMGVLLLFVLALTTLQ   49
                                        ::   :**:**.:**::*:*  :  :

pseudointermedius   YPASTWWVFVVLILLIGNVEITAFKHSKNDEKGVRILNMMSLFILVIYIVLVAVFI 95
Streptococcus       FPKENWWVFIVLLLLVGNVEVTGFKMLKKDLKGVNILNLMSLFIFVIYIFILTIVLF 81
aureus              FPKENWWVFIVLLLLVGNVEVTGFKMLKKDLKGVNILNLMSLFIFVIYIFILTIVLF 105
Vibrio              FPKENWWVFIVLLLLVGNVEVTGFKMLKKDLKGVNILNLMSLFIFVIYIFILTIV-- 81
argenteus           FPKENWWVFIVLLLLVGNVEVTGFKMLKKDLKGVNILNLMSLFIFVIYIFILTIVLF 105
pettenkoferi        FPAANWWVFVVLFLLVANVEITGFKHSKHDKKGVRLLNILTVILFVIYILTIVMY 105
cohnii              FPAINWWVFIVLLLIIGNVEITAFKNSKNDQKAVQILNIMSVILFVIYVILTLVLY 105
lugdunensis         FPTENWWVFIVLLLLVGNVEVTGFKAIKQDRKGLLILNLLTLLLYIVYLILVFIMY 116
warneri             FPNENWWVFIVLLLLVGNVEVTAFKSLKNDVKGVNILNILSIGLF---------- 94
haemolyticus        FPAENWWVFVVLALLVGNVEVTAFKALKHDAKAVSILNILSVILFVIYIILIFVMY 105
                    :*  .****:**  *::.***:*.**   *:* *.: :**::::  :
```

B

C

```
ATG CAA TTT TAT CTG ATT TTA CTA GCA ATA CTT TAT CTA ATT GTT AGT TTT ATT AGT ATT TTT AAA ATG GAA GTT GTA

TTT ACT CGC ATT TTG AGA ATT ATT ATG GGT GTG TTG TTA TTA TTC GTC TTA GCA TTA ACG ACG ATG AGT TTT CCA AAA
                L   R        I   M             L   L           V                                       P

GAG AAT TGG TGG GTA TTT ATC GTC TTA TTA CTC TTA GTC GGT AAT GTC GAA GTG ACA GGA TTT AAA ATG CTT AAA AAA
        W   W   V   F       V   L   L   L           V   E       T       F   K           K

GAT CTA AAA GGC GTA AAC ATC TTG AAT TTA ATG TCA TTA TTT ATC TTT GTC ATA TAT TTC ATC TTA ACC ATC GTA TTA
D       K                   L   N

TTC TAA
```

Fig. 5.2: MspA protein structure (a) Alignment of deduced amino acid sequence of the *Staphylococcus aureus* MspA protein with those of other bacteria. Asterisk (*) refers to conserved amino acids that can be replaced for alanine. (b) Positions of amino acids within the domain and loop structures to be replaced by alanine. (c) MspA sequence with amino acids in the loops shown in blue, while those in the domains are shown in red. Codon in green refers to the amino acid detected from GWAS data.
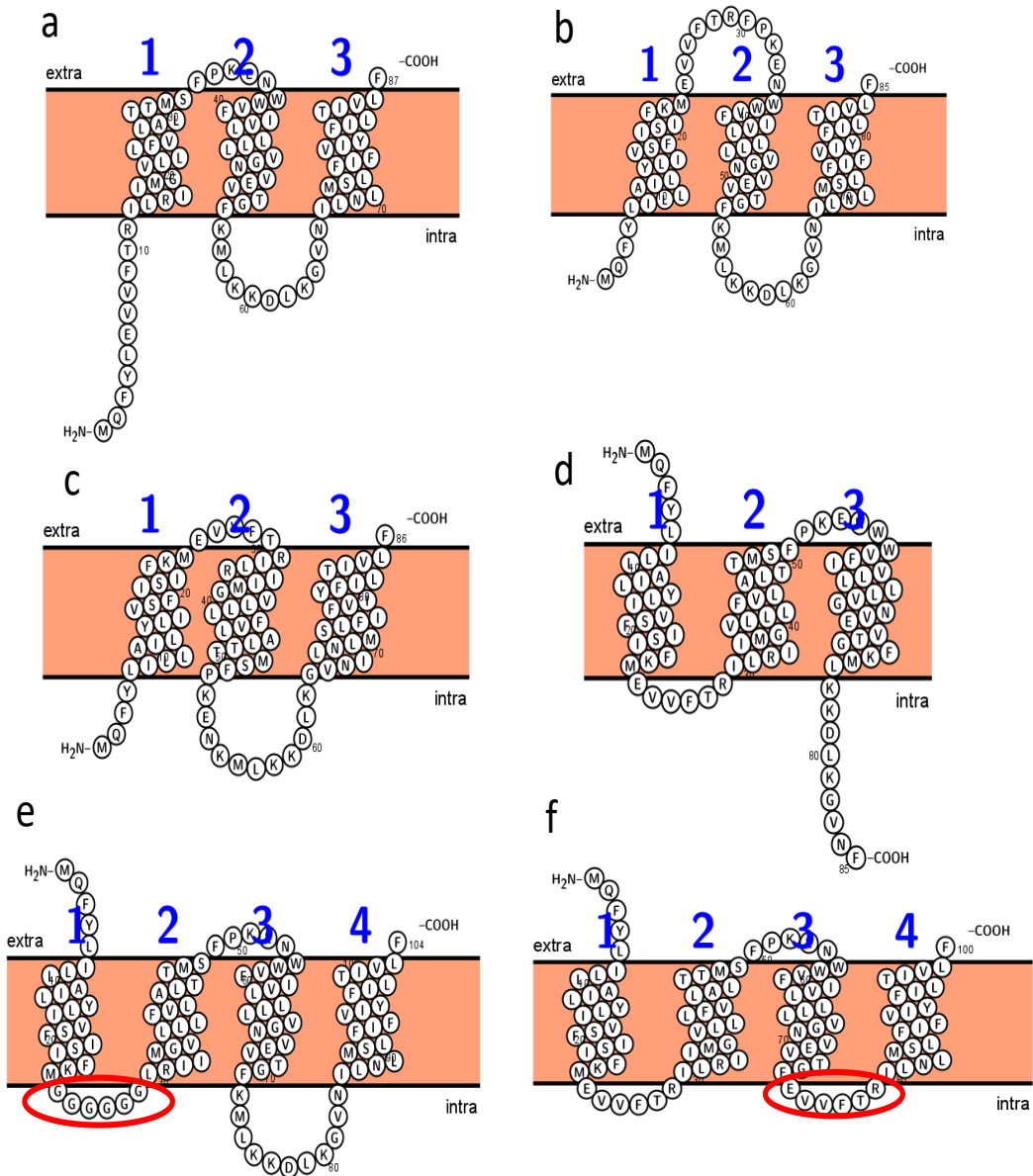
Fig. 5.3: MspA protein with different structures. Predict 2D MspA protein structures using Protter software after removing the first (a), second (b) third (c) or the last domain (d) or after substituting amino acids of the first loop with glycine (e) or substituting loop 3 for loop 1 (f).

5.2. Results

5.2.1. Substitution of conserved MspA residues with alanine

5.2.1.1. Variant creation and proof of the correct replacement of individual amino acids

To identify individual amino acids critical to the activity of MspA, the plasmid pRMC2-*mspA*^{wt} was used. The mutated *mspA* gene (*mspA*^{mut}), encoding a protein with a change of an individual amino acid, was amplified using site directed mutagenesis with individual primer pairs described in chapter 2. These primer pairs were designed to substitute an individual amino acid with alanine, one at a time. All the steps for creating the different variants are explained in Chapter 2. For simplicity I will refer to the strain containing the wild type *mspA* gene as CS^{wt} (for complemented strain with the wild type *mspA* gene), and those with a mutated *mspA* gene as CS^{mut} (for complemented strain with a mutated *mspA* gene)

5.2.1.2. Gradient PCR and *DpnI* digestion of the original pRMC2-*mspA*^{wt} plasmid

The gradient PCR reactions involved different optimal melting point for most of the primers between 59 and 62°C for all reactions (Fig. 5.6). Then, the best PCR product, referring to the pRMC2-*mspA*^{mut}, as well as the original plasmid (template), pRMC2-*mspA*^{wt} were digested with *DpnI* to remove the methylated T nucleotide (T^{m}) within the recognition site of the enzyme, i.e, GAT^{m}C (Fig. 5.7).
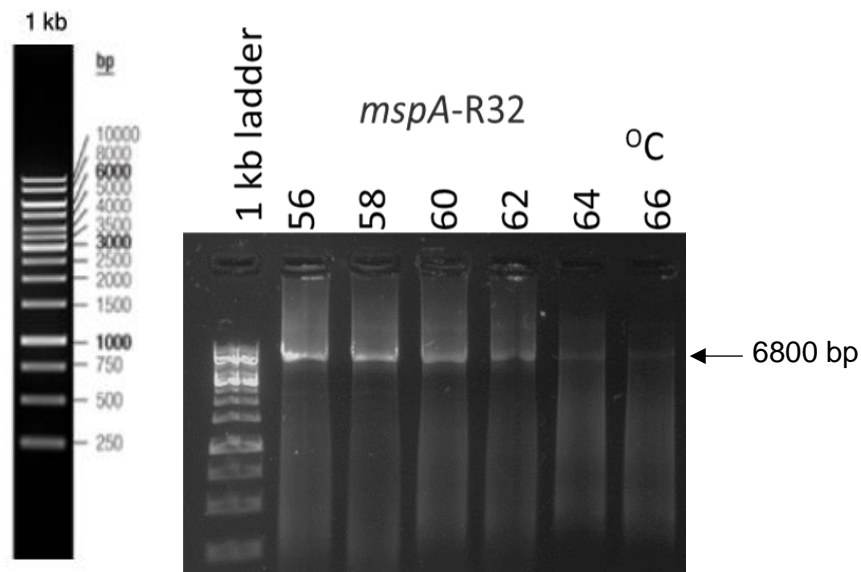
Fig. 5.4: Gradient PCR products (6800 bp) for the variant of amino acid position R32 used as a model.
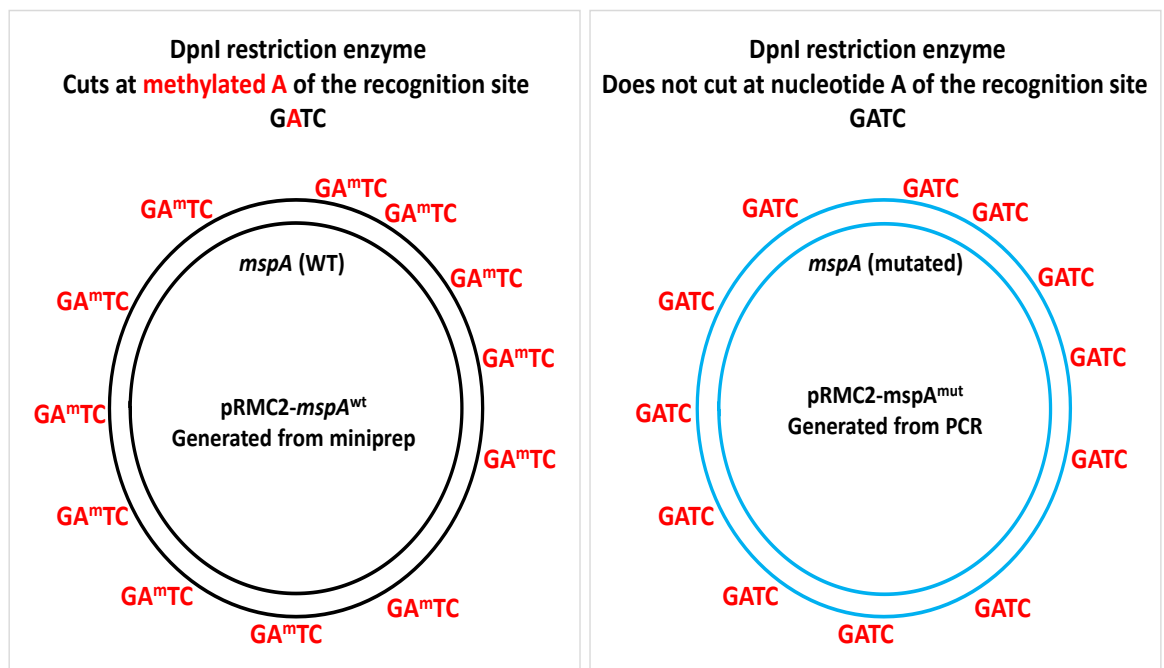
Fig. 5.5: Schematic representation describing the process by which pRMC2-*mspA*^{mut} was selected for further generation of variants via three steps of genetic transformation. *DpnI* is known to digest methylated DNA (ex., pRMC2-*mspA*^{wt}), a process that takes place in plasmids extracted from bacteria, while amplicon (ex., pRMC2-*mspA*^{mut}) generated by PCR is not methylated and thus is not digested with *DpnI* enzyme.

5.2.1.3. Transformation of *E. coli* and verification of the new pRMC2-*mspA*^{mut} plasmids by PCR and sequencing

The plasmids created via PCR, i.e, pRMC2-*mspA*^{mut}, with no methylated nucleotides (*DpnI*-undigested) were transformed in to *E. coli* strain DH-5α. Grown bacteria was spread on LB agar media containing ampicillin (100 µg/ml) for colony pick up (Fig. 5.8).
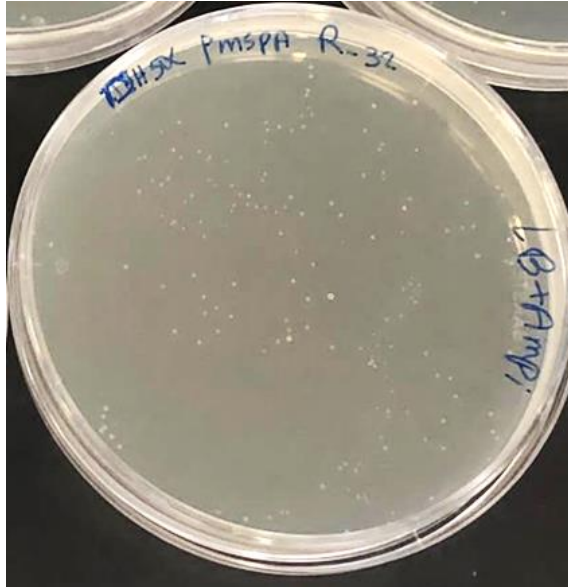
Fig. 5.6: Bacterial colonies on LB media after being transformed with the plasmid harbouring the variant R32.

5.2.1.4. Verification of the new pRMC2-*msA*^{mut} plasmids by PCR and sequencing

After plasmid miniprepreparation, the new plasmids were verified by PCR using pRMC2 primers described in chapter 2 (Fig. 5.9). Plasmids that produced the correct PCR product were further verified by sequencing (Eurofins, Germany) to confirm thatthe mutagenesis had worked (Fig. 5.10).
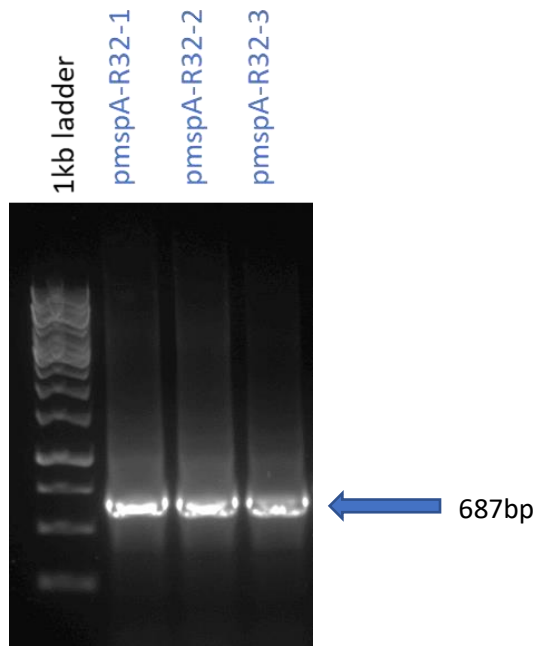
Fig. 5.7: PCR for three selected colonies. The arrow refers to the size of the generated amplicons (687 bp).

R32-1 wild type gene (*mspA*wt)

TCTGATTTTACTAGCAATACTTTATCTAATTGTTAGTTTTATTAGTATTTTTAAAATGGA
AGTTGTATTTACTCGCATTTTGAGAATTATTATGGGTGTGTTGTTATTATTCGTCTTAGC
ATTAACGACGATGAGTTTTCCAAAAGAGAATTGGTGGGTATTTATCGTCTTATTACTCTT

R32-2 mutated gene (*mspA*mut)

TTTATCTGATTTTACTAGCAATACTTTATCTAATTGTTAGTTTTATTAGTATTTTTAAAA
TGGAAGTTGTATTTACTCGCATTTTGGCAATTATTATGGGTGTGTTGTTATTATTCGTCT
TAGCATTAACGACGATGAGTTTTCCAAAAGAGAATTGGTGGGTATTTATCGTCTTATTAC

R32-3 wild type gene (*mspA*wt)

ATCTGATTTTACTAGCAATACTTTATCTAATTGTTAGTTTTATTAGTATTTTTAAAATGG
AAGTTGTATTTACTCGCATTTTGAGAATTATTATGGGTGTGTTGTTATTATTCGTCTTAG
CATTAACGACGATGAGTTTTCCAAAAGAGAATTGGTGGGTATTTATCGTCTTATTACTCT

Fig. 5.8: The sequencing results to confirm the correct mutation. The mutated codon, i.e, GCA, of alanine in the second sequence, i.e, R32-2, replacing the arginine codon, AGA.

5.2.1.5. Transformation of the pRMC2-*mspA*^{mut} plasmids harbouring the correct mutation in to *S. aureus* strain RN4220 then in to JE2 *mspA::tn* and confirmation of the correct plasmids by PCR

The plasmids with the correct mutation were electroporated in to *S. aureus* strain RN4220 in 0.2 cm cuvettes. Then, the bacteria were plated on TSA medium with 10 µg/ml chloramphenicol (Fig. 5.11). The plasmids from *S. aureus* RN4220 were, then, isolated using lysostaphin (100 µg/ml) as previously described and electroporated into JE2*mspA::tn* to generate the complement strain (CS^{mut}) and plated on to TSA containing chloramphenicol (10 µg/ml) and erythromycin (5 µg/ml) (Fig. 5.12). Finally, colony PCR using pRMC2 primers was used to confirm the strain contained the correct plasmids (Fig. 5.13). All the steps that we have done to create MspA-R32 variant have been explained here and all the data of the other 25 AA variants are available in the Appendix section.
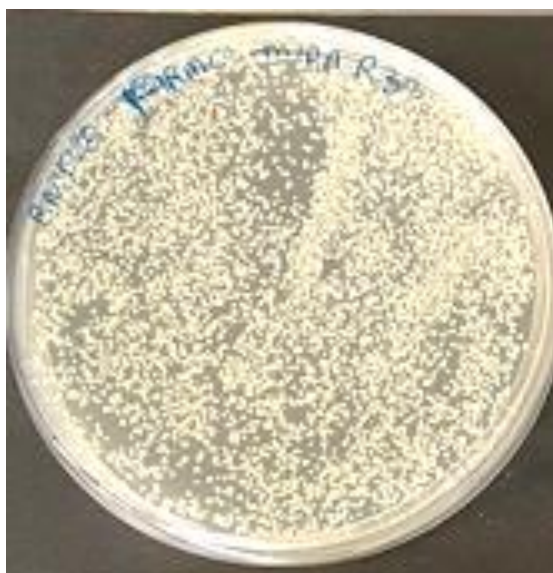
Fig. 5.9: The transformed bacteria RN4220-pRMC2^{R32}, grown on TSA containing 10 µg/ml chloramphenicol.
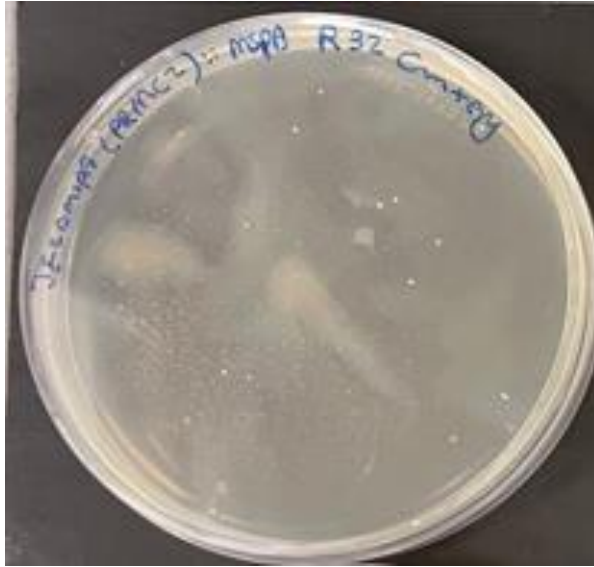
Fig. 5.10: The transformed bacteria JE2*mspA::tn* -pRMC2^{R32} complement strain grown on TSA containing 10 μg/ml chloramphenicol and 5 μg/ml erythromycin.
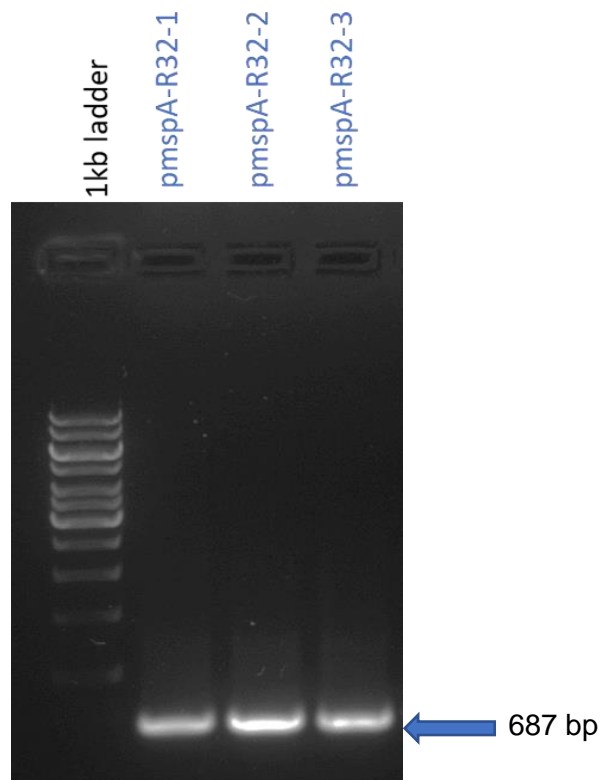
Fig. 5.11: Results of PCR for three CS^{mut} strains using pRMC2 primers to confirm the plasmid insertion.

5.2.2. The contribution of individual amino acid variants to THP-1 cell death

To determine the effect of the mutations in the 26 CS^mut strains on toxicity as compared with the wild type CS^wt strain, we performed a toxicity assay (explained in chapter 2) to assess the ability of the two types of CS strains to lyse THP-1 cells. The wild type JE2 and isogenic *mspA* knockout mutant were used as controls. The CS^wt was also used as a control after normalizing each sample with this strain in each assay. The wild type and mutated *mspA* genes were expressed following induction of the of tetracycline inducible promoter they were under the control of. Statistical analysis compared the toxicity of the CS^wt versus each of the 26 CS^mut strains. This work demonstrated that 18 amino acid variants showed a significant decrease in their toxicity effects when compared with the complement strain CS^wt (Fig. 5.14). The most affected mutant was variant E69A (mean=54.609), of which almost half of the THP-1 cells survived the toxicity assay. We therefore assume that the glutamic acid in this position is more important for toxicity than the other mutated AAs. The variants in positions L31A and M35A showed significant differences compared with the complement strain CS^wt (mean=56.3237 for variant L31A and mean=47.4423 for variant L35A). The toxicity of 15 other variants was also significantly affected, but to a lesser degree (R32A, L38A, V57A, L62A, F73A, K74A, D79A, L86A, N87A, I34A, V42A, L61A, V68A, T71A and K77A). The toxicity of variants L39A, P51A, W55A, W56A, F58A, V60A, L63A and K81A showed no significant difference compared with the CS^wt strain suggesting these AAs are not involved in this MspA activity.
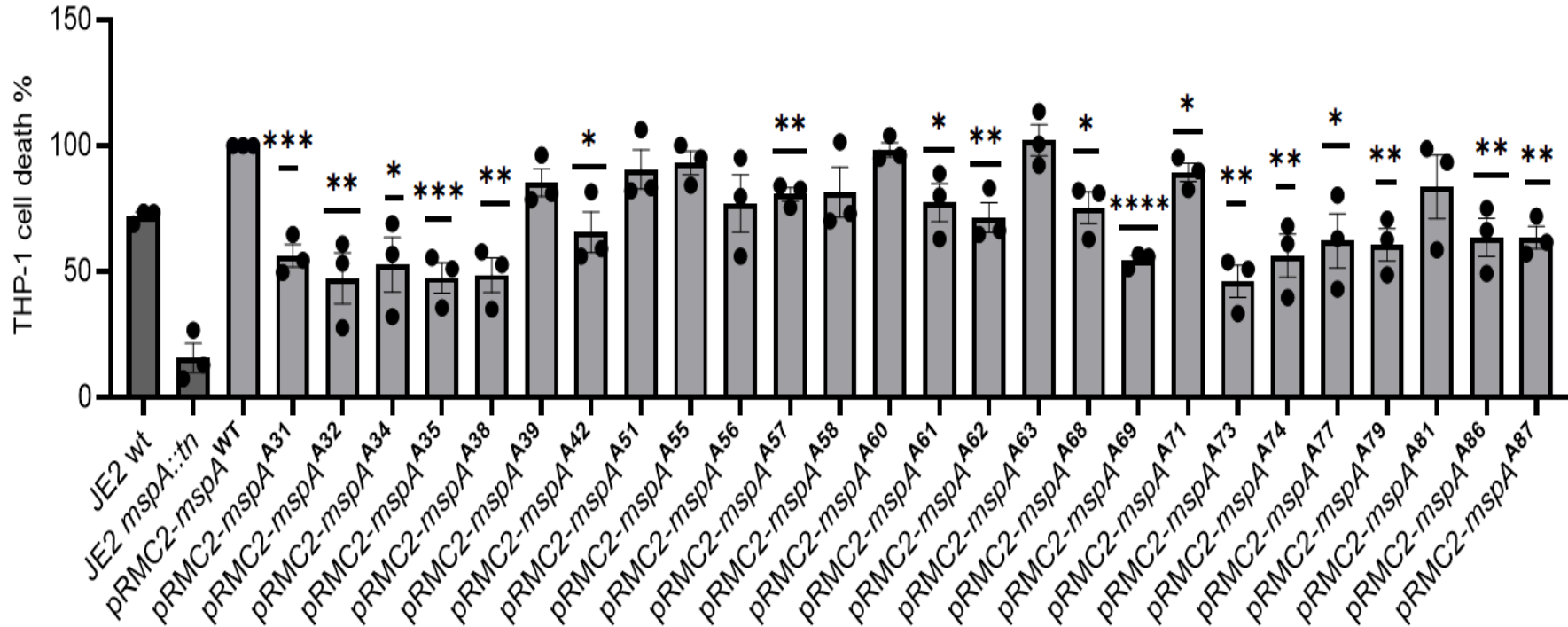
Fig. 5.12: Results of the THP-1 cell lysis assay for the 26 variants to determine which residues of MspA are required compared to the knockout mutant complemented with the wild type *mspA* gene (CS^wt). The wild type strain JE2 and its isogenic *mspA* knockout mutant were used as controls. All the gray light bars are JE2 *mspA*::tn with the different types of pRMC2 plasmid induced with 200 ng/ml tetracycline. Statistics were performed with t-test. The significance levels are *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, and ****$P \leq 0.0001$. Data represent the arithmetic mean of three biological repeats with two technical repeats , and error bars referring to standard error of the mean.

5.2.3. The contribution of individual amino acid variants to staphyloxanthin biosynthesis

To determine the effects of the mutations of the 26 AAs of MspA on staphyloxanthin production, we quantified the pigmentation (described previously in chapter 2) of the CS^{wt} and CS^{mut} strains. The wild type JE2 and isogenic *mspA* mutant were used as controls. The results indicated that only one variant (L39A) showed a significant reduction in staphyloxanthin production (mean=91.2205) compared to CS^{wt} and thusreduced carotenoid production. On the other hand, variant F58A, showed a significant increase in staphyloxanthin production compared to CS^{wt} (mean=117.242) (Fig. 5.15). Although statistically significant, these changes were quite small and so care needs to be taken when interpreting them.
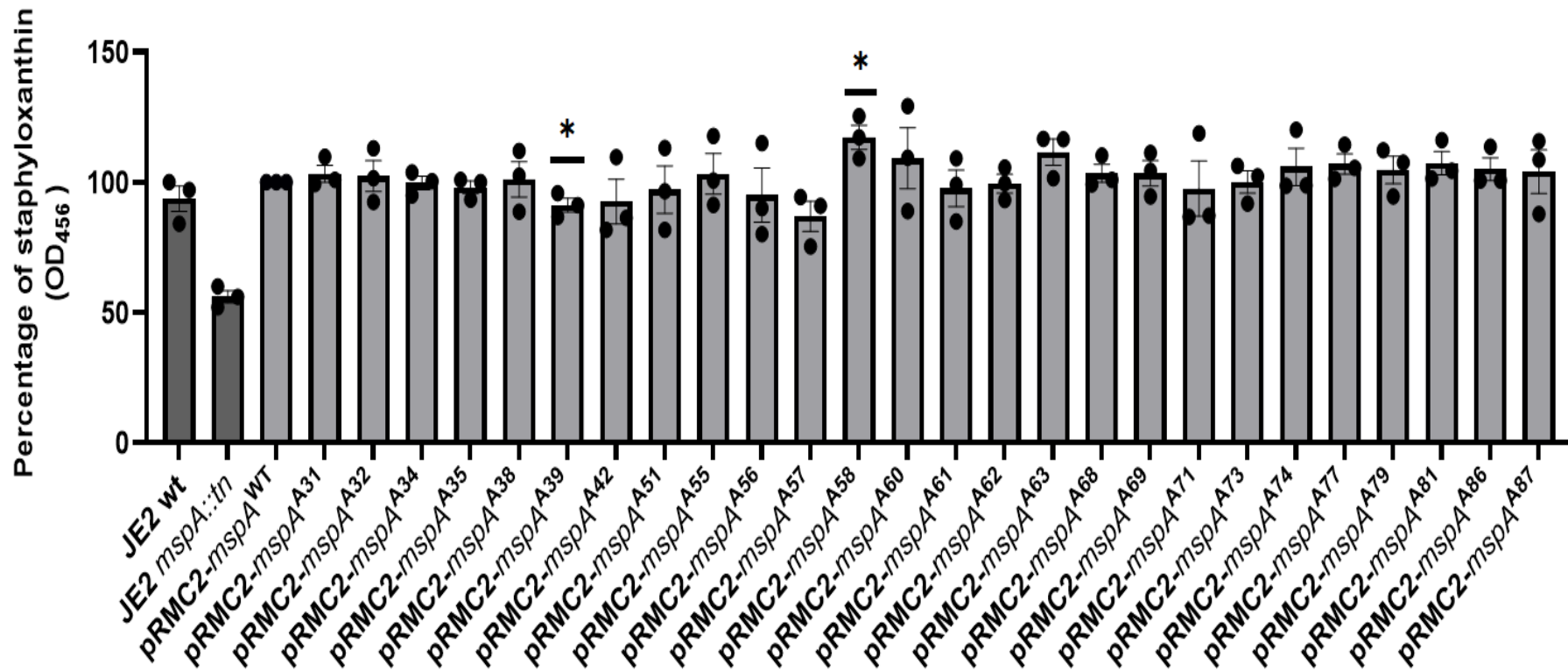
Fig. 5.13: Results of staphyloxanthin production assay for the 26 variants to determine which residues of MspA are required compared to the knockout mutant complemented with the wild type *mspA* gene (CS^wt). The wild type strain JE2 and its isogenic *mspA* knockout mutant were used as controls. All the gray light bars are JE2 *mspA*::tn with the different types of pRMC2 plasmid induced with 200 ng/ml tetracycline. Statistics were performed with t-test. The significance level was *P ≤ 0.05. . Data represent the arithmetic mean of three biological repeats with two technical repeats , and error bars referring to standard error of the mean.

5.2.4. The contribution of individual amino acid variants to oxacillin resistance

To determine the effects that mutation of the 26 amino acids of MspA had on *S. aureus* in terms of oxacillin resistance, we performed an oxacillin disk diffusion assay (explained previously in chapter 2) to quantify the resistance of the CS^{wt} and CS^{mut} strains. The wild type JE2 and isogenic *mspA* mutant were used as controls. Fig. 5.16 shows that 14 variants had significantly reduced in oxacillin resistance (*$P \leq 0.05$). They were R32A, M35A, L39A, P51A, W56A, F58A, V60A, L63A, E69A, K74A, K77A, D79A, L86A and N87A. One variant, W55A, had a significantly higher resistance when compared to CS^{wt} (**$P \leq 0.01$).

Finally, summary of the amino acid (AA) variant activities, e.g., toxicity, staphyloxanthin and Inhibition zone against oxacillin are shown in Table 5.1.
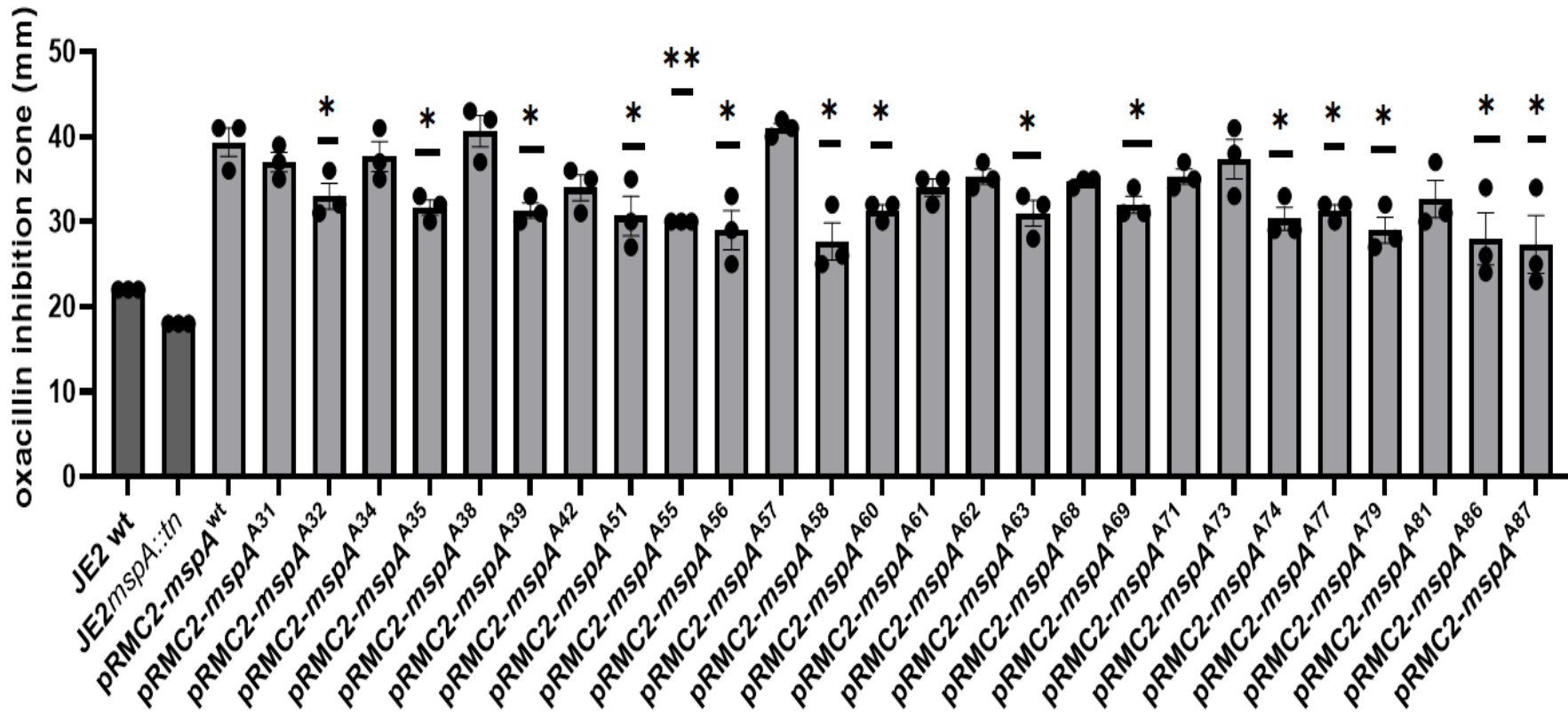
Fig. 5.14: Results of oxacillin resistance assay for the 26 variants to determine which residues of MspA are required compared to the knockout mutant complemented with the wild type *mspA* gene (CS^wt). The wild type strain JE2 and its isogenic *mspA* knockout mutant were used as controls. All the gray light bars are JE2 *mspA*::tn with the different types of pRMC2 plasmid induced with 200 ng/ml tetracycline. Statistics were performed with t-test. The significance levels are *P ≤ 0.05 and **P ≤ 0.01. Data represent the arithmetic mean of three biological repeats with two technical repeats , and error bars referring to standard error of the mean.

Table 5.1. Summary of the amino acid (AA) variant activity. The table shows the significant difference between the variants and the complement strain harbouring the wild type gene (CS^{wt}). The significance levels are P > 0.05 (not significant), *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, and ****P ≤ 0.0001. No AA participated in all three activities. Light grey refers to AAs that participated in one activity, while dark grey refers to AAs that participated in two activities. Only one AA (K or lysine of the variant A81) participates in none of the three activities (yellow colour).

genotype	variant	Toxicity (% THP-1 cell death)		Staphyloxanthin (OD456)		Inhibition zone against oxacillin (mm)	
		Mean	P value	Mean	P value	Mean	P value
JE2 wt		90	-	80	-	22	-
JE2mspA::tn	variant	30	-	50	-	18	-
CSwt (JE2 mspA::tn /pRMC2-mspAwt)		100	-	100	-	39	-
CSmut (JE2 mspA::tn /pRMC2-mspA31)	L31A	56.3237	0.0006	103.27	0.3724	37	0.3139
CSmut (JE2 mspA::tn /pRMC2-mspA32)	R32A	47.3356	0.006	102.301	0.7181	33	0.0487
CSmut (JE2 mspA::tn /pRMC2-mspA34)	I34A	52.7471	0.0121	99.6957	0.9118	37	0.5300
CSmut (JE2 mspA::tn /pRMC2-mspA35)	M35A	47.4423	0.0010	98.0677	0.4676	30	0.0153
CSmut (JE2 mspA::tn /pRMC2-mspA38)	L38A	48.5899	0.0017	101.093	0.8795	40.6667	0.6213
CSmut (JE2 mspA::tn /pRMC2-mspA39)	L39A	85.3598	0.0566	91.2205	0.0295	31.3333	0.0132
CSmut (JE2 mspA::tn /pRMC2-mspA42)	V42A	65.6941	0.0132	92.5351	0.4358	31	0.0777
CSmut (JE2 mspA::tn /pRMC2-mspA51)	P51A	90.6355	0.2999	97.1474	0.7695	30.6667	0.0391
CSmut (JE2 mspA::tn /pRMC2-mspA55)	W55A	93.2266	0.2216	103.191	0.7020	30	0.0050
CSmut (JE2 mspA::tn /pRMC2-mspA56)	W56A	77.1078	0.1145	95.023	0.6570	29	0.0222
CSmut (JE2 mspA::tn /pRMC2-mspA57)	V57A	80.7457	0.0019	86.871	0.0880	41	0.3982
CSmut (JE2 mspA::tn /pRMC2-mspA58)	F58A	81.5952	0.1394	117.242	0.0210	26	0.0132
CSmut (JE2 mspA::tn /pRMC2-mspA60)	V60A	96.18997	0.6164	109.185	0.4748	30	0.0112
CSmut (JE2 mspA::tn /pRMC2-mspA61)	L61A	77.3781	0.0414	97.69	0.7582	32	0.0517
CSmut (JE2 mspA::tn /pRMC2-mspA62)	L62A	71.4354	0.0085	99.4521	0.8869	35.333	0.1012
CSmut (JE2 mspA::tn /pRMC2-mspA63)	L63A	102.193	0.7418	111.545	0.0830	32	0.0211
CSmut (JE2 mspA::tn /pRMC2-mspA68)	V68A	75.4284	0.0175	103.422	0.3767	34.6667	0.0516
CSmut (JE2 mspA::tn /pRMC2-mspA69)	E69A	54.6096	<0.0001	103.428	0.5199	32	0.0196
CSmut (JE2 mspA::tn /pRMC2-mspA71)	T71A	89.3852	0.0444	97.5438	0.8282	34	0.1012
CSmut (JE2 mspA::tn /pRMC2-mspA73)	F73A	46.0808	0.0011	100.112	0.9806	37.3333	0.5239
CSmut (JE2 mspA::tn /pRMC2-mspA74)	K74A	56.3176	0.0070	105.849	0.4591	30.3333	0.0135
CSmut (JE2 mspA::tn /pRMC2-mspA77)	K77A	62.2255	0.0250	107.032	0.1440	31.3333	0.0112
CSmut (JE2 mspA::tn /pRMC2-mspA79)	D79A	60.768	0.0037	104.748	0.4218	29	0.0103
CSmut (JE2 mspA::tn /pRMC2-mspA81)	K81A	83.711	0.2649	107.27	0.1818	31	0.0723
CSmut (JE2 mspA::tn /pRMC2-mspA86)	L86A	63.5929	0.0087	104.989	0.3144	28	0.0312
CSmut (JE2 mspA::tn /pRMC2-mspA87)	N87A	63.5744	0.0012	104.037	0.6556	23	0.0335

5.2.5. Deletion of MspA domains and replacement of MspA loops

5.2.5.1. PCR and *DpnI* digestion of the original pRMC2-*mspA*^{wt} plasmid

After the plasmid pRMC2-*mspA*^{wt} was extracted (explained previously), the *mspA*^{wt} gene was amplified using primers described in chapter 2 (Table 2.4) to result in a change in either a domain or loop structure (Fig. 5.18). In Table 2.4, the primers were designed to remove every domain (domain 1 to domain 4, one at a time), to substitute loop 1 entirely with the AA glycine (G) and to substitute loop 3 with the AA sequence originally present in loop 1. In this kind of reaction, we applied similar PCR conditions to those for generating the AA variants, except that the last domain (domain 4) required digestion with restriction enzyme and further ligation, as the forward primer was very close to the end of the *mspA* gene. Under this condition, the PCR product was cut with *SacI* (procedure explained in chapter 2) flanking the domain and then self-ligated before adding *DpnI* (explained previously). Then, PCR products were digested with *DpnI* and new plasmids (pRMC2-*mspA*^{mut}) were run on a gel to check the new variants. *DpnI* was expected to digest the plasmid template (pRMC2-*mspA*^{wt}) because it is methylated but not the PCR-generated unmethylated plasmids (pRMC2-*mspA*^{mut}).
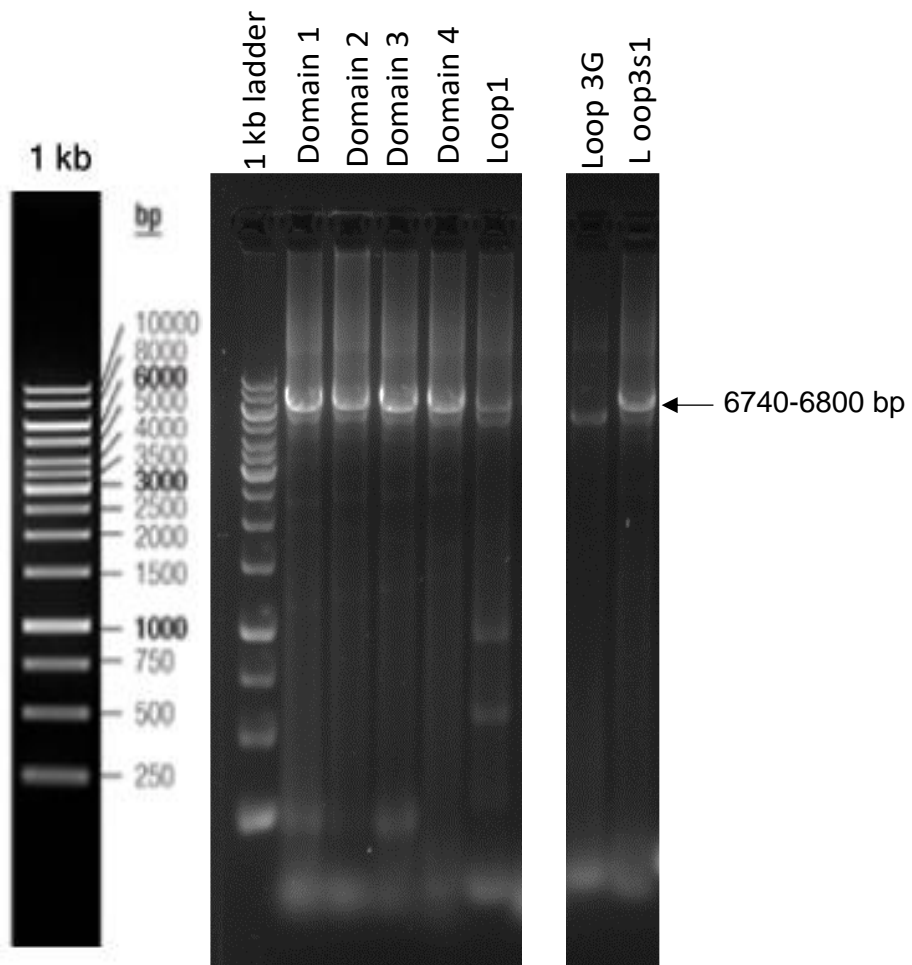
Fig. 5.15: PCR products using the primers in Table 2.4 for either domain removal or loop substitution. The size of pRMC2-*mspA*mut after removal of domain 1 was 6746 bp, while sizes were 6740 bp, 6743 bp and 6740 bp after removal of domains 2, 3 and 4, respectively. Sizes of pRMC2-*mspA*mut after substituting AAs of loop 1 for glycine (G) and substituting loop 3 with the AA sequence originally presented in loop 1 are 6800 bp and 6786 bp, respectively.

5.2.5.2. Transformation of *E. coli* and subsequent transformations into *S. aureus*

The transformation methods were explained previously in sections 5.2.1.4 and 5.2.1.5, with the exception that the *Dpnl*digested new plasmids (pRMC2-*mspA*^{mut}) were transformed into *E. coli* strains DH5-α. Fig. 5.19 shows transformed bacteria spread on LB agar media with ampicillin (100 µg/ml) and incubated in 37°C for 18 h. The plasmids were verified by colony PCR using pRMC2 primers (Fig. 5.20), as previously described, and then plasmids were sent to Eurofins (Germany) for sequencing to confirm the correct mutation (Fig. 5.21). As explained previously, the plasmids with the correct mutations were electroporated into *Staphylococcus aureus* strain RN4220 (Fig. 5.22). Then, the plasmids were moved into JE2*mspA::tn* knockout mutant by electroporation to generate complemented strains with the mutated gene (CS^{mut}) and plated on to TSA containing chloramphenicol (10 µg/ml) and erythromycin (5 µg/ml) (Fig. 5.23). Finally, colony PCR using pRMC2 primers was used to confirm the incorporation of the plasmids in the JE2 *mspA::tn* background (Fig. 5.24).
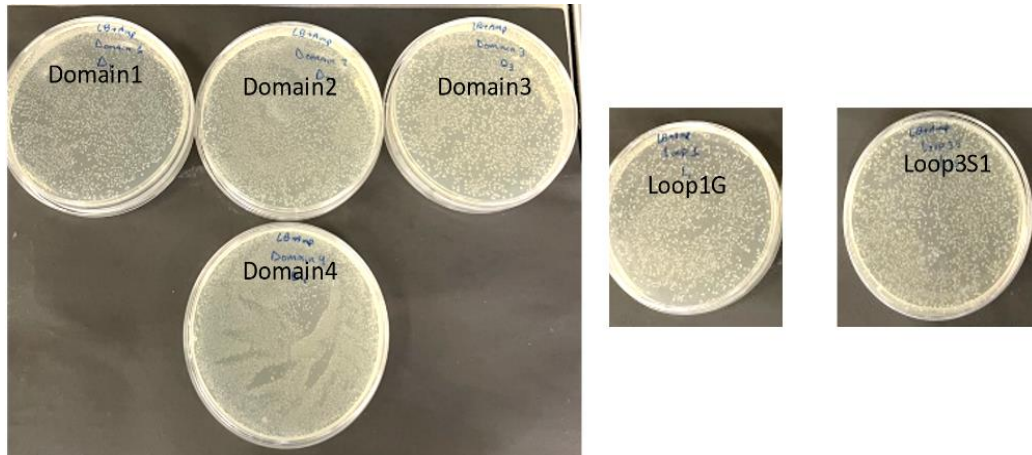
Fig. 5.16: Generated colonies on LB media after transformation of pRMC2-*mspA*mut into *E. coli*.
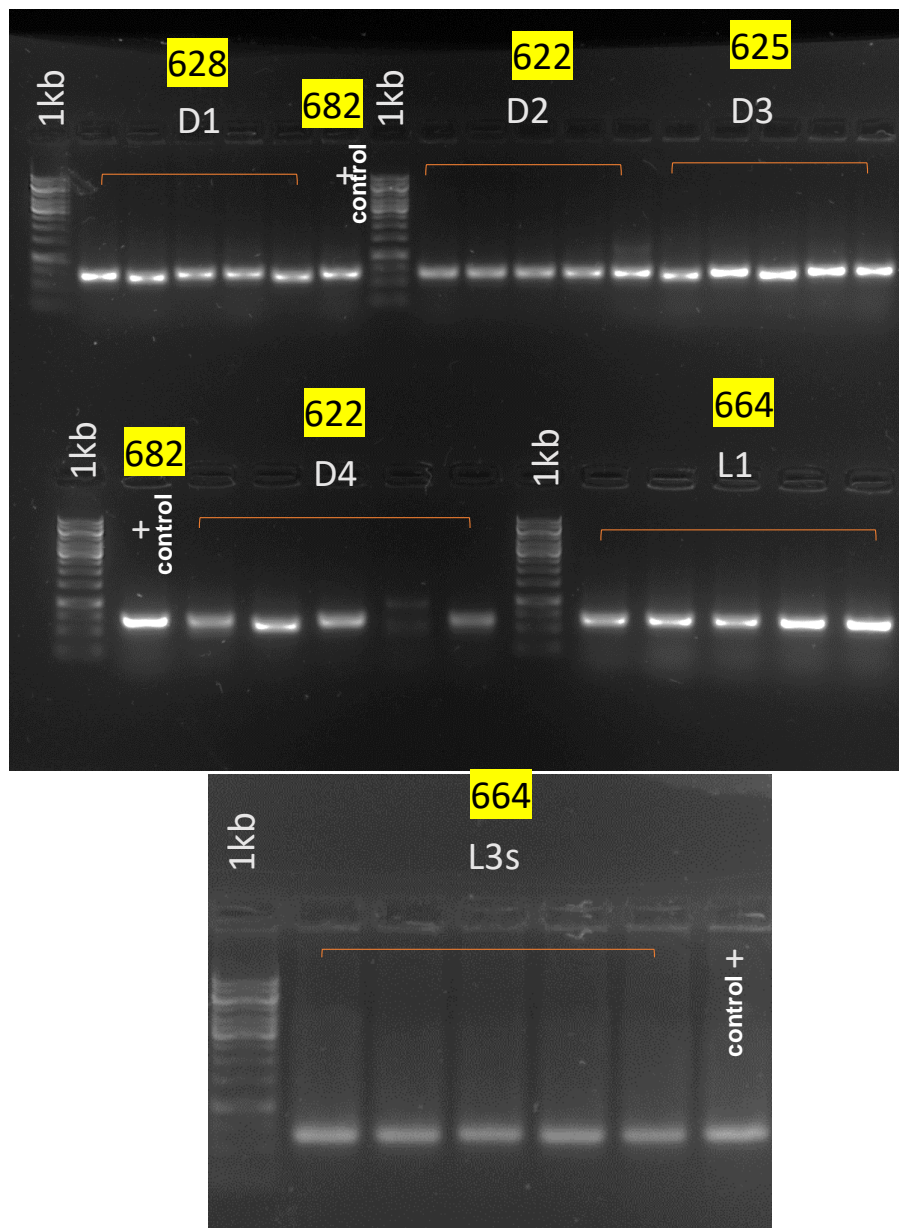
Fig. 5.17: Colony PCR for colonies harbouring new plasmid (CSmut) with domain removed or loop substituted. Yellow highlighted numbers refer to amplicon size in bp.

Fig. 5.18: The sequencing results to confirm the correct mutations of domain removal or loop substitution. Benchling software was used (https://benchling.com). The pRMC2-*mspA*^{wt} was used in the alignment.
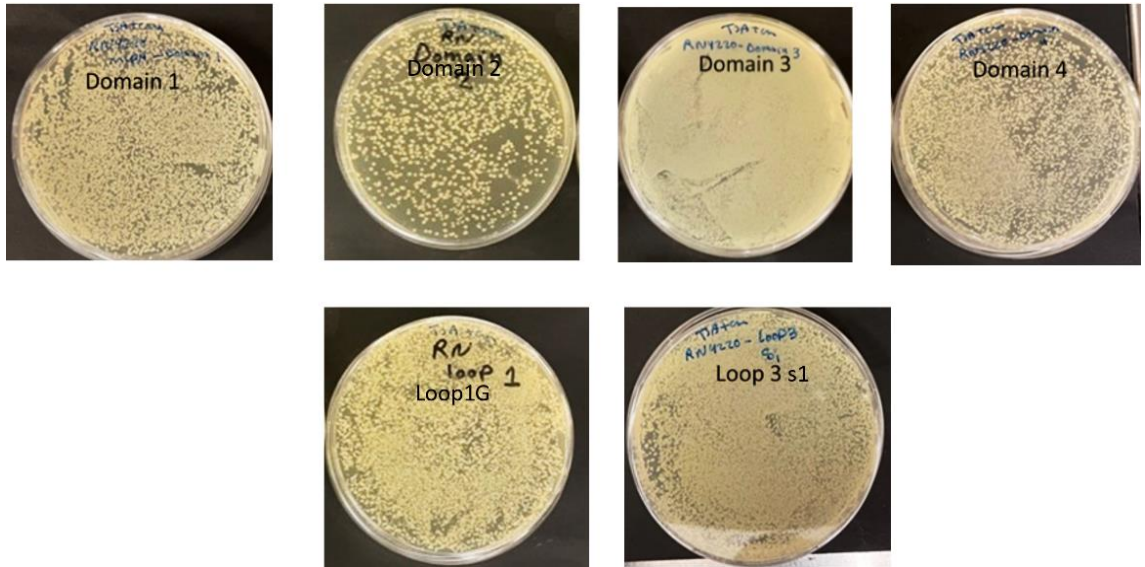
Fig. 5.19: Transformation of pRMC2-*mspA*^{mut} into RN4220. Colonies grown on TSA media containing 10 µg/ml chloramphenicol.
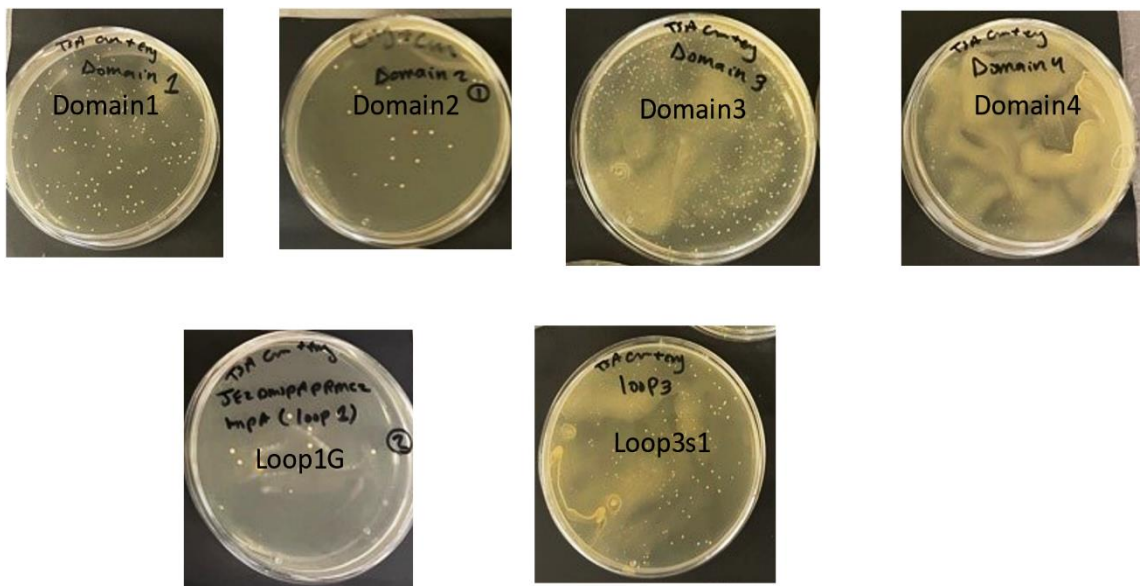
Fig. 5.20: Transformed JE2*mspA::tn* JE2 mutant strain with the pRMC2-*mspA*^{mut} to generate CS^{mut}. Coloniesgrown on TSA containing 10 µg/ml chloramphenicol and 5 µg/ml erythromycin.
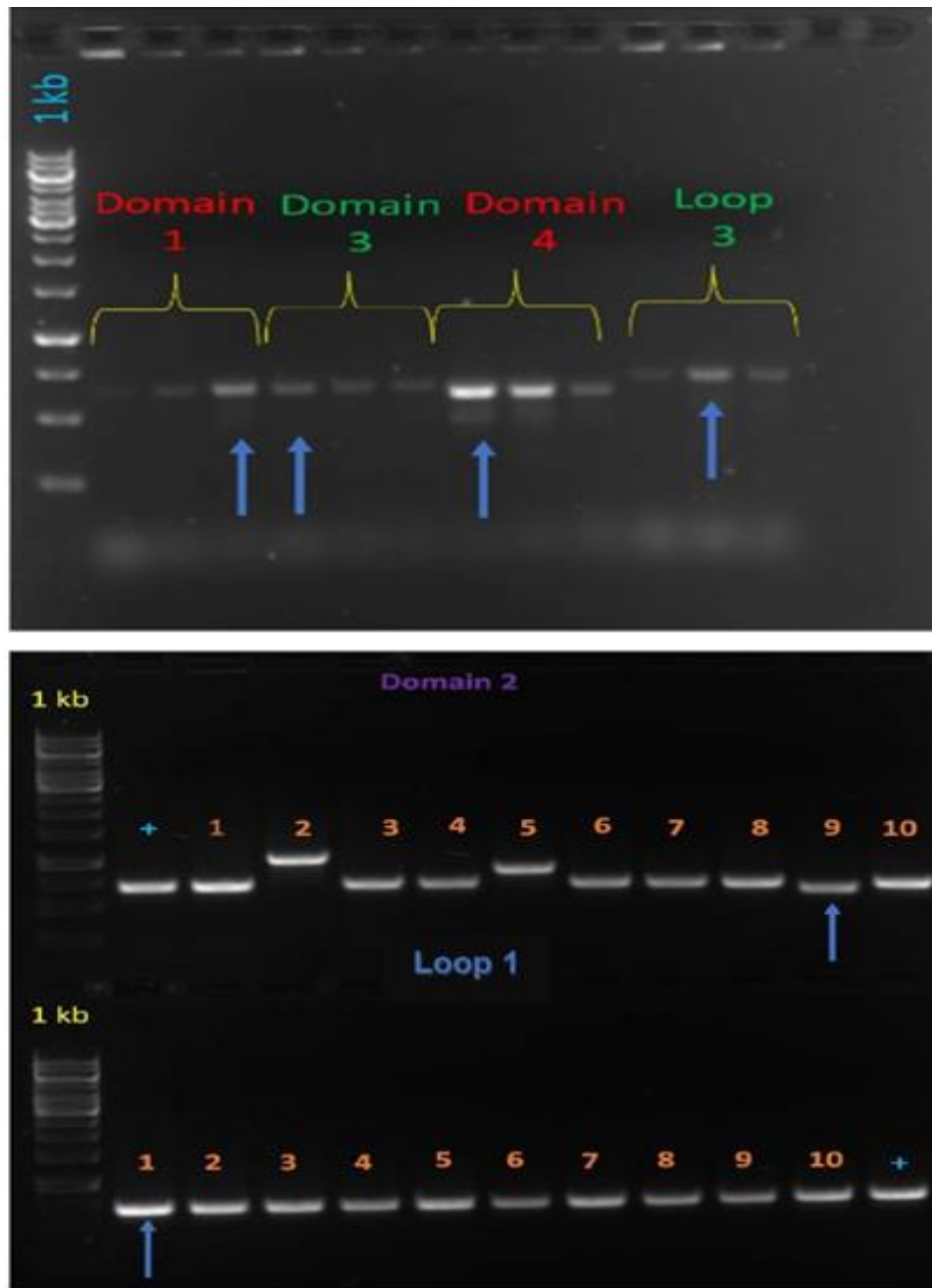
Fig. 5.21: Colony PCR for CS^mut with domain removed or loop substituted. Three colonies are shown for domains 1, 3 and 4 and three colonies for loop 3. Ten other colonies are shown for domain 2 and loop1 in addition to the control colony, i.e, CS^wt (+). The blue arrow indicates the colony that has been used in the subsequent assays.

5.2.6. The contribution of the variants with domain removal or loop substitution to THP-1 cell death

To determine the effects mutation of the domains and loops of MspA had on *S. aureus* toxicity, we performed a THP-1 cell lysis assay (explained previously) comparing the ability of the CS^mut and CS^wt strains to lyse THP-1 cells. The wild type JE2 and isogenic *mspA* mutant were used as controls. We found that the removal of any of the four domains of MspA affected the ability of the bacteria to lyse THP-1 cells. There was also an effect, albeit lower in scale, when the loops were substituted (Fig 5.25). Results of the influence of domains or loops in causing THP-1 cell death are summarized in Table 5.2.
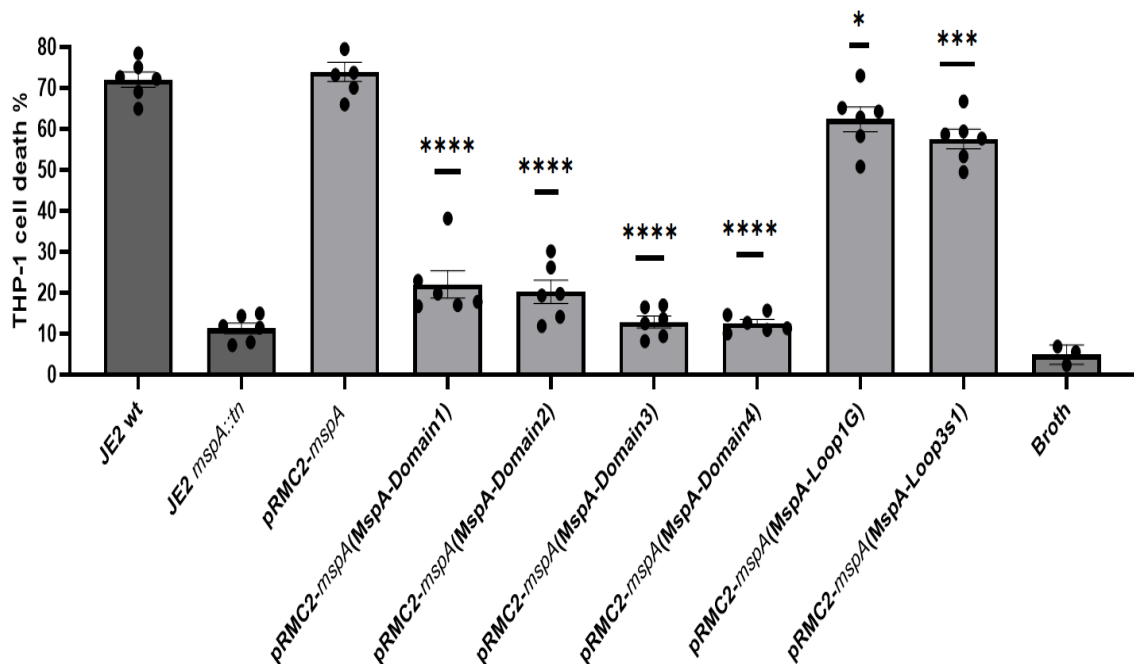
Fig. 5.22: Results of THP-1 cell lysis assay for the domain and loop variants to determine which of them in MspA are required compared to the knockout mutant complemented with the wild type *mspA* gene (CS^wt). The wild type strain JE2 and its isogenic *mspA* knockout mutant were used as controls. All the gray light bars are JE2 *mspA*::tn with the different types of pRMC2 plasmid induced with 200 ng/ml tetracycline. Statistics were performed with t-test. The significance levels are *P ≤ 0.05, ***P ≤ 0.001, and ****P ≤ 0.0001. . Data represent the arithmetic mean of six biological repeats , and error bars referring to standard error of the mean.

5.2.7. The contribution of the variants with domain removal or loop substitution to staphyloxanthin production

To determine the effects that mutation of the domains and loops of MspA had on *S. aureus* staphyloxanthin production, we quantified the pigmentation (explained previously) by comparing the ability of the CS^mut strains with the mutated *mspA* genes to produce staphyloxanthin to the CS^wt strain. The wild type JE2 and isogenic *mspA* mutant were used as controls. We found that each of the four domains was also very important for the process of carotenoid biosynthesis. We could still see a significance different for the variant with loop 3 substituted for loop 1 but not for loop 1 substituted for poly-glycine(Fig. 5.26).
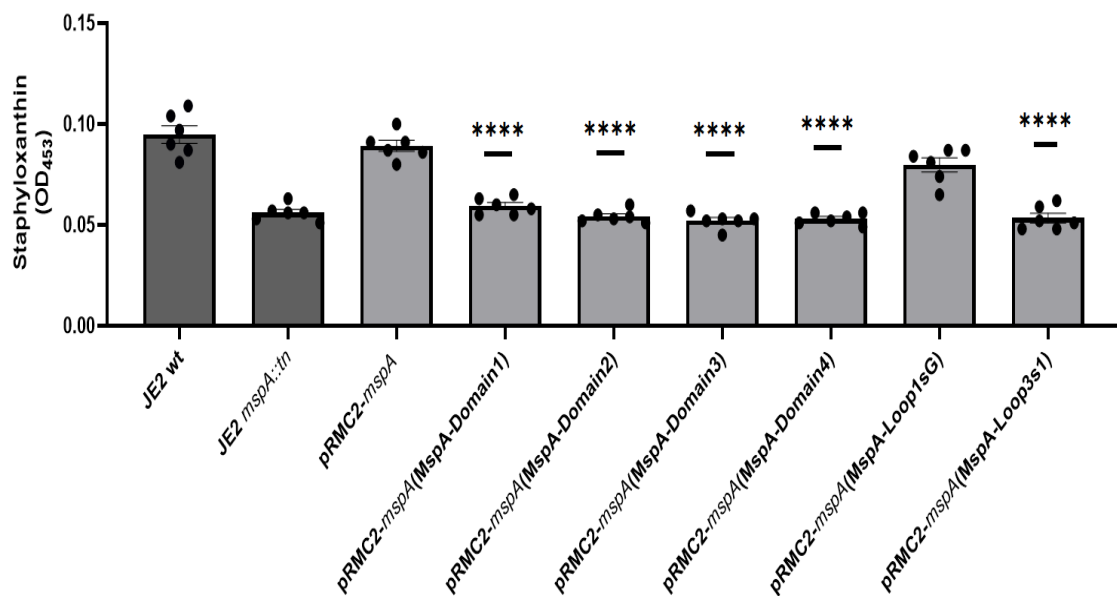
Fig. 5.23: Results of the staphyloxanthin production assay for the domain and loop variants to determine which of them in MspA are required compared to the knockout mutant complemented with the wild type *mspA* gene (CS^wt). The wild type strain JE2 and its isogenic *mspA* knockout mutant were used as controls. All the gray light bars are JE2 *mspA*::tn with the different types of pRMC2 plasmid induced with 200 ng/ml tetracycline. Statistics were performed with t-test. The significance level is ****P ≤ 0.0001. Data represent the arithmetic mean of six biological repeats , and error bars referring to standard error of the mean..

5.2.8. The contribution of the variants with domain removal or loop substitution to oxacillin resistance

To determine the effects that mutation of the domains and loops of MspA had on *S. aureus* oxacillin resistance, we performed an oxacillin disk diffusion assay (explained previously) and compared the ability of the CS^{wt} and CS^{mut} strains to resist oxacillin. The wild type JE2 and isogenic *mspA* mutant were used as controls. We found that all the variants were showed significant differences compared to the complement stain with wild type gene (Fig. 5.27).
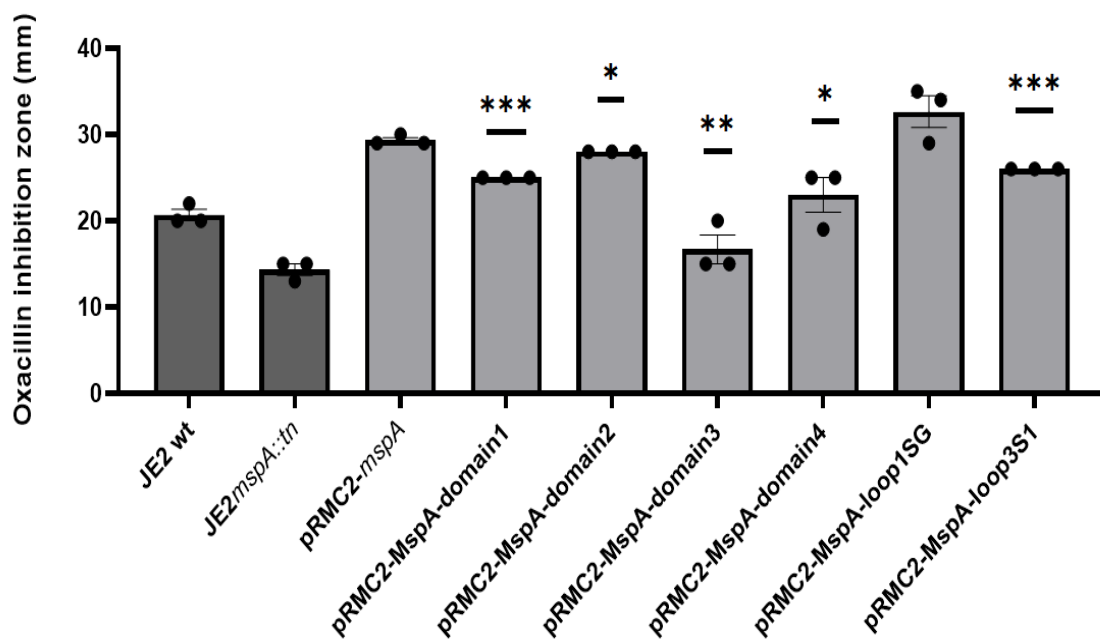
Fig. 5.24: Results of the oxacillin resistance assay for the domain and loop variants to determine which of them in MspA are required compared to the knockout mutant complemented with the wild type *mspA* gene (CS^{wt}). The wild type strain JE2 and its isogenic *mspA* knockout mutant were used as controls. All the gray light bars are JE2 *mspA*::tn with the different types of pRMC2 plasmid induced with 200 ng/ml tetracycline. Statistics were performed with t-test. The significance levels are *P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001. . Data represent the arithmetic mean of three biological repeats with two technical repeats , and error bars referring to standard error of the mean.

Table 5.2. Summary of the variant's activity after removal of MspA domains or substitution of loops. The table shows the significant differences between the variants (CS^{mut}) and the complement strain harbouring the wild type gene (CS^{wt}).

genotype	Toxicity (% THP-1 cell death)		Staphyloxanthin (OD456)		Inhibition zone oxacillin (mm)	
	Mean	P value	Mean	P value	Mean	P value
JE2 wt	72.014	-	0.0946667	-	22	-
JE2mspA::tn	11.2475	-	0.056	-	18	-
CSwt (JE2 mspA::tn /pRMC2-mspAwt)	73.8865	-	0.0891667	-	39	-
CSmut (JE2 mspA::tn /pRMC2-mspA-domain1)	22.0272	<0.0001	0.059333	<0.0001	25	0.0002
CSmut (JE2 mspA::tn /pRMC2-mspA-domain2)	14.13043	<0.0001	0.0541667	<0.0001	28	0.0161
CSmut (JE2 mspA::tn /pRMC2-mspA-domain3)	12.8228	<0.0001	0.052	<0.0001	16.6667	0.0017
CSmut (JE2 mspA::tn /pRMC2-mspA-domain4)	12.62136	<0.0001	0.053	<0.0001	23	0.0354
CSmut (JE2 mspA::tn /pRMC2-mspA-loop1G)	62.3474	0.0128	0.0796667	0.0593	32.6667	0.1518
CSmut (JE2 mspA::tn /pRMC2-mspA-Loop3s1)	57.5147	0.0006	0.053333	<0.0001	26	0.0006

5.3. Discussion

The MspA protein consists of 105 amino acids comprising four transmembrane domains and three loops. To identify the active domains, loops and amino acids that participate in the detected MspA protein activities, two site-directed mutagenesis approaches were undertaken. The conserved amino acid residues (26) were identified by aligning the *S. aureus* MspA protein with homolouges from other bacterial genera identified in the NCBI database. The domains and loops were defined and mutated based on their predicted structure and howMspA sits in the bacterial membrane. The overlap between the AA and domains/loops is such that none of the conserved AAs were found in the first domain and first loop, whereas seven of the conserved amino acids exist in domain 2; 13 in domain 3; 4 in loop 3; and only 2 were situated in domain 4 (Fig. 5.28 & Table 5.1). Based on this conservation, this would suggest that the central region of the MspA protein is likely to be its active region. However, this did not prove to be the case, as I will now discuss in relation to each of the three activities tested: toxicity, staphyloxanthin production and oxacillin resistance.
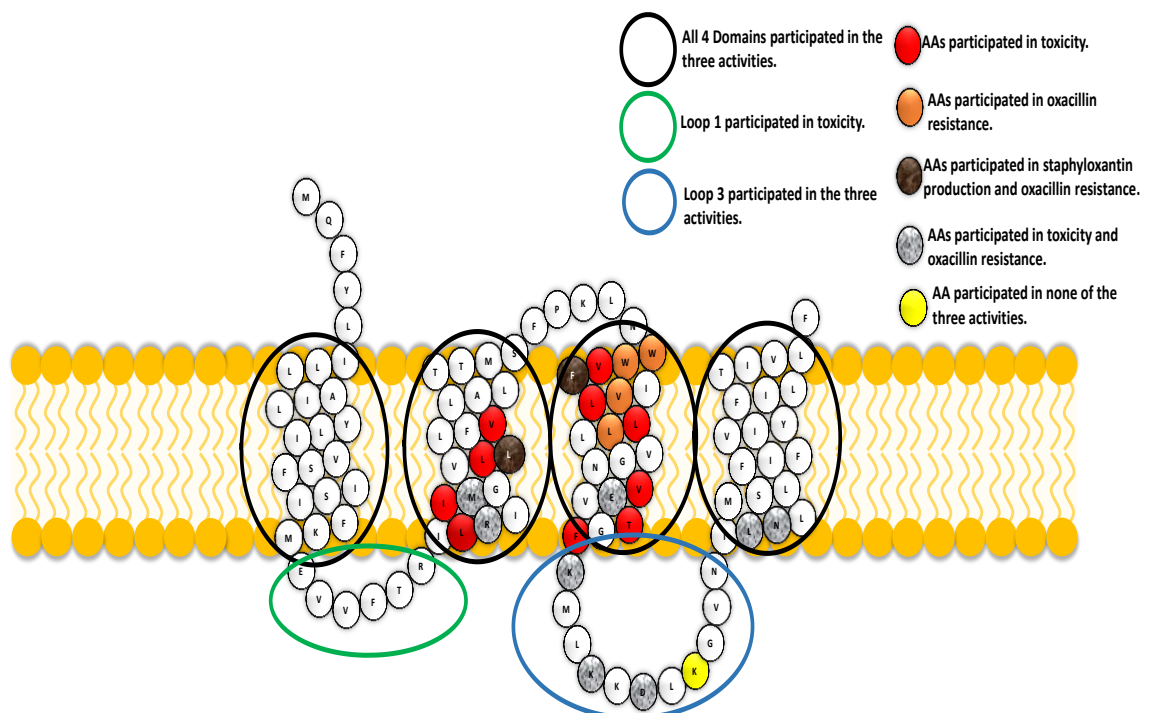
Fig 5.25: 2D structure of the MspA protein that shows the conserved AAs and the domains/loop structures that participate in toxicity, staphyloxanthin (or carotenoid) biosynthesis and oxacillin resistance.

Toxicity: There were 18 AAs that appeared to significantly contribute to the effect MspA has on toxicity, and as summarised on Fig 5.28, these all seem to cluster in the middle portion of the protein. Interestingly, deletion/mutation of any of the larger regions of the protein, domains or loops, all affected toxicity. Given the number of AAs in domains 2, 3 and 4 and loop 3 that contributed to toxicity, it is unsurprising that the deletion of these domains also caused a loss in toxicity. However, this work was limited by the fact that we based our selection of which AAs to mutate on their level of conservation, and as such we may have missed other important residues elsewhere in the protein, in particular in domain 1. In addition to the effect that the loss of important residues had on MspA activity, these deletions were predicted to cause major structural changes to how the protein sits in the bacterial membrane (Fig. 5.3), likely the effects observed following mutation of the individual AAs. We were able to take greater care when we mutated the loops, such that these were replaced as opposed to being deleted, and as such no major structural changes should have been caused to the protein. Despite this, $_{mutation}$ of both loops affected toxicity, suggesting that in addition to those characterised here, there are critical AA still to be identified elsewhere in the protein. Further work could adopt a blanket approach and change each of the 105 residues to identify all of the AAs that contribute to this activity.

Staphyloxanthin production: Quite a different story has emerged with respect to the role of individual AAs in the effect MspA has on staphyloxanthin production. Only, two AAs affected this activity, L39 and F58, and even then with quite a modest effect (Fig 5.15). The deletions of the domains and loop 3 all reduced staphyloxanthin production by almost a half (Fig 5.26). The lack of much AA specific activity would suggest one of two things, the first that perhaps had we mutated all 105 AAs, we would have identified those specifically involved in this activity. The second is that perhaps the effect of MspA on this activity is a more general structural role, not involving the activity of any specific AAs.

Oxacillin resistance: The AAs involved in the oxacillin resistance conferring activity of MspA were largely located across the C terminal half of the protein, although as with toxicity, this is likely to be biased by the fact that there were few conserved AA in the N terminal region, so these did not get mutated . As for both toxicity and staphyloxanthin production, the deletion of the domains affected oxacillin resistance, as did the substitution of loop 3, but not loop 1. This suggests that as for toxicity, there are AAs that are critical to this activity, suggesting the role MspA plays in it is likely to be quite specific.

There was a high degree of overlap between the AAs that affected toxicity and oxacillin resistance, but little in common with staphyloxanthin production. Of the 13 AAs that contributed to oxacillin resistance, eight of them also contributed to toxicity. This suggests that the means by which MspA affects this activity has some common features. However, that these functions did not fully overlap, that is, that some AAs only affected toxicity and some only oxacillin resistance, suggests that MspA utilises different processes to affect these activities. I hypothesise that perhaps MspA interacts with multiple targets utilising different regions of the protein, some of which affect both toxicity and oxacillin resistance and some that only affect one activity or the other.

The major limitation of this work is that we did not mutate all 105 AAs of MspA, as this would have given us a fuller picture of the important regions of this protein. Also, we were unable to check whether the mutations and deletions had any effect on MspA protein expression, as despite effost we have been unable to geneter an antiMspA antibody. In hindsight, we believe it is likely that domains 1 and 4 and loops 1 and 3 may contain important AAs, as when we deleted/substituted these entire regions, activity was affected. This would be the first thing I would do with more time in the laboratory. The other experiment I would like to have had time to perform is to see if MspA acts as a multimer. As it is such a small protein it is challenging to imagine that it can affect so many processes acting as a monomer. There are several ways this could be examined, but I would have liked to have tried using bimolecular fluorescence complementation (BiFC) using a plasmid that contains a yellow fluorescence protein gene that is split into two portions (VS155 & VN155). Each of them would be attached to a copy of the *mspA* gene and the yellow emission would be generated if these two gene portions attached. Finally, I think it is critical that we identify what other proteins or molecules MspA interacts with, as this is likely to be key to its activity. There are several ways we could do this. If we had antibodies to MspA, we could perform immunoprecipitation to pull out partner proteins. Alternatively, we could use the system described above, but instead clone a library of random *S. aureus* DNA fragments into the second vector to look for interacting proteins. Both of these experiments are huge undertakings and beyond the scope of this PhD, but likely to produce some very interesting results that could be key to understanding how this small protein has such pleiotropic effects on core *S. aureus* processes.

We should acknowledge that a limitation of the mutagenesis strategy, particularly for the domain mutants, that affects MspA protein expression was not checked.

6. Summary

Staphylococcus aureus, a notorious multidrug-resistant bacterium, is a facultative aerobic Gram-positive pathogen of phylum Firmicutes that causes a number of serious clinical diseases in humans. We need to understand how it causes diseases if new approaches to prevent and treat infections are to be developed. This work aimed to contribute to this goal by focussing on a novel membrane bound protein that a GWAS approach identified as contributing to the ability of *S. aureus* to lyse human cells. During this GWAS study, the research group discovered several genes that positively correlated with toxicity. This included the membrane stabilizing protein A gene, or *mspA*, that encodes a small membrane-bound protein of 105 amino acids (AA). In this thesis, we aimed to verify the contribution of this protein to the toxicity of the bacteria, characterise the mechanism by which MspA protein affects toxicityand investigate contribution of the protein to iron homeostasis. We also studied its contribution to β-lactam resistance, expression of PBP encoding genes, autolytic activity of the bacteria and influence on cell wall thickness. Finally, we decided to detect the bacterial response to changing conserved amino acids (AAs) of the MspA protein and to manipulating the protein's domain and loop structures to detect their contribution to toxicity, defensive capabilities against the human immune system via staphyloxanthin production and to resistance to oxacillin.

These characteristics were studied across the three results chapters of this thesis. The results of chapter 3 are summarised in Fig. 6.1, where we showed that the loss of the *mspA* gene resulted in the bacteria being less able to produce cytolytic toxins and lyse THP-1 cells. The effects were complemented by expressing the gene from an inducible plasmid. Next, we sought to determine whether MspA protein loss resulted in a reduction in expression of PSMs. We compared the level of production of the PSMs group of toxins in the wild-type and *mspA* mutant and found the latter produced a reduced amount of these toxins. One hypothesis was that MspA could affect the expression of the *agr* quorum sensing system, and we verified this using a reporter fusion system, which showed that inactivation of the MspA protein resulted in a down-regulation, or lack of activation of the *agr* system. To identify regulatory changes in cytolytic activity due to the loss of the MspA protein, proteomic analysis (tandem mass tagging coupled to mass spectroscopy or TMT-MS) was performed at the level of whole cell lysates of JE2 wild-type *S. aureus* and *mspA* mutant. The results indicated that 63 proteins showed differential abundance in the *mspA* mutant

with two or more-fold difference. Among these proteins was CrtM, which is involved in the biosynthesis of the carotenoid pigment staphyloxanthin, which was lower in abundance in the mutant strain. This explained our observation that the *mspA* mutant was less golden in colour than the wild-type strain. The Hla protein (alpha toxin) was proven to be more abundant in the cell lysate of the mutant than in the wild-type bacteria. As Hla was more abundant in whole cell lysate of the mutant according to the proteomic data, we additionally performed Western immunoblot and found a slight increase in Hla protein in the whole cell lysate and a slight reduction in the supernatant of the mutant compared to the wild type. Four other proteins involved in iron homeostasis were more abundant in the *mspA* mutant. These were HrtA and HrtB are involved in transport (efflux) of iron from the bacteria, and IsdB and IsdC, which are involved in the acquisition (influx) of iron.

Levels of cytosolic iron and heme should be balanced by *S. aurues* to meet the nutritional requirements, on the one hand, and to protect bacteria from potential iron toxicity, on the other hand. Iron homeostasis occurs by balancing the influx/efflux processes in an orchestrated fashion. Hemic iron acquired from the host can be released by Isd (Iron-regulated surface determinant) system through their oxygenase activity, where heme can be exploited as a cofactor in the bacterial heme-proteins, e.g., cytochromes (Skaar et al., 2004; Reniere et al., 2007; Tiburzi et al., 2009). Such an action is energetically favourable to the bacteria, which justifies preference for heme as an iron source in *S. aureus*. As indicated earlier, cytosolic heme homeostasis is maintained through the action of the efflux pump HrtAB (heme regulated transporter). Gene encoding this pump are regulated by the heme concentration sensory system HssRS (heme-sensing two-component regulator) (Torres et al., 2007; Stauff et al., 2007; 2008). In addition, *S. aureus* can store the excess of inorganic ferric iron inside the ferritin-like protein FtnA to avoid toxicity (Horsburgh et al., 2001). In turn, ferritins can sustain maximal bacterial growth in iron starvation and protect cells against Fenton chemistry during oxidative bursts. The staphylococcal ferritin gene *ftnA* is also able to oxidize Fe(II) to Fe(III) when assembled in a 24-mer protein (Andrews et al., 2003; Zühlke et al., 2016). Expression of this gene is attenuated by the action of PerR (peroxide-responsive repressor) based on iron level required for *S. aureus* growth (Morrissey et al., 2004). PerR also drives expression of enzymes responsible for hydroxyperoxides scavenging in order to lock reactive iron during oxidative bursts (Nobre and Saraiva 2013).

As we have shown that MspA is active in two distinct genetic backgrounds (i.e., JE2: MRSA & SH1000: MSSA), we believe that blocking expression of the *mspA* gene

could be a novel tool to treat *S. aureus* infections. Although it still warrants further investigation, such an alternative control strategy could be used alongside or in place of conventional antibiotic approaches. Encouragingly, Koch et al. (2017) has highlighted the successful use of small-molecule inhibitors to disrupt membrane protein function, which resulted in attenuated virulence and reduced mortality in murine models of infection. Therefore, identifying a means of repressing MspA production or knocking out the *mspA* gene could incapacitate the bacteria, rendering them susceptible to immune clearance. This novel approach can be considered as a potential target for future therapeutic development.

As we detected differences in the intracellular abundance of iron in the bacterial cytoplasm, we used the antibiotic streptonigrin that indirectly measures intracellular iron. The *mspA* mutant appeared to be more susceptible to this antibiotic, which confirmed that the *mspA* mutant harbours more intracellular iron. Then, we performed an iron adaptation assay to know whether MspA helps the bacteria to adapt in iron rich media. The results suggest that the *mspA* gene is positively involved in the adaptation of *S. aureus* to high iron environments (Fig. 6.1). We also showed that the toxic effect of increased intracellular iron levels may be contributing to lowering of the toxicity phenotype observed for the *mspA* mutant. Much of this work has been published, alongside mice infection experiments that demonstrated how non-pathogenic *S. aureus* is without a functioning *mspA* gene (Duggan et al., 2020).

In Chapter 4, we compared the susceptibility levels of the wild type JE2 strain and its isogenic *mspA* mutant to three β-lactam antibiotics, i.e, oxacillin, amoxycillin with clavulanic acid and ampicillin, using a disk diffusion assay and found that the *mspA* mutant was more resistant to the three β-lactam antibiotics. This effect was, however, limited to the JE2 background, with no difference in resistance observed for the SH1000 strain. We next sought to determine whether this effect on JE2 was due to the increased hetero- or homo-resistance level of the MspA mutant population. Accordingly, we compared the heteroresistance levels of the wild type JE2 strain and its isogenic *mspA* mutant using a PAP-AUC assay and the results indicated that the two genotypes showed low levels of heteroresistance , since bacterial growth was maintained up to high concentrations (4 mg/ml for the wild type, while 8 mg/ml for the *mspA* mutant strain) of the antibiotic, then dropped soon to zero. Then, we hypothesised that the higher level of resistance of the *mspA* mutant to β-lactam antibiotics may be due to the production of the β-lactamase enzyme. However, using a nitrocefin assay we demonstrated that neither the wild type nor mutant strain produced any β-lactamase enzyme.
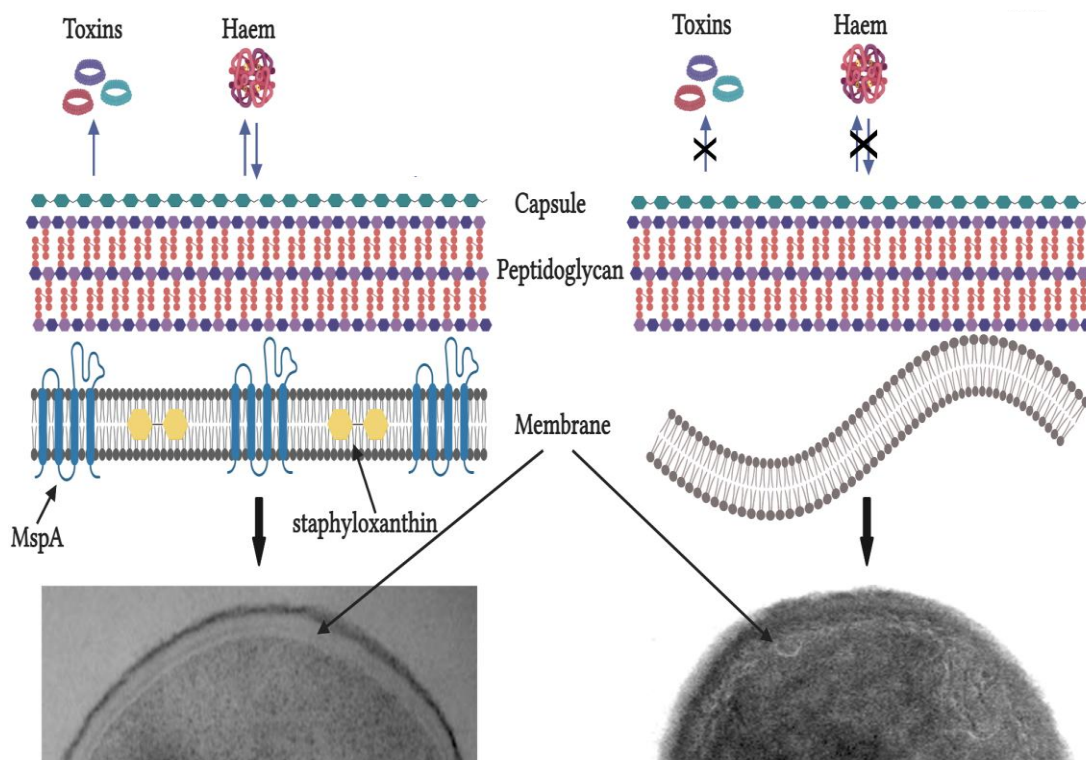
Fig. 6.1: Summary of the effect the loss of MspA has at the bacterial cell envelope. In the presence of MspA there is a normal level of toxin secretion, iron (haem) uptake and efflux and staphyloxanthin biosynthesis. When MspA is absent all of these processes are negatively affected. Recent findings generated by another researcher (Dr. Seana Duggan) in Prof. Massey's lab group suggest that the membrane in the MspA mutant is quite unstable (see the membrane ruffles and invagination in the TEM above).

Then, we performed qRT-PCR for the five genes involved in peptidoglycan biosynthesis, i.e, *pbp1, pbp2, pbp3, pbp4* and *pbp2a,* to examine their expression levels in the *mspA* mutant. We found that *pbp1* and *pbp4* were expressed at significantly lower levels in the *mspA* mutant compared to their levels in the wild type JE2 strains, whereas *pbp2* and *pbp3* were expressed at significantly higher levels. Interestingly, there was no significant difference between the wild type and *mspA* mutant strain in the level of *pbp2a* expression, which is the enzyme that specifically confers oxacillin resistance. This led us to speculate that *pbp2* and *pbp3* might make a stronger contribution to conferring resistance in the mutant strain than other *pbps* genes. The effects of this dysregulation of the peptidoglycan biosynthetic genes the level were tested for its contribution to of penicillin binding proteins (PBPs) in the bacterial membrane was tested using bocillin, and the results demonstrated that the *mspA* mutant bound significantly more bocillinand thus was producing more PBPs than the wild type strain. Then, we compared the autolytic activity in the wild type and mutant strain as an indicator of the sensitivity of peptidoglycan within the cell wall to the bacterial autolytic enzymes. The results indicated that the *mspA* mutant was less

autolytic compared to the wild type strains which suggests that the peptidoglycan in the cell wall of the *mspA* mutant has changed structurally, making it less sensitive to autolytic enzymes and β-lactam antibiotics.

To examine whether any major structural changes in the cell wall could explain our results, we performed transmission electron microscopy (TEM) on the wild type and *mspA* mutant. This revealed that the cell wall of the *mspA* mutant was significantly thicker than that of the wild type strain across both backgrounds. We also exposed the bacteria to sub-inhibitory levels of oxacillin, to induce an increase in the expression of the resistance machinery. These TEMs demonstratesd that the cell wall of the *mspA* mutant was more stable under these conditions. The combined results of chapters 3 and 4 are summarised in Fig. 6.2.
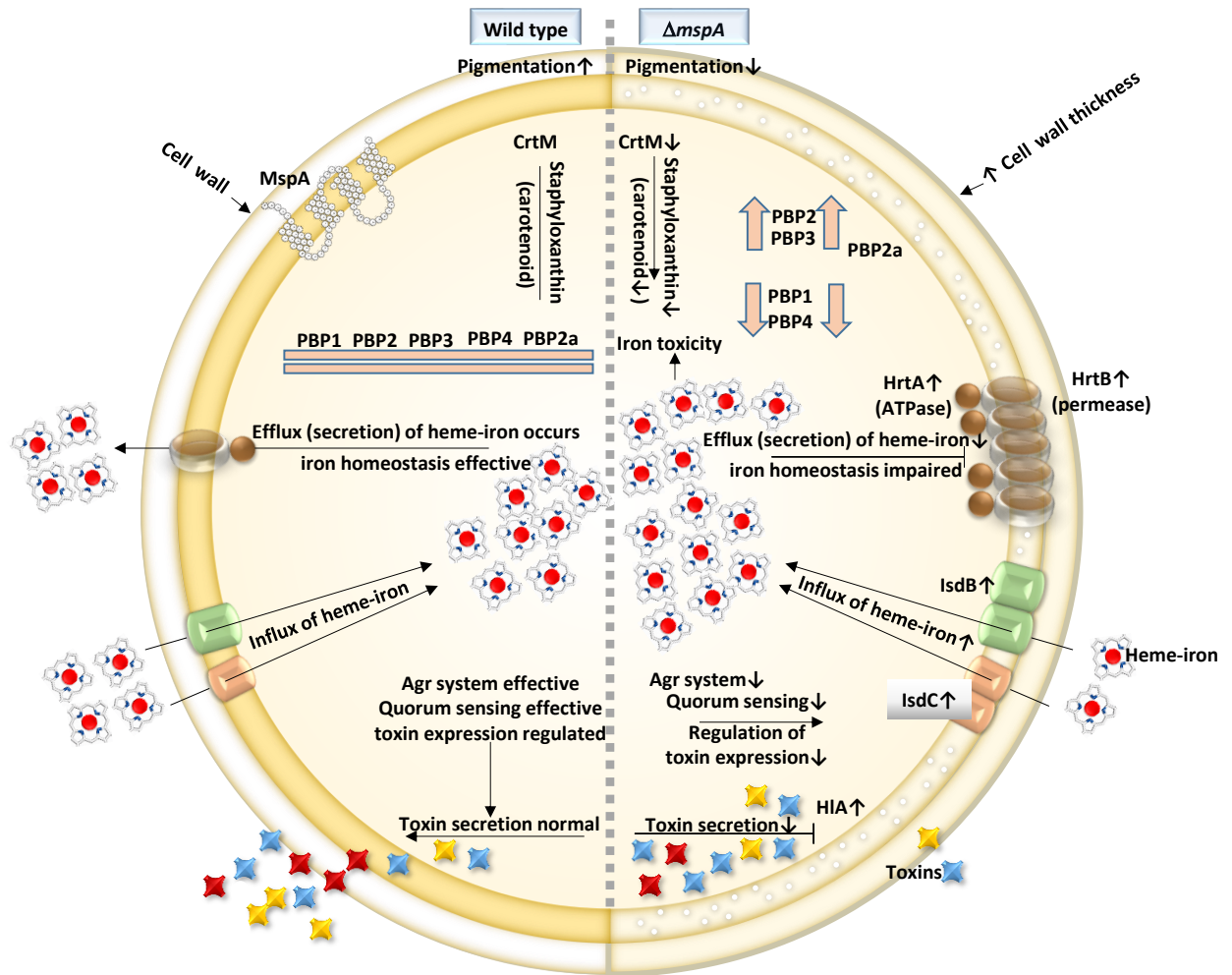
Fig 6.2: Summary of the effects that inactivation of *mspA* has on *S. aureus* at a cellular level. In the absence of MspA, the bacteria produce fewer staphyloxanthin, secrete less toxins, have a defective Agr response, and their ability to control their iron homeostasis is affected. However, inactivation of MspA also results in an increased resistance to β-lactam antibiotics, possibly as a result of increased amounts of PBP2 and PBP3, which we believe causes an increase in the thickness of the cell wall.

In Chapter 5, we applied site-directed mutagenesis (SDM), a procedure to create specific or targeted changes in DNA and subsequent protein, to identify regions and amino acids critical to the activity of MspA. Firstly, we performed an AA sequence alignment including the MspA protein of *Staphylococcus aureus* alongside other bacteria and we detected 26 conserved AAs. These AAs were substituted, one at a time, with alanine, while one AA (L62) from the GWAS data was also substituted with alanine. Then, we removed the four domains, one at a time, and made substitutions of two loops (loop 1 was substituted for poly-glycine, and loop 3 was substituted for loop 1).

The results of the toxicity effects in terms of the ability of the complement strains with the AA variants (CS^mut) to lyse THP-1 cells, demonstrated that 18 AAs showed a

significant decrease when compared to the CS with the wild type gene (CS^{wt}). They were L31A, R32A, I34A, M35A, L38A, V42A, V57A, L61A, L62A, V68A, E69A, T71A, F73A, K74A, K77A, D79A, L86A and N87A. To determine whether the AA variants contributed to the production of staphyloxanthin or carotenoid, we quantified the pigmentation of these CS^{mut} variants compared to CS^{wt}. The results indicated that the CS^{mut} variant L39A showed a significant reduction in staphyloxanthin production compared to CS^{wt}, while variant F58A showed a significant increase. In terms of resistance to oxacillin for the 26 CS^{mut} variants, the results of the disk diffusion assay indicated that 15 AA variants, i.e, R32A, M35A, L39A, P51A, W55A, W56A, F58A, V60A, L63A, E69A, K74A, K77A, D79A, L86A and N87A, showed a significant reduction compared to CS^{wt}. Interestingly, none of the 18 AA residues that were involved in toxicity were involved in staphyloxanthin production, while two AA residues, L39 and F58, seemed to participate in the latter activity and also to contribute to oxacillin resistance.

The results of the domain/loop CS^{mut} variants showed the importance of each of the four domains to the protein's ability to lyse THP-1 cells, while the loops showed a lower influence compared to CS^{wt}. The results of the domain/loop variants showed that each of the four domains as well as the loop 3 substituted for loop 1 are very important for the process of carotenoid biosynthesis. No influence for loop 1 substituted for poly-glycine was detected. In terms of the influence of the domains/loops, all the variants, except for that with loop 1 substituted for poly-glycine, showed a significant reduction in oxacillin resistance compared to CS^{wt} (Fig. 6.3).

Fig 6.3: 2D structure of the MspA protein referring to the conserved AAs and the domains/loop structures that participate in toxicity, staphyloxanthin (or carotenoid) biosynthesis and oxacillin resistance.

We are still quite far away from understanding how this small protein can have all of these pleiotropic effects. It seems unlikely that it is directly interacting with all of these distinct processes and instead, we hypothesise that it could be involved in some core process that keeps the membrane stable, which would be required for other membrane proteins to act optimally. For example, one hypothesis is that MspA might be involve in lipoteichoic acid (LTA) biosynthesis. LTA is responsible for anchoring the bacterial membrane to the cell wall (Corrigan et al., 2011) and so if negatively affected, could result in a destabilised membrane. To test this, we could extract and quantify the cellular LTA and compare it between the mutant and the wild type. Alternatively, we could utilise a bacterial two-hybrid system or perform a crosslinking experiment to identify proteins that can physically interact with MspA.

Given its small size, we also speculate that MspA may multimerise to become active. With more time and resources, to investigate this question I would apply a method call bimolecular fluorescence complementation (BiFC, Fig. 6.4). This would involve using a plasmid that contains a yellow fluorescence protein gene that is split into two portions (VS155 & VN155, Fig. 6.4), each of which will be attached to a copy of the *mspA* gene so that yellow emission will be generated when these two gene portions attach together. This will determine whether MspA protein is a monomer or dimer (Lai & Chiang, 2013). We could take this further by using BiFC-fluorescence resonance energy transfer (FRET) or BiFC-FRET combined to see if MspA is trimer or a tetramer (Vidi & Watts, 2009). These results would provide further information on how MspA can affect so many *S. aureus* processes.
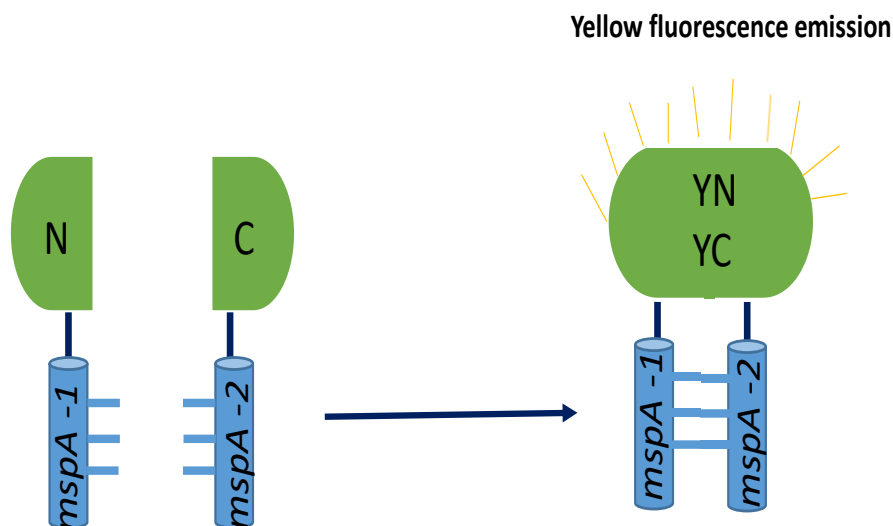
Fig 6.4. Schematic representation of (BiFC) Fluorescence emission is generated due to merging between the two yellow fluorescent protein portions YN and YC the two copies of MspA protein (dimer) being attached.

In conclusion, this study has characterised the activity of a previously unknown protein that we have called MspA, as we believe it is involved in stabilising the *S. aureus* cell membrane. Prior information regarding this gene in other bacteria (see Fig 5.2) is that it encodes a membrane-stabilizing protein, but no prior reports to emphasize its role in bacterial pathogenesis. However, there are reports to emphasize the influence of Lipoteichoic acid (LTA) inhibition when the gene encoding LTA (*ltaS*) is mutated on its possible use in future therapeutic applications human infections caused by *S. aureus* or other bacterial pathogens (Gründling and Schneewind 2007). This gene in addition to *mspA* deserve further study either individually or in combination.

The inactivation of MspA has pleitropic effects on the bacteria that appear to stem from the activity of proteins that reside in the membrane (e.g., Agr, Hrt, PBPs). As pleotropic effects of this gene seem to be extended to pathogenesis via manipulating toxin production and secretion, then, we highly recommend that it might have therapeutic potential to treat or protect humans from the disease. In addition to gaining insight into how these activities are affected, here we have identified critical regions and residues of the protein. Given the importance of the activities of this protein to the ability of *S. aureus* to cause disease, we propose that it warrants further in-depth characterisation, given its potential as a target for therapeutic intervention.

Appendix

A. 1. PCR products using the primers in Table 2.3

We were using a 1kb ladder, and if we got a faint PCR band, we repeated the PCR using the old PCR as a template with the same primers that we used to create the mutant; in the end, we obtained a thick amplicon. Some of the mutants were not in order because I repeated the steps if I did not get the correct mutation.

Check *Dpn1* integrity with pRMC2-*mspA*

Gradient PCR for L-31 variant (56-58-60-64-66)°C- we chose the amplicon at 58°C.
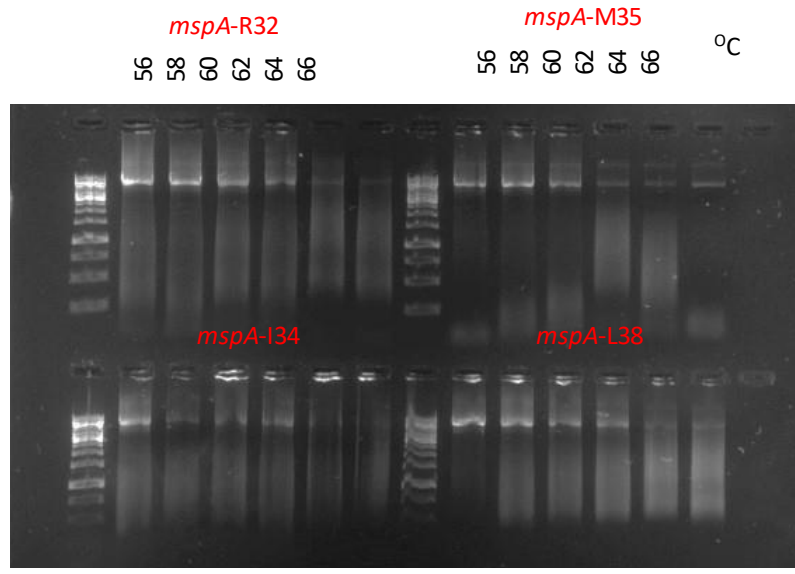
Gradient PCR for variants R-32/I34/M35/L38 (56-58-60-64-66)°C- we chose the amplicon at 56°C for all of them.
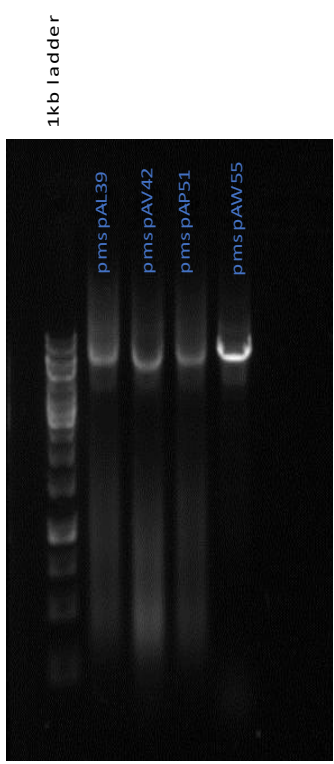
PCR for variants L39/V42/P51/W55- we used a 59°C annealing temperature for these variants.
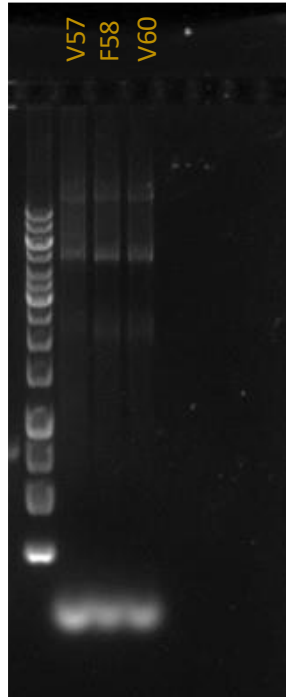
PCR for variants V57/F58/V60- we used a 59°C annealing temperature for these variants.
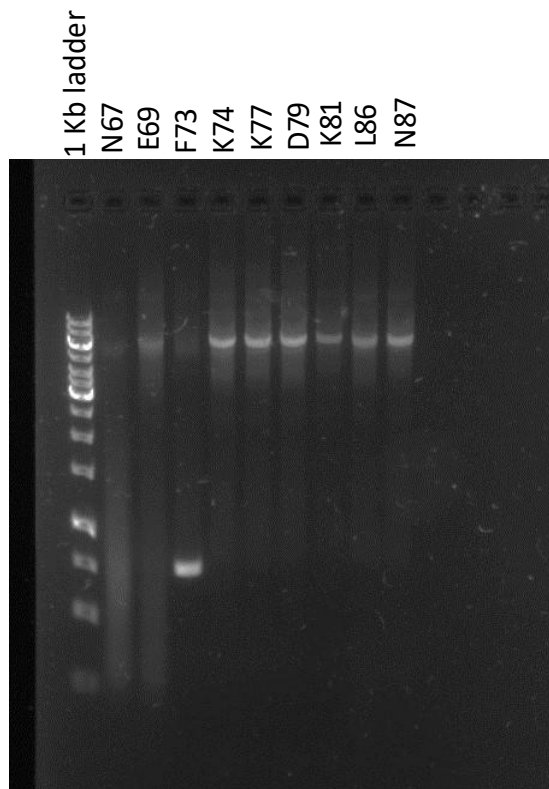
PCR for variants N67/E69/F73/K74/K77/D79/K81/L86 and N87- we used a 58°C annealing temperature for these variants. We repeated variant F73 as it was not amplifying the expected region.
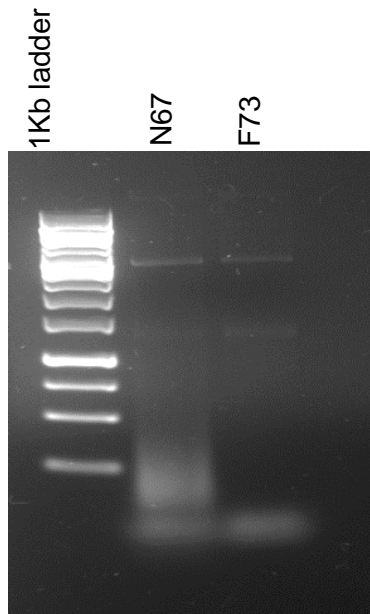
PCR for variants N67and repeated F73- we used a 60°C annealing temperature for these variants.
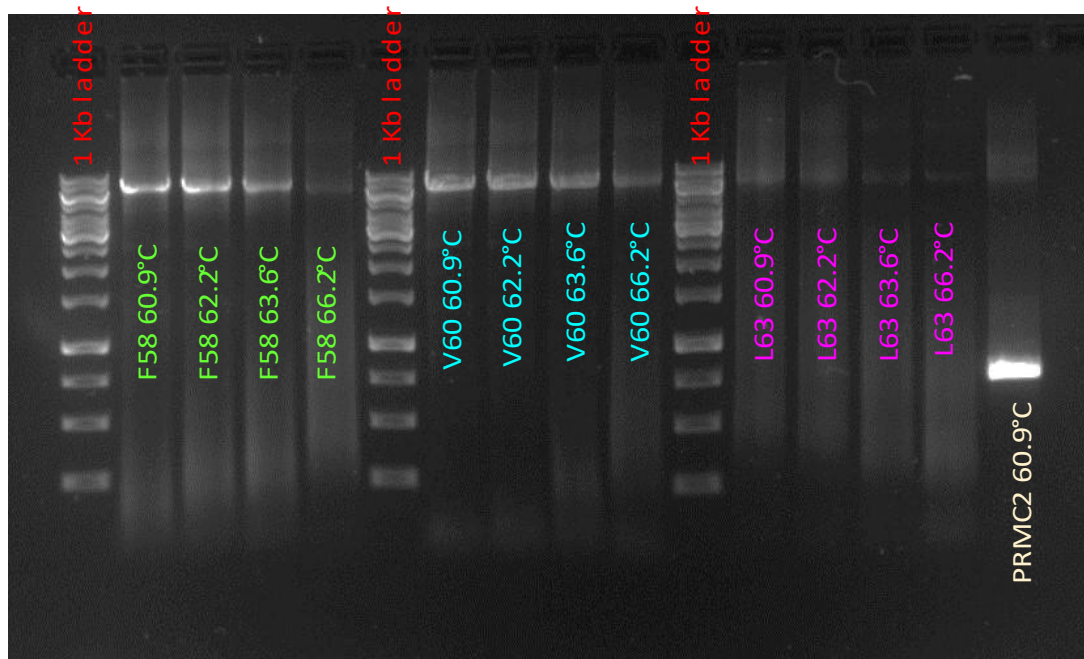
Gradient PCR for variants F58/V60 and L63 (60.9-62.2-63.6-66.2) °C. We added pRMC2 plasmid with pRMC2 primers as a positive control. We chose the amplicon at 60.9°C. for these variants and the PCR was repeated for variant L63 (using this amplicon as a template with the same primers that we used to create the mutation) to increase the amplification.

Gradient PCR temperature
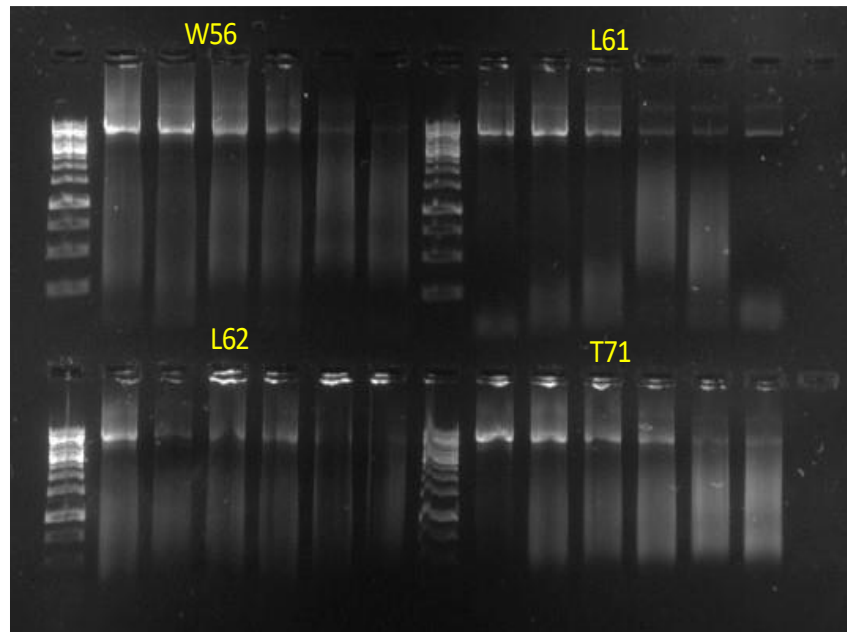
Gradient PCR for variants W56/L61/L62 and T71 (56-58-60-64-66)°C- the amplicon at 56°C was chosenfor all the variants.

A. 2. *E. coli* transformants

After we generated the PCR product and digested with *DpnI*, we transformed *E. coli* DH5-α or Mach1 using LB media supplemented with ampicillin (100 µg/ ml)

L31
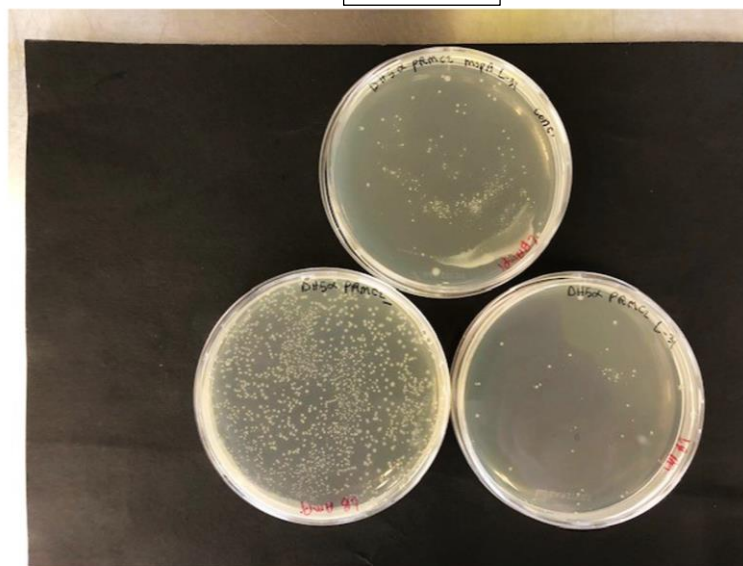
Transformation for L-31 variant.

R32-I34-M35-L38
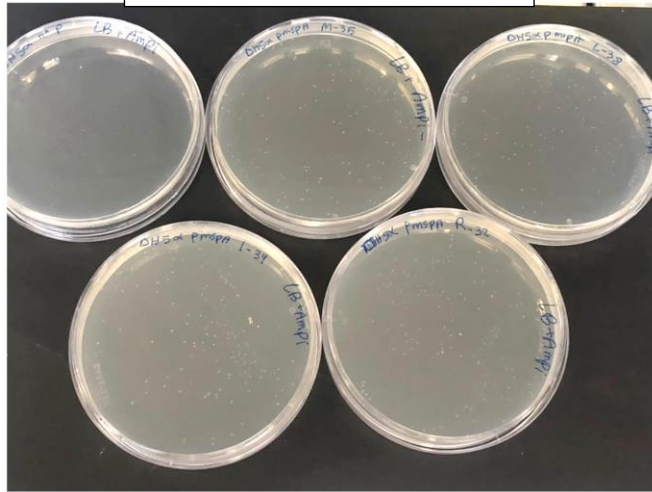
Transformation for R-32/I-34/M-35/L-38 variants. The negative control was transformed with water .

L39-V42-P51-W55-W56
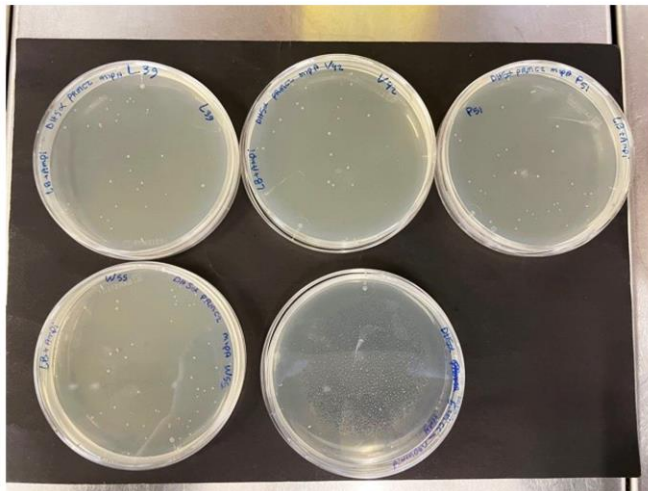
Transformation for L-39/V-42/P-51/W-55/W56 variants. The negative control was transformed with water.

V57-F58-V60-L61-L62-L63-V68-E69-T71
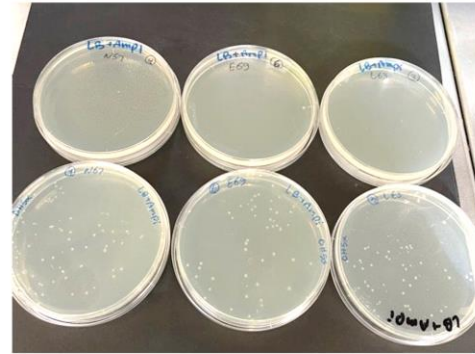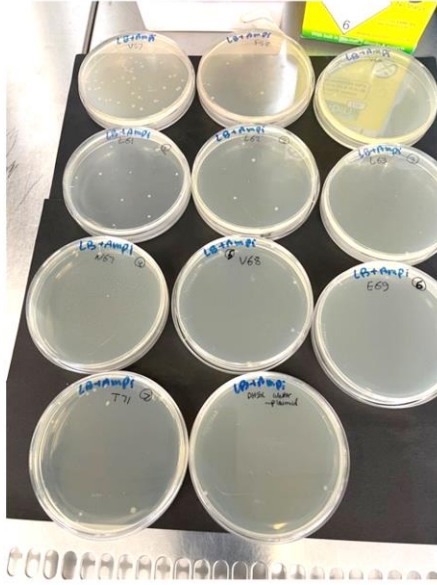
Transformation for V57-F58-V60-L61-L62-L63-V68-E69-T71 variants. The negative control was transformed with water .

F73-K74-K77-D79-K81-L86-N87
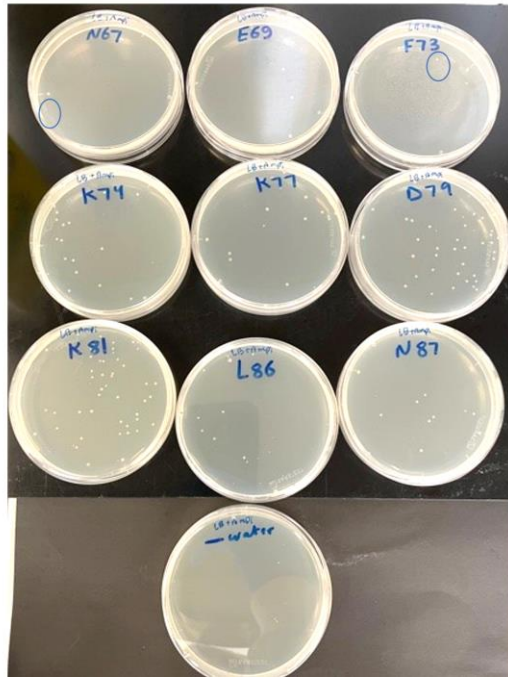
Transformed to DH5 α
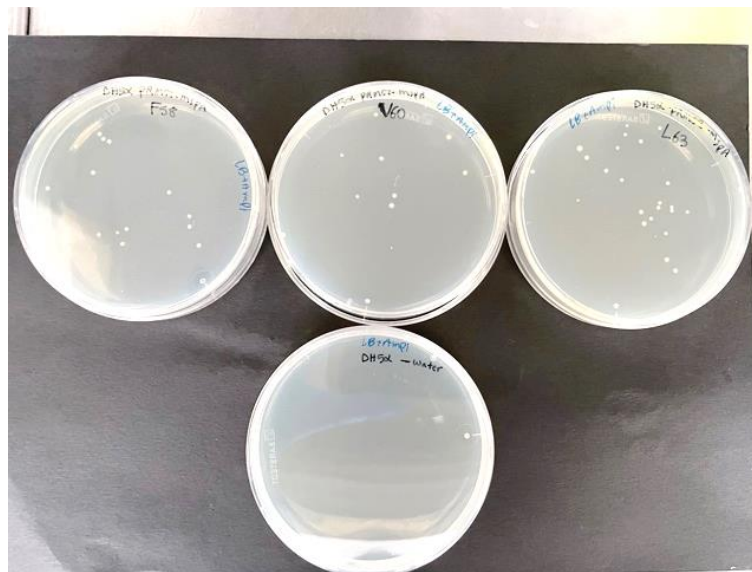
Transformation for F73-K74-K77-D79-K81-L86-N87 variants. The negative control was transformed with water .

A.3. Checking the right insertion by PCR before sending for sequencing

After transformation in to *E. coli,* the plasmid was checked by PCR before being extracted and sent for sequencing.
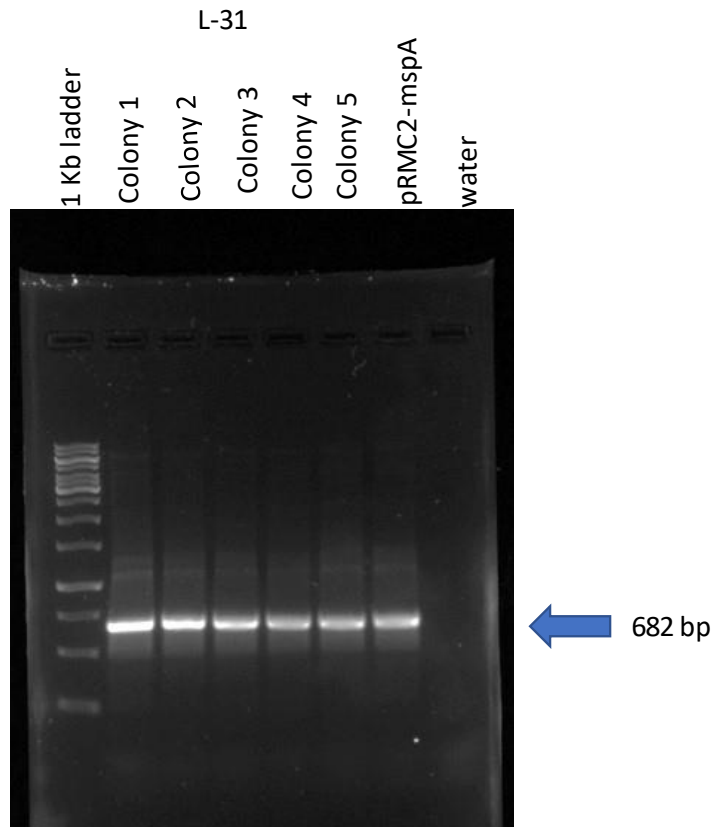
PCR for variant L-31, using pRMC2 primers at a 60°C annealing temperature. pRMC2 plasmid was a positive control and water was a negative control. The amplicon expected size was 682 bp.
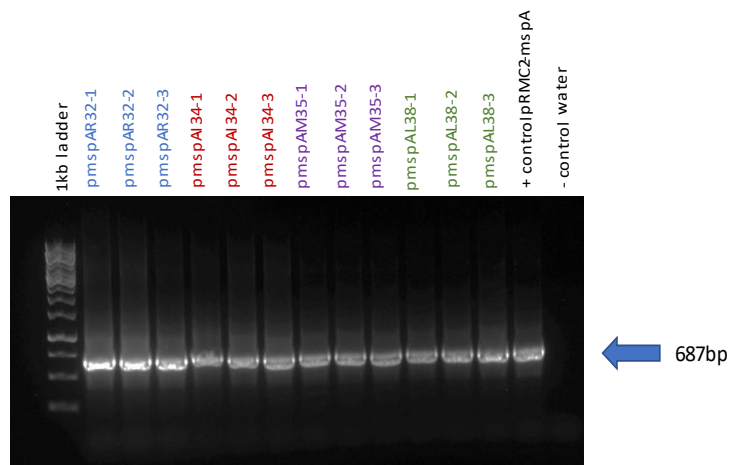
PCR for variants R-32/I-34/ M-35/ L-38 using pRMC2 primers at a 60°C annealing temperature. pRMC2 plasmid was a positive control and water was a negative control. The amplicon expected size was 682 bp.
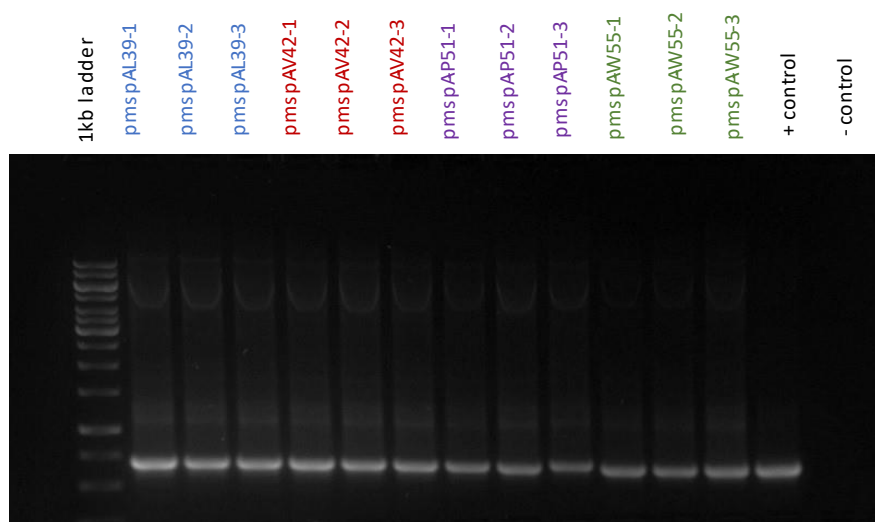
PCR for variants L-39/V-42/ P-51/ W-55 using pRMC2 primers at a 60°C annealing temperature. pRMC2 plasmid was a positive control and water was a negative control. The amplicon expected size was 682 bp.
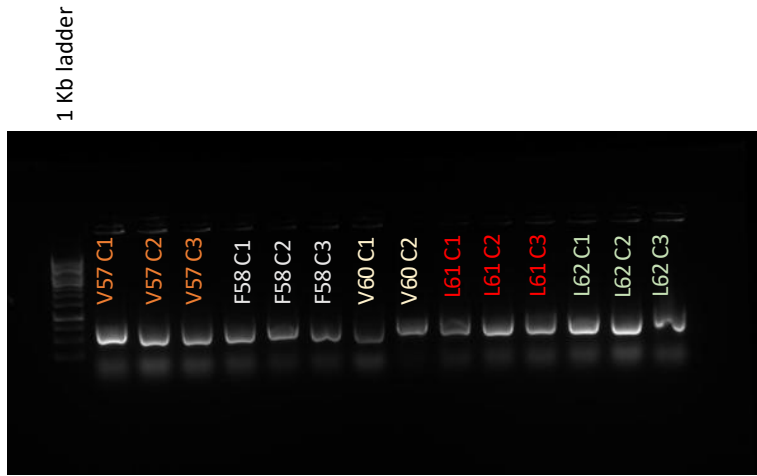
PCR for variants V-57/F-58/ V-60/ L-61/L-62 using pRMC2 primers at a 60°C annealing temperature. pRMC2 plasmid was a positive control and water was a negative control. The amplicon expected size was 682 bp.
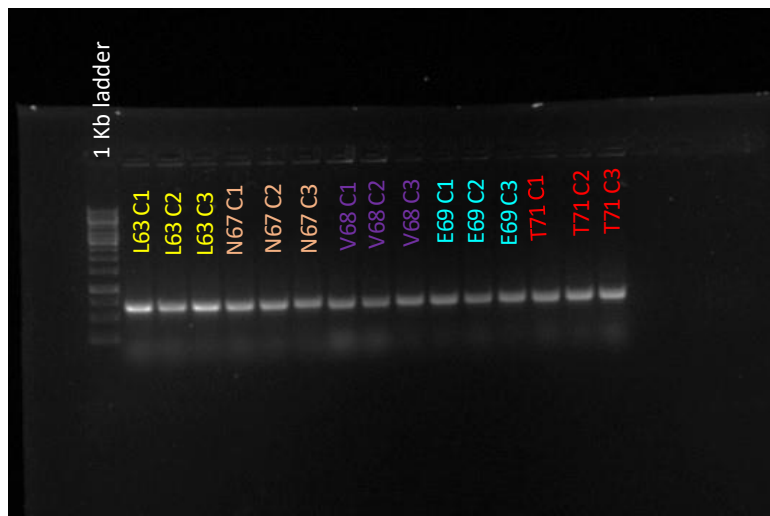
PCR for variants L-63/N-67/V-68/E-69/T-71 using pRMC2 primers at a 60°C annealing temperature. pRMC2 plasmid was a positive control and water was a negative control. The amplicon expected size was 682 bp.
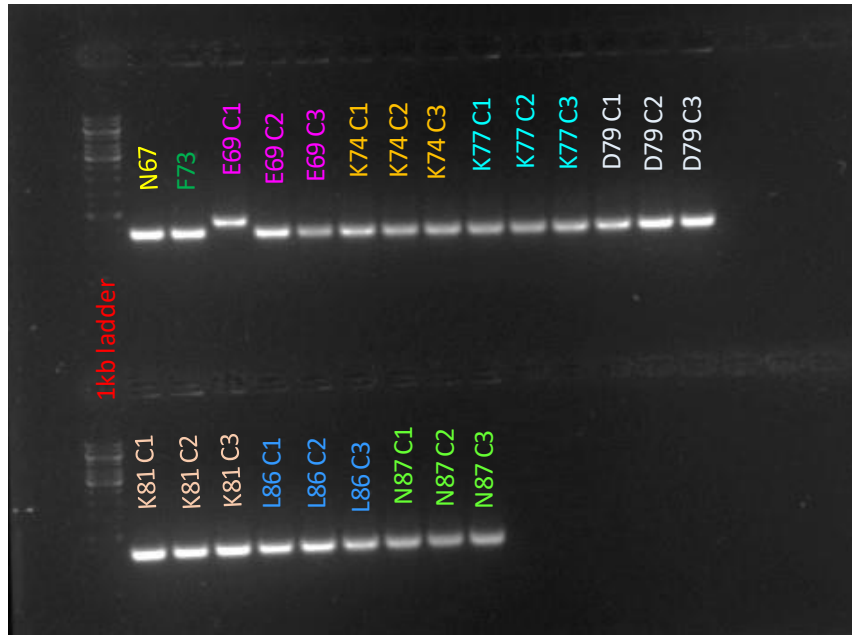
PCR for variants N67/F73/E69/K74/K77/D79/K81/L86 and N87 using pRMC2 primers at a 60°C annealing temperature. pRMC2 plasmid was a positive control and water was a negative control. The amplicon expected size was 682 bp.

A.4. Sequencing results for 26 variants

We have already shown sequencing for that variant R32. Here we show all the other.

sequencing results had been done byEurofins.

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mspA L31 C5

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>mspA L38-3
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>mspA-L39 C1

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>mspA-V42 C1

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>mspA-P51 C3

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213

>mspA-W55 C1

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>mspA-W56 C4

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mspA-F58 C3

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mspA-V60 C1

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mspA-L61 C3

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mspA-L62 C3
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mspA-L63 C3

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A

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A.5. RN4220 transformants

The plasmid with the correct mutation was electroporated in to RN4220 and potential transformants were spread onto a TSA plate supplemented with chloramphenicol .

RN4220 *mspA* L-31

Transformation for variant L-31. The negative control was transformed with water.

R32-I34-M35-L38
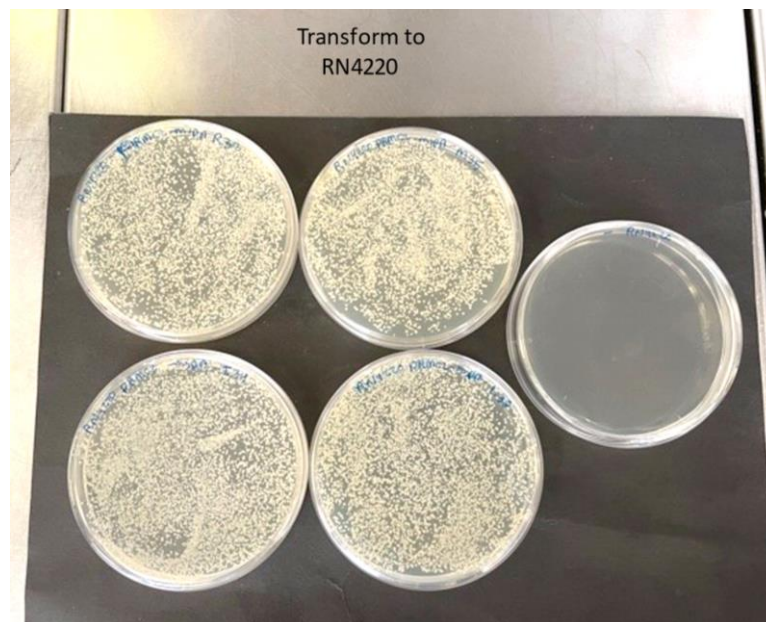
Transformation for variants R-32/I-34/M-35/L-38.The negative control was transformed the with water.
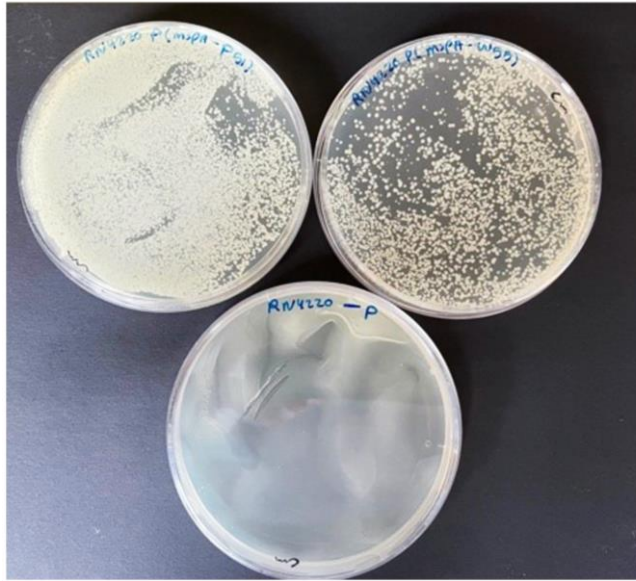
P51-W55

Transformation for variants P-51/W-55. The negative control was transformed with water.

L39-V42-W56
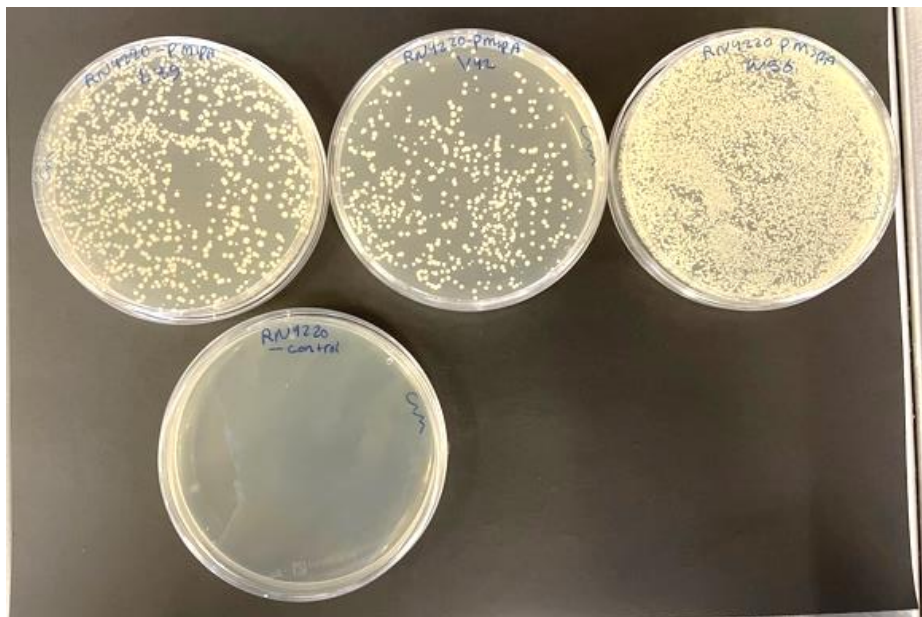
Transformation for variants L39-V42-W56. The negative control was transformed with water.
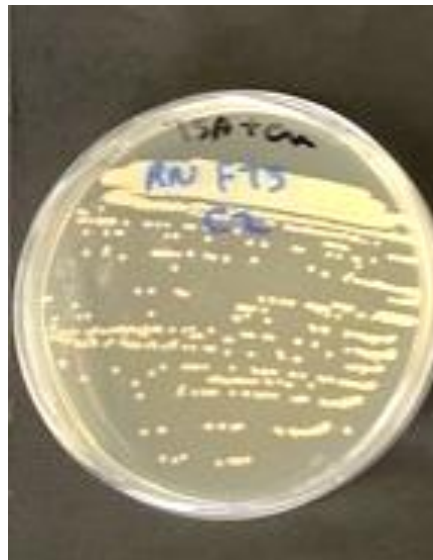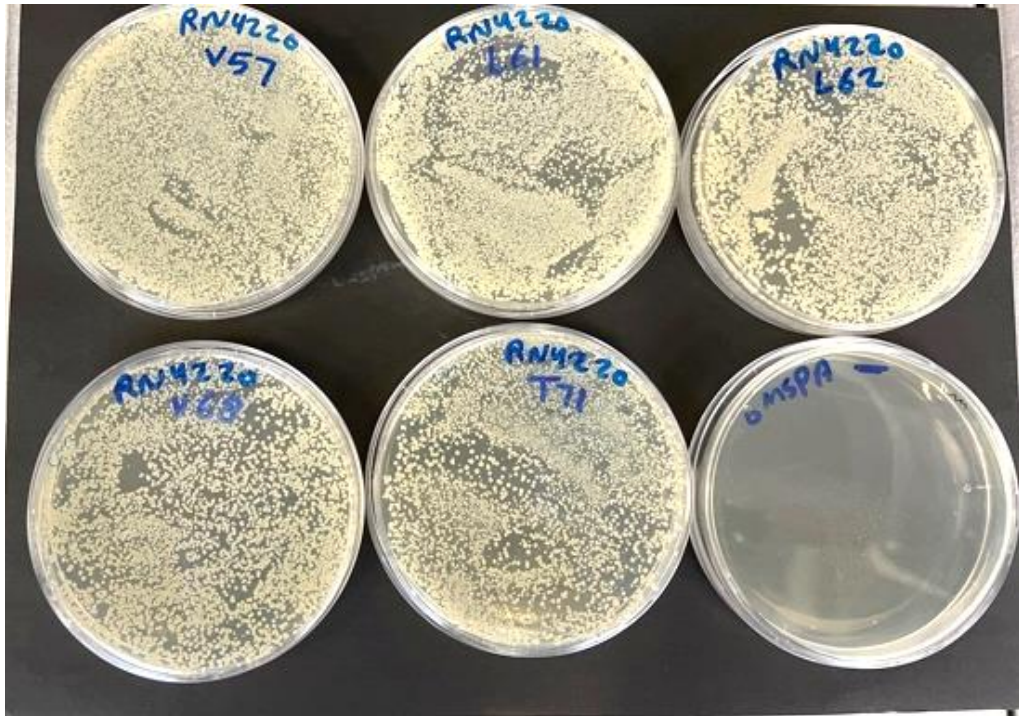
Transformation for variants V57/L61/L62/V68/T7/F73. The negative control was transformed water.
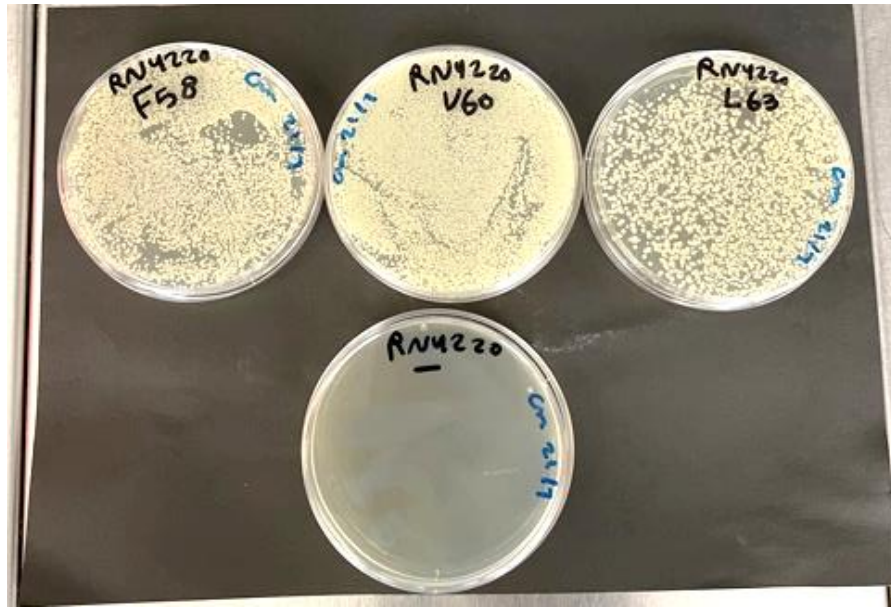
Transformation for variants F58/V60/L63. The negative control was transformed with water.
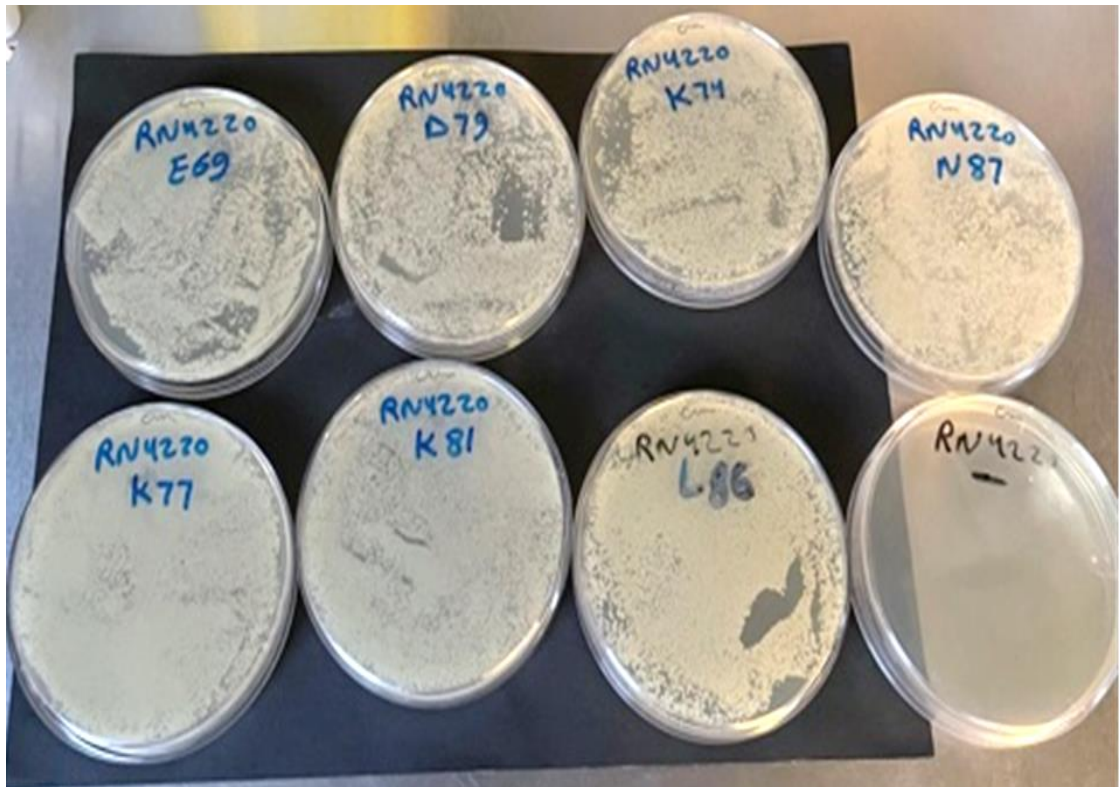
Transformation for variants E69/D79/K74/N87/K77/K81/L86. The negative control was transformed with water.

A.6 JE2*mspA::tn* transformants and colony PCR check

The plasmid was extracted from RN4220 strain using lysostaphin electroporated in to JE2*mspA::tn,* on to TSA supplemented with chloramphenicol (10 µg/ml) and erythromycin (5 µg/ml).
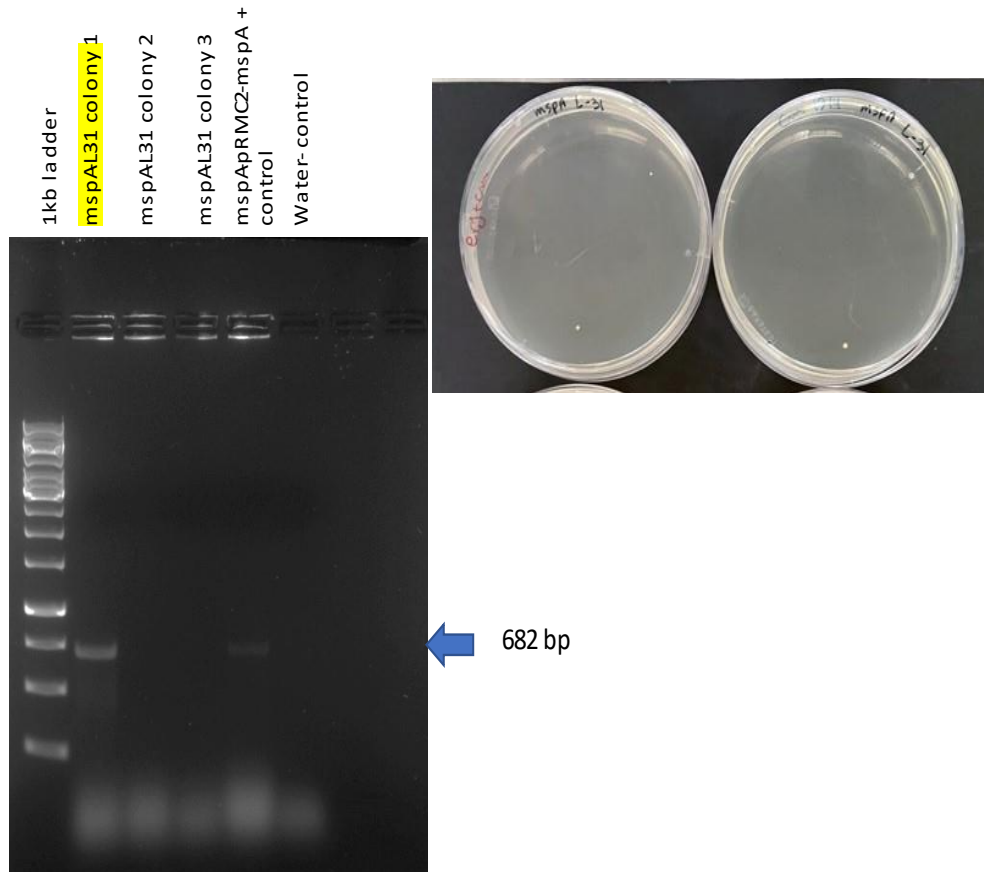
Transformation for variant L-31 . The negative control was transformed with water. The colony PCR was applied to check the correct insertion.
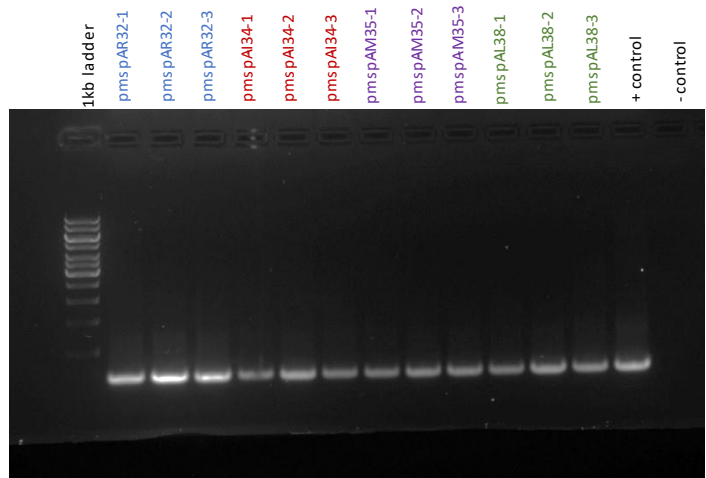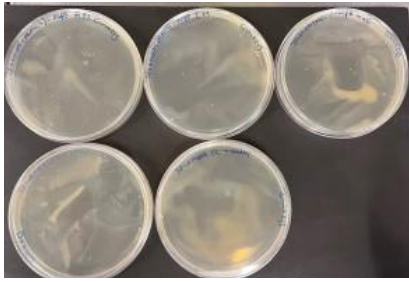
Transformation for variants R-32/I-34/M-35/L38. The negative control was transformed with water. The colony PCR was applied to check the correct insertion.
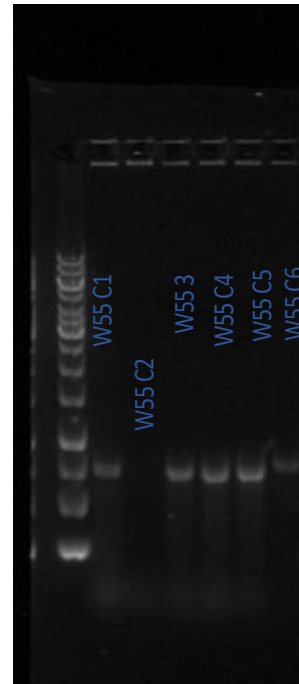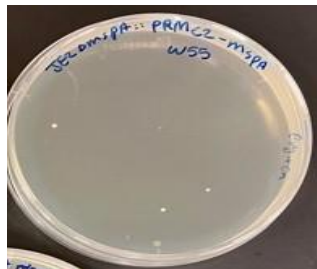
Transformation for variant W55. The negative control was transformed with water. The colony PCR was applied to check the correct insertion.
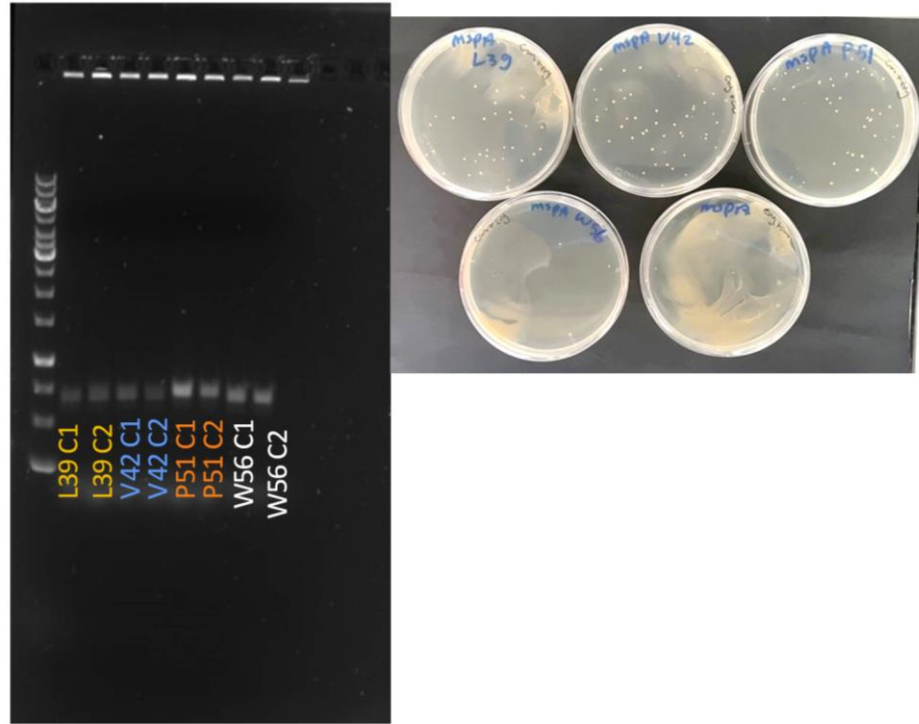
Transformation for variants L-39/V-42/P-51/W-56 . The negative control was transformed with water. The colony PCR was applied to check the correct insertion.
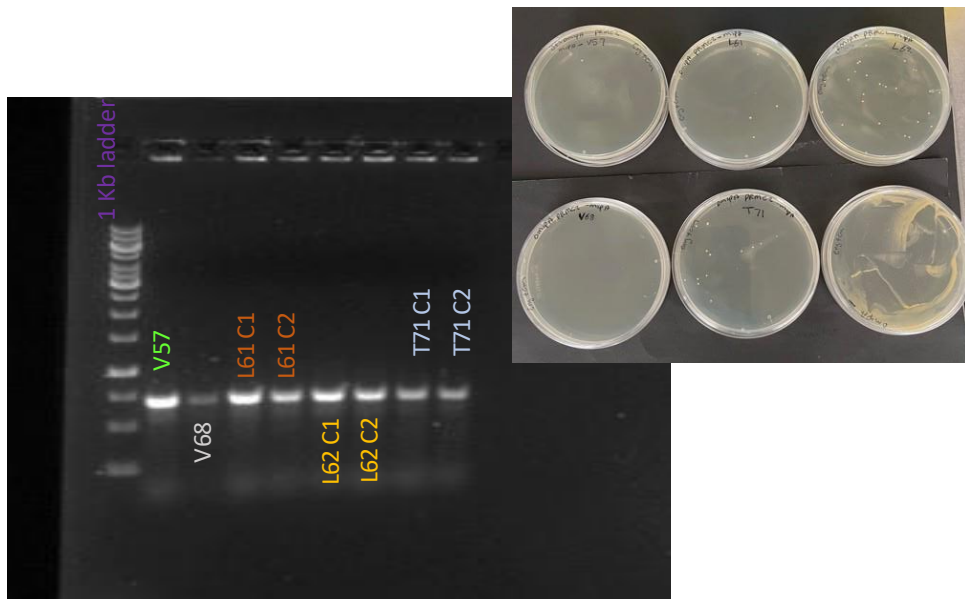
Transformation for variants V-57/V-68/L-61/T-71. The negative control was transformed with water. The colony PCR was applied to check the correct insertion.
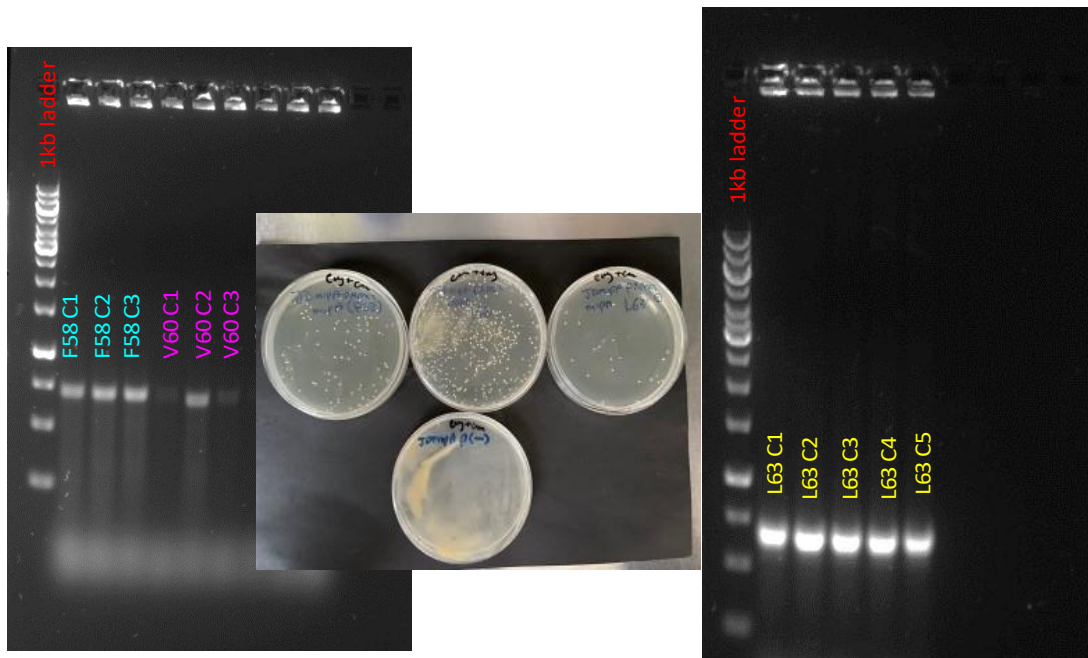
Transformation for variants F-58/V-60. The negative control was transformed with water. The colony PCR was applied to check the correct insertion.
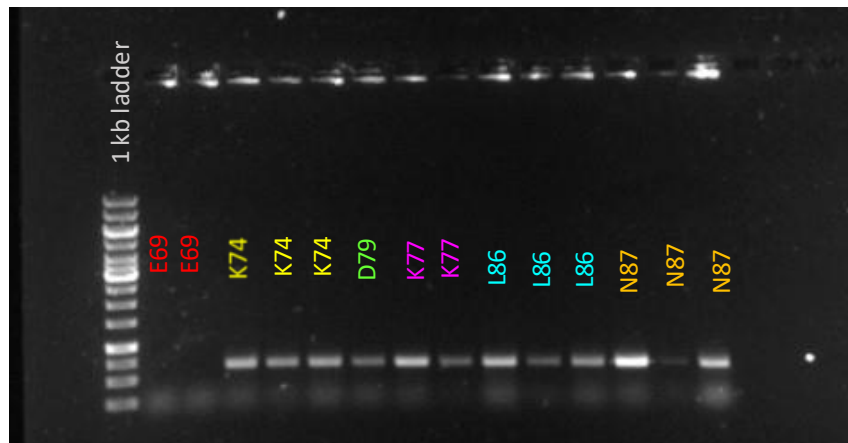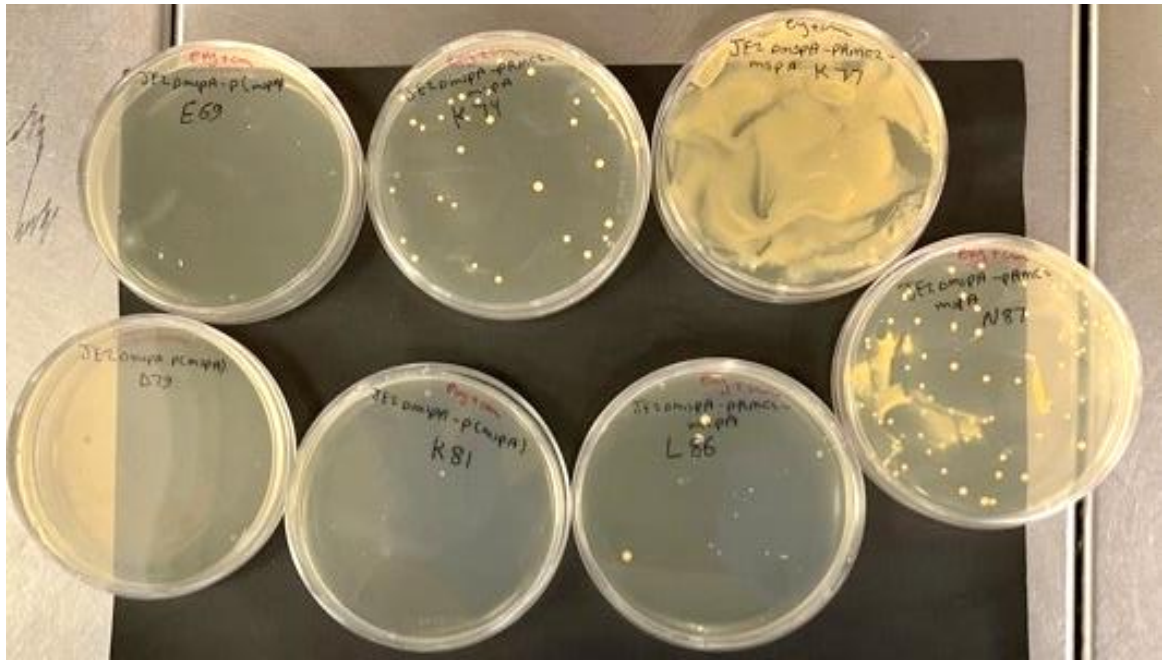
Transformation for variants E-69/K-74/D-79/K-77/L-86/N-87. The negative control was transformed with water. The colony PCR was applied to check the correct insertion.
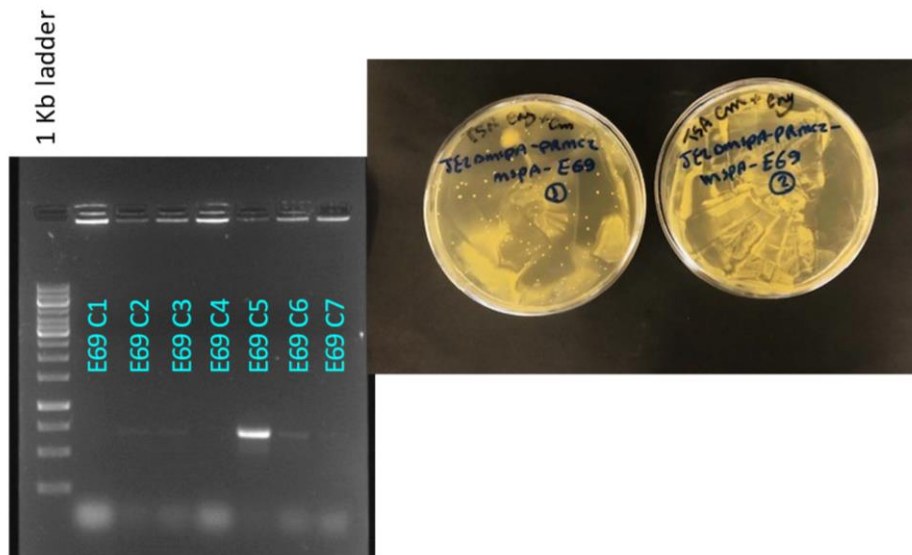
Transformation for variant E-69. The negative control was transformed with water. The colony PCR was applied to check the correct insertion.

Colony PCR to check K81
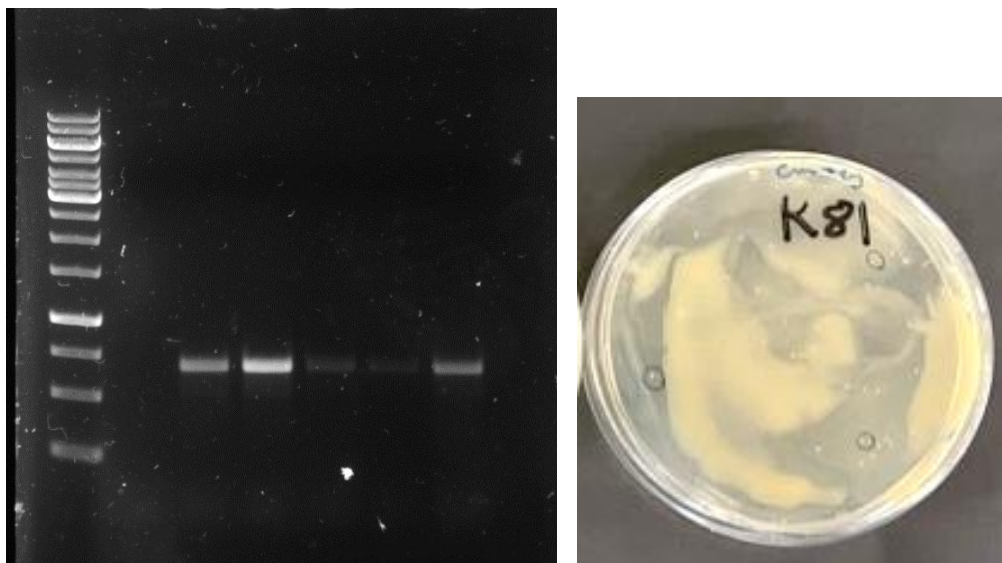
Transformation for variant K-81. The negative control was transformed with water. The colony PCR was applied to check the correct insertion.
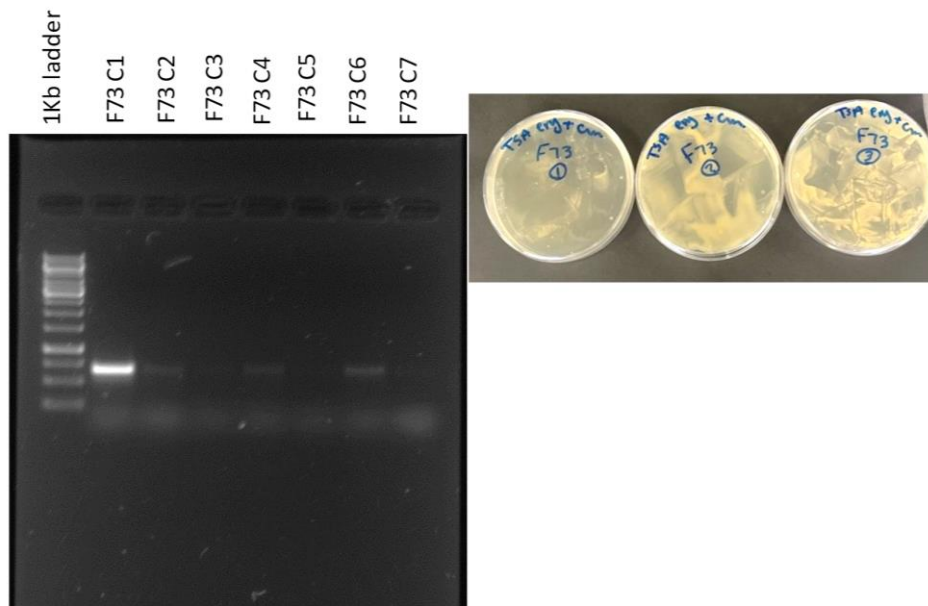
Transformation for variant F-73. The negative control was transformed with water. The colony PCR was applied to check the correct insertion.

A.7. Oxacillin disc diffusion tests

Below are some plates showing the disc diffusion method for the variant.

In this method, the variants were grown overnight with chloramphenicol (10 µg/ml) and erythromycin (5 µg/ml) and the next day this assay was performed by supplementing the Muller Hinton agar with tetracycline (200 µg/ml) and chloramphenicol (10 µg/ml) and then the oxacillin disc was applied.
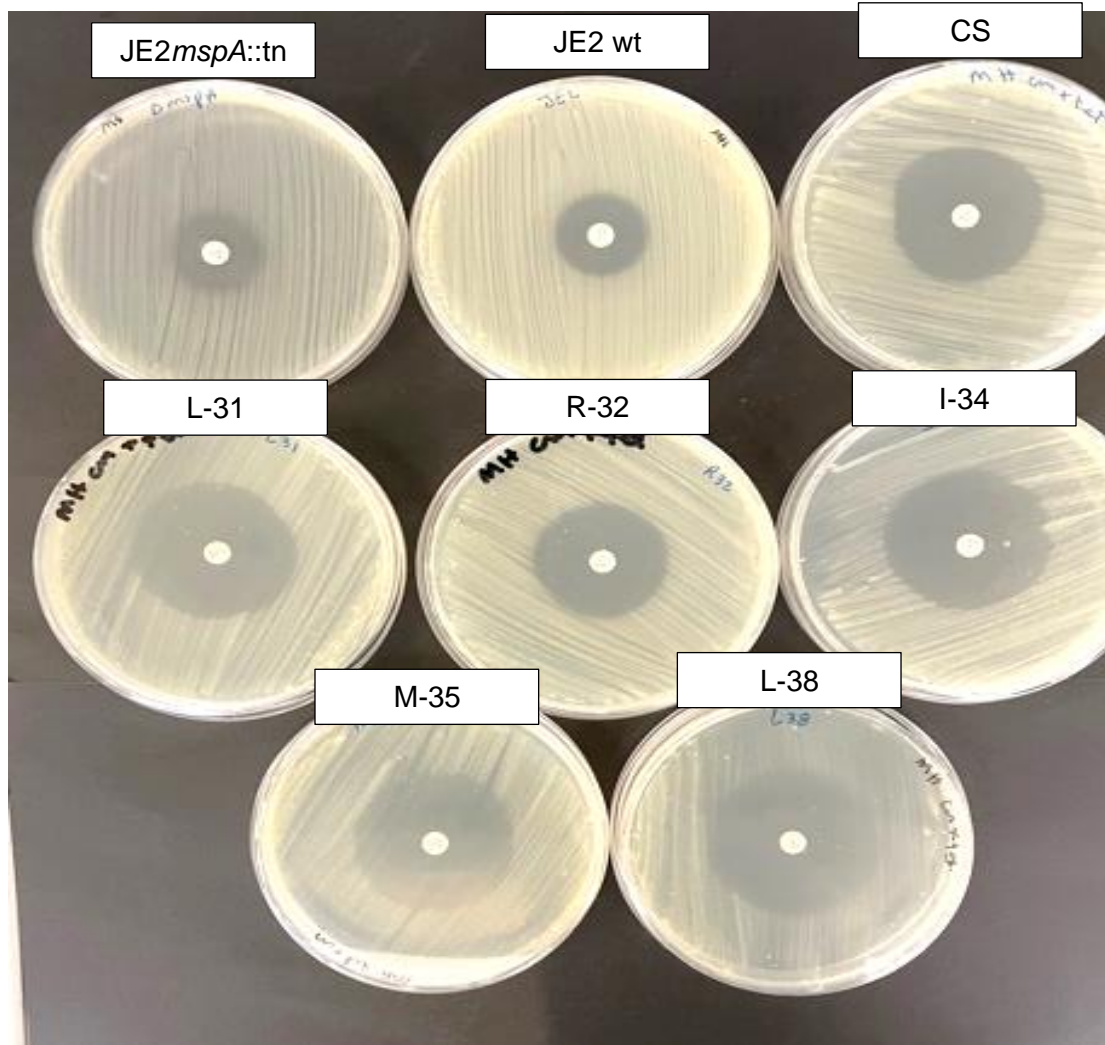
Oxacillin disc diffusion test for variants L-31/R-32/I-34/M-35/L-38 .
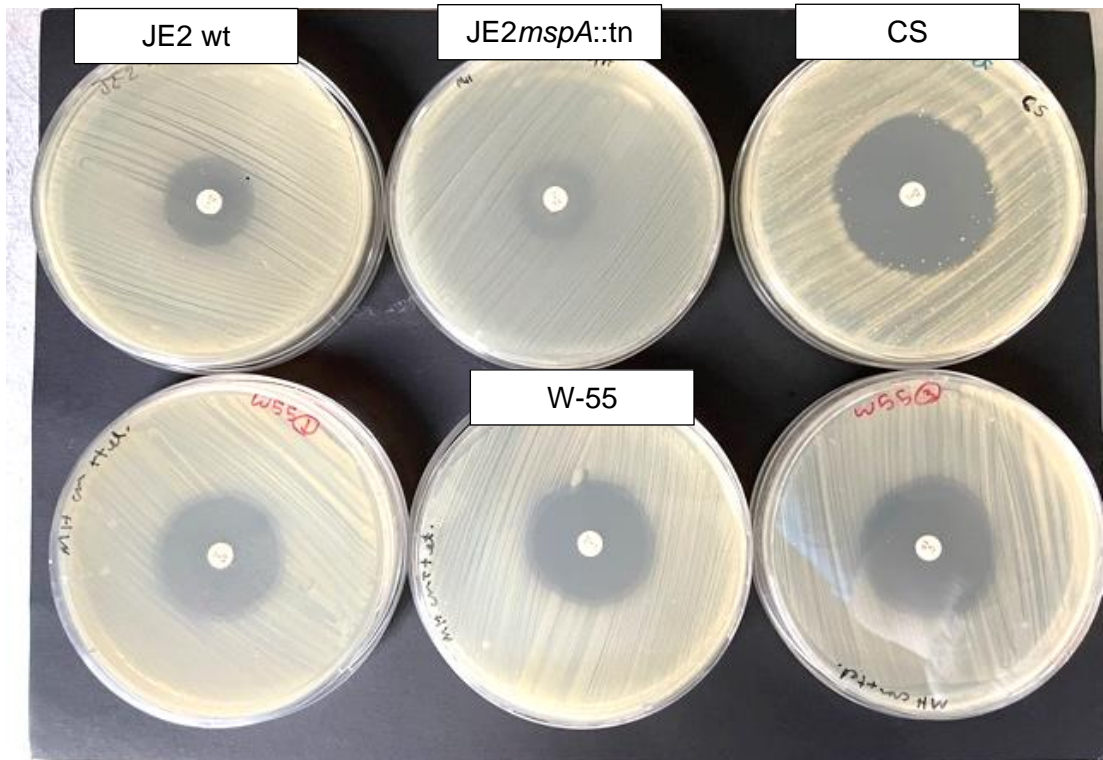
Oxacillin disc diffusion test for variant W-55 .
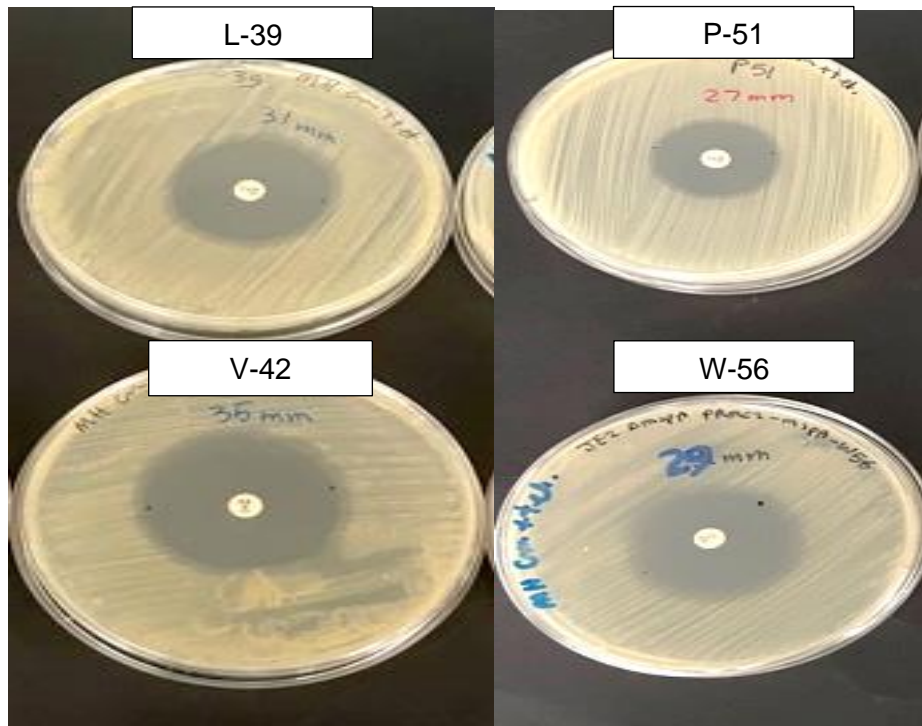
Oxacillin disc diffusion test for variants L-39/P-51/V-42/W-56.
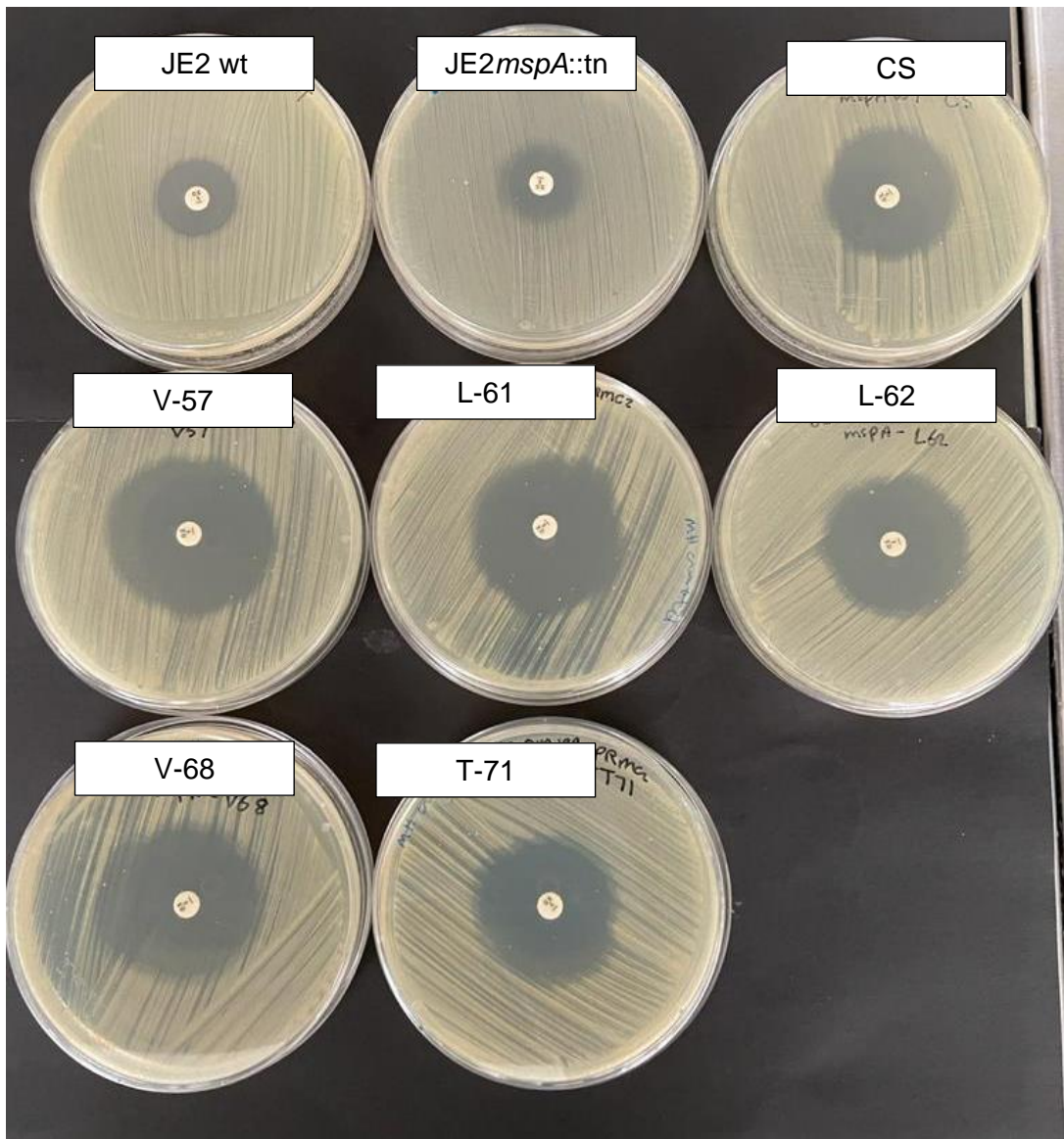
Oxacillin disc diffusion test for variants V-57/L-61/L-62/V-68/T-71.
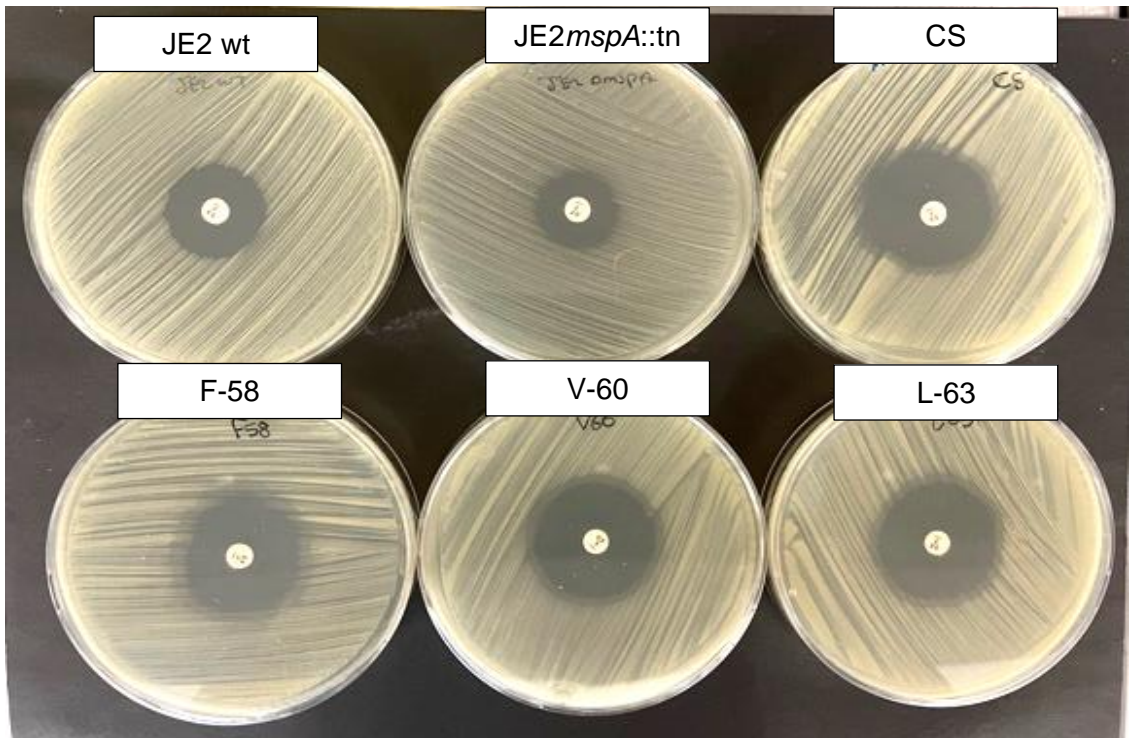
Oxacillin disc diffusion test for variants F-58/V-60/L-63 .
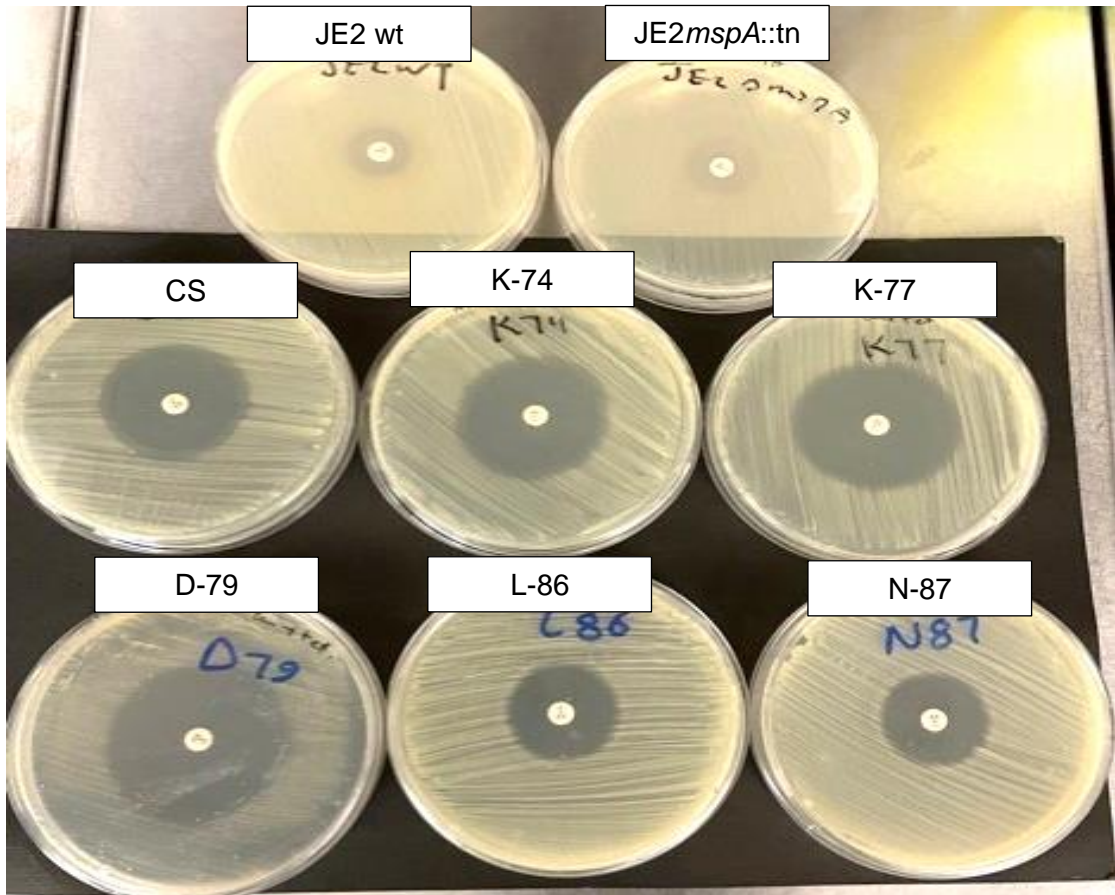
Oxacillin disc diffusion test for variants K-74/K-77/D-79/L-86/N-87 .
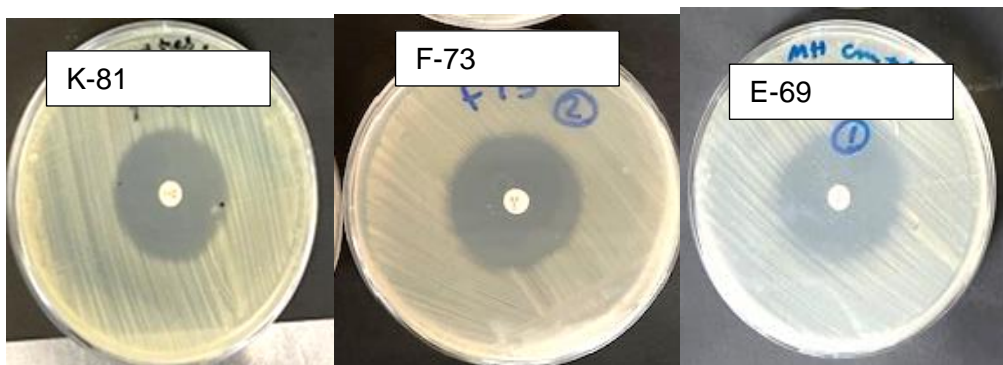
Oxacillin disc diffusion test for variants K81/F73/E69 .
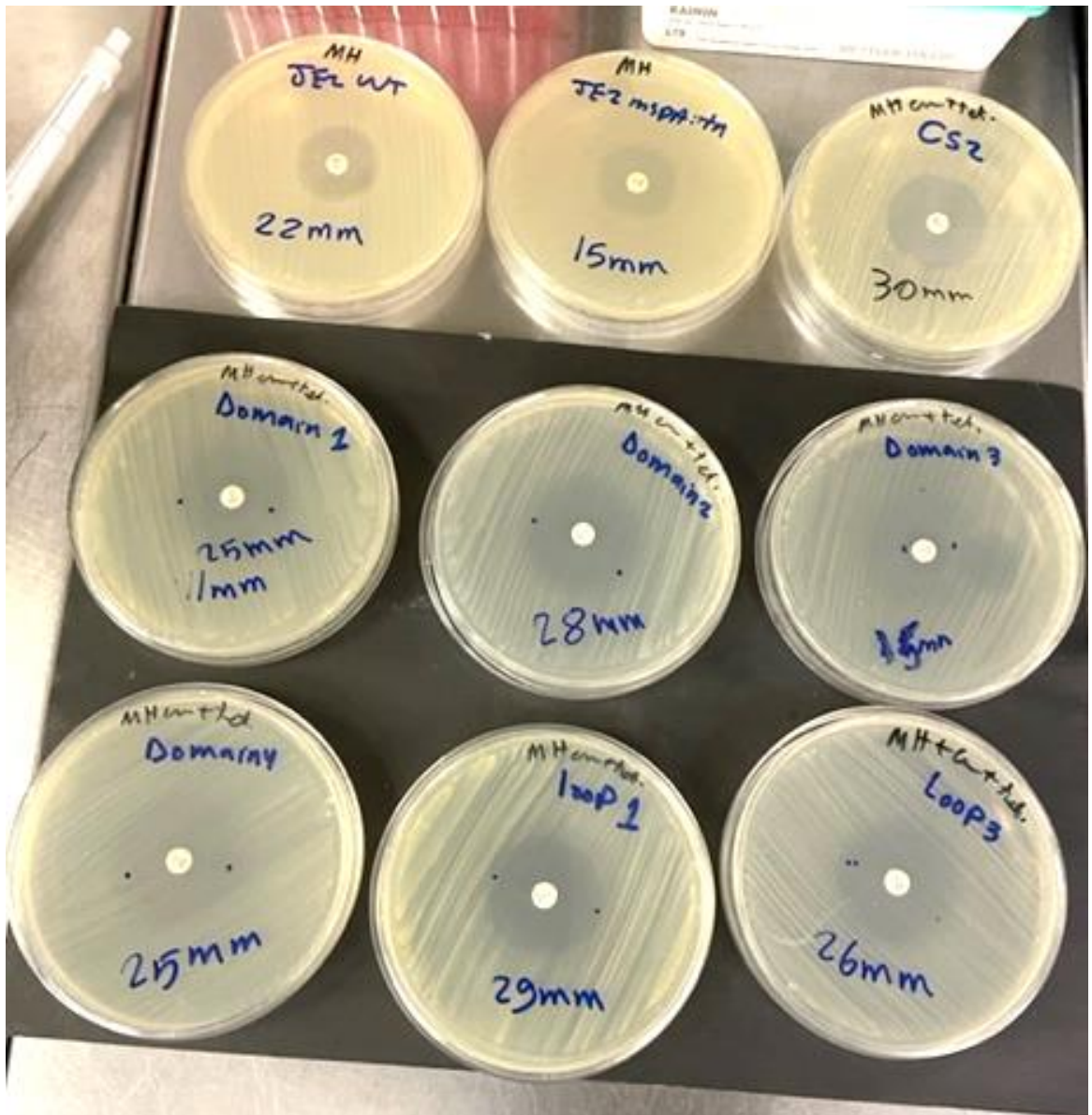
Oxacillin disc diffusion test for the domain removal and loop substitution variants .

Statistical Data (ANOVA)

Raw Data for Figure 3.6

JE2 WT	JE2 *mspA*::tn	JE2 *mspA*::tn p*mspA* 0tet.	JE2 *mspA*::tn p*mspA* 100ng/ml tet.	JE2 *mspA::tn* p*mspA* 200ng/ml tet.	JE2 *mspA*::tn p*mspA* 250ng/ml tet.	Broth
27.10	4.41	4.92	11.30	15.48	39.53	0.81
36.39	4.48	3.73	14.93	19.57	31.94	0.69
57.29	2.94	5.08	23.38	23.40	22.95	0.00
60.83	6.45	10.87	18.67	31.94	32.48	
41.79	7.41	6.78	22.52	12.00	16.28	
35.53	4.27	8.40	24.71	19.38	22.64	

Statistical Data (ANOVA)

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
JE2 WT vs. JE2*mspA*::tn	38.16	25.08 to 51.24	Yes	****	<0.0001
JE2 WT vs. JE2 *mspA*::tn p*mspA* 0tet.	36.53	23.45 to 49.61	Yes	****	<0.0001
JE2 WT vs. Column D	23.91	10.83 to 36.99	Yes	****	<0.0001
JE2 WT vs. Column E	22.86	9.786 to 35.94	Yes	****	<0.0001
JE2 WT vs. Column F	15.52	2.440 to 28.60	Yes	*	0.0118
JE2 WT vs. Broth	42.66	26.64 to 58.68	Yes	****	<0.0001
JE2*mspA*::tn vs. JE2 *mspA*::tn p*mspA* 0tet.	-1.637	-14.72 to 11.44	No	ns	0.9997
JE2*mspA*::tn vs. Column D	-14.25	-27.33 to -1.176	Yes	*	0.0255
JE2*mspA*::tn vs. Column E	-15.3	-28.38 to -2.221	Yes	*	0.0136
JE2*mspA*::tn vs. Column F	-22.64	-35.72 to -9.566	Yes	***	0.0001
JE2*mspA*::tn vs. Broth	4.495	-11.52 to 20.51	No	ns	0.9727
JE2 *mspA*::tn p*mspA* 0tet. vs. Column D	-12.62	-25.70 to 0.4611	No	ns	0.0644
JE2 *mspA*::tn p*mspA* 0tet. vs. Column E	-13.66	-26.74 to -0.5838	Yes	*	0.036
JE2 *mspA*::tn p*mspA* 0tet. vs. Column F	-21.01	-34.09 to -7.929	Yes	***	0.0003
JE2 *mspA*::tn p*mspA* 0tet. vs. Broth	6.132	-9.886 to 22.15	No	ns	0.8877
Column D vs. Column E	-1.045	-14.12 to 12.03	No	ns	>0.9999
Column D vs. Column F	-8.39	-21.47 to 4.688	No	ns	0.4245
Column D vs. Broth	18.75	2.731 to 34.77	Yes	*	0.0135
Column E vs. Column F	-7.345	-20.42 to 5.733	No	ns	0.5796
Column E vs. Broth	19.79	3.776 to 35.81	Yes	**	0.0079
Column F vs. Broth	27.14	11.12 to 43.16	Yes	***	0.0001

Raw Data for Figure 3.7

SH1000 WT	SH1000 *mspA*::tn	SH1000 *mspA*::tn p*mspA* 0tet.	SH1000 *mspA*::tn p*mspA* 50ng/ml tet.	SH1000 *mspA*::tn p*mspA* 100ng/ml tet.	SH1000 *mspA*::tn p*mspA* 200ng/ml tet.	SH1000 *mspA*::tn p*mspA* 250ng/ml tet.
16.36	3.16	4.31	14.05	17.08	34.49	41.26
19.55	1.94	6.05	17.48	30.27	37.50	46.67
33.83	6.67	6.16	12.80	18.92	28.68	27.78
40.43	7.57	7.64	16.97	23.00	37.20	24.84
57.91	2.81	2.95	23.91	25.14	28.01	33.39
34.56	3.03	6.27	6.13	10.33	17.21	15.88

Statistical Data (ANOVA)

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
SH1000 WT vs. SH1000 *mspA*::tn	29.58	14.28 to 44.87	Yes	****	<0.0001
SH1000 WT vs. SH1000 *mspA*::tn p*mspA* 0tet.	28.21	12.91 to 43.51	Yes	****	<0.0001
SH1000 WT vs. Column D	18.55	3.252 to 33.85	Yes	**	0.0093
SH1000 WT vs. Column E	12.98	-2.315 to 28.28	No	ns	0.1412
SH1000 WT vs. Column F	3.259	-12.04 to 18.56	No	ns	0.9937
SH1000 WT vs. Column G	2.137	-13.16 to 17.43	No	ns	0.9994
SH1000 *mspA*::tn vs. SH1000 *mspA*::tn p*mspA* 0tet.	-1.365	-16.66 to 13.93	No	ns	>0.9999
SH1000 *mspA*::tn vs. Column D	-11.03	-26.32 to 4.271	No	ns	0.2947
SH1000 *mspA*::tn vs. Column E	-16.59	-31.89 to -1.296	Yes	*	0.0263
SH1000 *mspA*::tn vs. Column F	-26.32	-41.61 to -11.02	Yes	****	<0.0001
SH1000 *mspA*::tn vs. Column G	-27.44	-42.74 to -12.14	Yes	****	<0.0001
SH1000 *mspA*::tn p*mspA* 0tet. vs. Column D	-9.661	-24.96 to 5.636	No	ns	0.4481
SH1000 *mspA*::tn p*mspA* 0tet. vs. Column E	-15.23	-30.53 to 0.06861	No	ns	0.0517
SH1000 *mspA*::tn p*mspA* 0tet. vs. Column F	-24.95	-40.25 to -9.655	Yes	***	0.0002
SH1000 *mspA*::tn p*mspA* 0tet. vs. Column G	-26.07	-41.37 to -10.78	Yes	***	0.0001
Column D vs. Column E	-5.568	-20.87 to 9.730	No	ns	0.9118
Column D vs. Column F	-15.29	-30.59 to 0.006686	No	ns	0.0502
Column D vs. Column G	-16.41	-31.71 to -1.116	Yes	*	0.0288
Column E vs. Column F	-9.723	-25.02 to 5.574	No	ns	0.4405
Column E vs. Column G	-10.85	-26.14 to 4.452	No	ns	0.313
Column F vs. Column G	-1.122	-16.42 to 14.18	No	ns	>0.9999

Raw Data for Figure 3.11

JE2wt	JE2 *mspA::Tn*	JE2 *crtM::Tn*	JE2 *floA::Tn*
0.036	0.017	0.014	0.041
0.04	0.025	0.006	0.054
0.044	0.019	0.004	0.047
0.048	0.022	0.002	0.057
0.041	0.015	0.007	0.037
0.05	0.021	0.003	0.046

Statistical Data (ANOVA)

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
JE2wt vs. JE2 *mspA::Tn*	0.02333	0.01462 to 0.03205	Yes	****	<0.0001
JE2wt vs. JE2 *crtM::Tn*	0.03717	0.02845 to 0.04588	Yes	****	<0.0001
JE2wt vs. JE2 *floA::Tn*	-0.00383	-0.01255 to 0.004881	No	ns	0.6151
JE2 *mspA::Tn* vs. JE2 *crtM::Tn*	0.01383	0.005119 to 0.02255	Yes	**	0.0013
JE2 *mspA::Tn* vs. JE2 *floA::Tn*	-0.02717	-0.03588 to -0.01845	Yes	****	<0.0001
JE2 *crtM::Tn* vs. JE2 *floA::Tn*	-0.041	-0.04971 to -0.03229	Yes	****	<0.0001

Raw Data for Figure 3.12

JE2 *wt*	JE2 *mspA::Tn*	JE2 *crtM::Tn*	JE2 *floA::Tn*
57.00	8.82	52.05	29.55
53.85	8.08	58.82	43.55
51.22	0.93	50.00	52.54
48.98	4.17	55.93	46.23
52.70	9.30	66.67	51.35
72.28	2.80	70.15	50.00

Statistical Data (ANOVA)

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
JE2 *wt* vs. JE2 *mspA::Tn*	50.32	38.33 to 62.31	Yes	****	<0.0001
JE2 *wt* vs. JE2 *crtM::Tn*	-2.934	-14.92 to 9.052	No	ns	0.9016
JE2 *wt* vs. JE2 *floA::Tn*	10.47	-1.517 to 22.45	No	ns	0.1006
JE2 *mspA::Tn* vs. JE2 *crtM::Tn*	-53.25	-65.24 to -41.27	Yes	****	<0.0001
JE2 *mspA::Tn* vs. JE2 *floA::Tn*	-39.85	-51.84 to -27.87	Yes	****	<0.0001
JE2 *crtM::Tn* vs. JE2 *floA::Tn*	13.4	1.417 to 25.39	Yes	*	0.025

Raw Data for Figure 3.14

	JE2 wt	JE2 wt hemin on	JE2 *mspA*::tn	JE2 *mspA*::tn hemin on
Zero	1.728	1.947	1.7	1.66
	1.858	1.743	1.896	1.671
	1.598	1.966	1.887	1.747
	1.843	1.811	1.829	1.825
	1.759	1.688	1.657	1.544
	1.857	1.682	1.494	1.448
	1.783	1.844	1.694	1.684
	1.742	1.835	1.694	1.634
	1.769	1.753	1.716	1.726
	1.727	1.763	1.686	1.598
	1.775	1.748	1.648	1.608
	1.723	1.727	1.755	1.628
	1.843	1.828	1.841	1.735
	1.844	1.843	1.761	1.752
	1.816	1.81	1.756	1.742
	1.835	1.787	1.776	1.698
	1.889	1.802	1.818	1.67
	1.826	1.794	1.674	1.675
	1.78972	1.798388889	1.737888889	1.669166667
40	0.985	1.655	0.708	1.339
	1.139	1.519	1.288	1.228
	1.182	1.508	0.952	1.35
	0.76	1.47	0.761	0.998
	0.955	1.465	0.687	1.003
	0.803	1.525	0.736	1.032
	1.188	1.408	0.333	1.168
	0.64	1.347	0.376	1.167
	0.946	1.463	0.181	0.981
	1.119	1.299	0.331	1.042
	1.139	1.348	0.334	0.973
	1.045	1.323	0.334	0.919
	0.458	1.265	0.4	2.24
	0.519	1.42	0.382	0.865
	0.441	1.315	0.383	0.754
	0.525	1.365	0.379	0.793
	0.431	1.233	0.386	0.734

Statistical Data (ANOVA) Figure 3.14

Statistical Data (Zero)

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
JE2 wt vs. JE2 wt hemin on	-0.00867	-0.08170 to 0.06437	No	ns	1
JE2 wt vs. JE2 *mspA*::tn	0.05183	-0.02120 to 0.1249	No	ns	0.3
JE2 wt vs. JE2 *mspA*::tn hemin on	0.1206	0.04752 to 0.1936	Yes	***	0
JE2 wt hemin on vs. JE2 *mspA*::tn	0.0605	-0.01254 to 0.1335	No	ns	0.1
JE2 wt hemin on vs. JE2 *mspA*::tn hemin on	0.1292	0.05618 to 0.2023	Yes	****	<0.0001
JE2 *mspA*::tn vs. JE2 *mspA*::tn hemin on	0.06872	-0.004316 to 0.1418	No	ns	0.1

Statistical Data (40)

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
JE2 wt vs. JE2 wt hemin on	-0.5816	-0.8184 to -0.3449	Yes	****	<0.0001
JE2 wt vs. JE2 *mspA*::tn	0.2996	0.06281 to 0.5363	Yes	**	0
JE2 wt vs. JE2 *mspA*::tn hemin on	-0.2591	-0.4959 to -0.02237	Yes	*	0
JE2 wt hemin on vs. JE2 *mspA*::tn	0.8812	0.6444 to 1.118	Yes	****	<0.0001
JE2 wt hemin on vs. JE2 *mspA*::tn hemin on	0.3225	0.08576 to 0.5592	Yes	**	0
JE2 *mspA*::tn vs. JE2 *mspA*::tn hemin on	-0.5587	-0.7954 to -0.3219	Yes	****	<0.0001

Raw Data for Figure 3.15

	SH1000 WT	SH1000 WT hemin on	SH1000 *mspA*::tn	SH1000 *mspA*::tn hemin on
zero	0.748	0.9	0.629	0.516
	0.736	0.901	0.594	0.402
	0.728	0.926	0.623	0.457
	0.758	0.69	0.61	0.58
	0.758	0.605	0.55	0.571
	0.793	0.673	0.545	0.496
	0.754	0.677	0.627	0.697
	0.795	0.647	0.622	0.657
	0.783	0.713	0.636	0.599
	0.917	0.58	0.646	0.633
	0.893	0.623	0.707	0.679
	0.852	0.671	0.64	0.619
	0.77	0.672	0.655	0.697
	0.794	0.628	0.666	0.63
	0.801	0.713	0.622	0.655
	0.813	0.937	0.728	0.769
	0.783	0.912	0.729	0.716
	0.763	0.908	0.712	0.883
	0.79105556	0.743111111	0.641166667	0.625333333
40	0.151	0.923	0.155	0.254
	0.152	0.917	0.157	0.271
	0.152	0.976	0.152	0.139
	0.161	0.631	0.162	0.227
	0.169	0.862	0.153	0.177
	0.156	0.663	0.141	0.435
	0.177	0.654	0.183	0.186
	0.168	0.826	0.166	0.17
	0.177	0.74	0.163	0.168
	0.149	0.733	0.149	0.371
	0.16	0.825	0.149	0.29
	0.153	0.771	0.15	0.267
	0.238	0.632	0.152	0.301
	0.191	0.825	0.157	0.214
	0.199	0.732	0.151	0.239
	0.145	0.816	0.364	0.401
	0.146	0.94	0.652	0.462

Statistical Data (ANOVA) Figure 3.15

Statistical Data (Zero)

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
SH1000 wt vs. SH1000 wt hemin on	0.04794	-0.03439 to 0.1303	No	ns	0.4233
SH1000 wt vs. SH1000 *mspA*::tn	0.1499	0.06756 to 0.2322	Yes	****	<0.0001
SH1000 wt vs. SH1000 *mspA*::tn hemin on	0.1657	0.08339 to 0.2481	Yes	****	<0.0001
SH1000 wt hemin on vs. SH1000 *mspA*::tn	0.1019	0.01961 to 0.1843	Yes	**	0.0092
SH1000 wt hemin on vs. SH1000 *mspA*::tn hemin on	0.1178	0.03545 to 0.2001	Yes	**	0.0019
SH1000 *mspA*::tn vs. SH1000 *mspA*::tn hemin on	0.01583	-0.06650 to 0.09817	No	ns	0.9573

Statistical Data (40)

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
SH1000 wt vs. SH1000 wt hemin on	0.03477	0.71770 to 0.53452	yes	****	<0.0001
SH1000 wt vs. SH1000 *mspA*::tn	0.581	-0.04648 to 0.1367	No	ns	0.568
SH1000 wt vs. SH1000 *mspA*::tn hemin on	0.1118	0.02025 to 0.20342	Yes	**	0.0104
SH1000 wt hemin on vs. SH1000 *mspA*::tn	0.581	0.48941 to 067259	Yes	***	<0.0000
SH1000 wt hemin on vs. SH1000 *mspA*::tn hemin on	0.514278	0.42269 to 0.60587	Yes	**	0.0019
SH1000 *mspA*::tn vs. SH1000 *mspA*::tn hemin on	0.066722	- 0.02487 to 0.15831	No	ns	0.2299

Raw Data for Figure 3.16

JE2 *WT/0*	JE2 *WT/25*	JE2 *WT/50*	JE2 *WT/100*
76.92308	59.55882	49.47368	40.67797
81.08108	76.47059	38.09524	36.23188
98.61111	63.63636	65.95745	47.22222

Statistical Data (ANOVA) Figure 3.16

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
JE2 *WT/0* vs. JE2 *WT/25*	18.98	-8.353 to 46.32	No	ns	0.1964
JE2 *WT/0* vs. JE2 *WT/50*	34.36	7.027 to 61.70	Yes	*	0.0161
JE2 *WT/0* vs. JE2 *WT/100*	44.16	16.83 to 71.50	Yes	**	0.0038
JE2 *WT/25* vs. JE2 *WT/50*	15.38	-11.96 to 42.72	No	ns	0.339
JE2 *WT/25* vs. JE2 *WT/100*	25.18	-2.158 to 52.51	No	ns	0.0714
JE2 *WT/50* vs. JE2 *WT/100*	9.798	-17.54 to 37.13	No	ns	0.6728

Raw Data for Figure 3.17

SH1000 *WT/0*	SH1000 *WT/2.5*	SH1000 *WT/5*	SH1000 *WT/10*
82.47	63.93	33.33	22.22
67.31	57.38	12.50	11.54
100.00	76.92	34.09	15.79

Statistical Data (ANOVA)

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
SH1000 *WT/0* vs. SH1000 *WT/2.5*	17.18	-13.36 to 47.72	No	ns	0.339
SH1000 *WT/0* vs. SH1000 *WT/5*	56.62	26.08 to 87.16	Yes	**	0.0016
SH1000 *WT/0* vs. SH1000 *WT/10*	66.74	36.20 to 97.28	Yes	***	0.0005
SH1000 *WT/2.5* vs. SH1000 *WT/5*	39.44	8.897 to 69.98	Yes	*	0.0139
SH1000 *WT/2.5* vs. SH1000 *WT/10*	49.56	19.02 to 80.10	Yes	**	0.0036
SH1000 *WT/5* vs. SH1000 *WT/10*	10.12	-20.41 to 40.66	No	ns	0.7205

Raw Data for Figure 3.19

JE2 WT	JE2 *hrtB::Tn*	JE2 *hrtA::Tn*	JE2 *fur::Tn*
60.54	31.67	49.18	69.09
68.16	28.74	50.00	64.88
36.47	36.21	35.82	41.90
38.28	27.72	59.30	46.41
50.00	35.51	51.24	51.92
51.72	29.13	52.00	45.07

Statistical Data (ANOVA)

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
JE2 WT vs. JE2 *hrtB::Tn*	19.37	4.256 to 34.48	Yes	**	0.0092
JE2 WT vs. JE2 *hrtA::Tn*	1.274	-13.84 to 16.38	No	ns	0.9952
JE2 WT vs. JE2 *fur::Tn*	-2.349	-17.46 to 12.76	No	ns	0.9717
JE2 *hrtB::Tn* vs. JE2 *hrtA::Tn*	-18.09	-33.20 to -2.983	Yes	*	0.0155
JE2 *hrtB::Tn* vs. JE2 *fur::Tn*	-21.72	-36.83 to -6.606	Yes	**	0.0034
JE2 *hrtA::Tn* vs. JE2 *fur::Tn*	-3.623	-18.73 to 11.49	No	ns	0.9067

Raw Data for Figure 3.22

	JE2 wt	JE2 wt hemin on	JE2 *hrtA*::tn	JE2 *hrtA*::tn hemin on	JE2 *hrtB*::tn	JE2 *hrtB*::tn hemin on
Zero	1.728	1.947	2.012	1.864	1.889	1.795
	1.858	1.743	1.912	1.892	1.912	1.849
	1.598	1.966	1.834	1.851	1.955	1.81
	1.843	1.811	1.895	1.773	1.749	1.703
	1.759	1.688	1.817	1.786	1.845	1.852
	1.857	1.682	1.801	1.839	1.907	1.794
	1.773833	1.806166667	1.8785	1.834166667	1.876166667	1.8005
40	0.985	1.655	0.391	1.438	0.461	1.445
	1.139	1.519	0.39	1.447	0.432	1.454
	1.182	1.508	0.305	1.474	0.623	1.41
	0.76	1.47	0.304	1.395	0.635	1.399
	0.955	1.465	0.297	1.387	0.742	1.384

Statistical Data (ANOVA) Figure 3.22

Statistical Data (Zero)

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
JE2 wt vs. JE2 wt hemin on	-0.03233	-0.1802 to 0.1156	No	ns	0.9845
JE2 wt vs. JE2 *hrtA*::tn	-0.1047	-0.2526 to 0.04324	No	ns	0.2891
JE2 wt vs. JE2 *hrtA*::tn hemin on	-0.06033	-0.2082 to 0.08757	No	ns	0.8134
JE2 wt vs. JE2 *hrtB::tn*	-0.1023	-0.2502 to 0.04557	No	ns	0.3121
JE2 wt vs. JE2 *hrtB::tn* hemin on	-0.02667	-0.1746 to 0.1212	No	ns	0.9935
JE2 wt hemin on vs. JE2 *hrtA*::tn	-0.07233	-0.2202 to 0.07557	No	ns	0.6745
JE2 wt hemin on vs. JE2 *hrtA*::tn hemin on	-0.028	-0.1759 to 0.1199	No	ns	0.9919
JE2 wt hemin on vs. JE2 *hrtB::tn*	-0.07	-0.2179 to 0.07790	No	ns	0.7034
JE2 wt hemin on vs. JE2 *hrtB::tn* hemin on	0.005667	-0.1422 to 0.1536	No	ns	>0.9999
JE2 *hrtA*::tn vs. JE2 *hrtA*::tn hemin on	0.04433	-0.1036 to 0.1922	No	ns	0.9405
JE2 *hrtA*::tn vs. JE2 *hrtB::tn*	0.002333	-0.1456 to 0.1502	No	ns	>0.9999
JE2 *hrtA*::tn vs. JE2 *hrtB::tn* hemin on	0.078	-0.06990 to 0.2259	No	ns	0.6023
JE2 *hrtA*::tn hemin on vs. JE2 *hrtB::tn*	-0.042	-0.1899 to 0.1059	No	ns	0.9522
JE2 *hrtA*::tn hemin on vs. JE2 *hrtB::tn* hemin on	0.03367	-0.1142 to 0.1816	No	ns	0.9814
JE2 *hrtB::tn* vs. JE2 *hrtB::tn* hemin on	0.07567	-0.07224 to 0.2236	No	ns	0.6323

Statistical Data (40)

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
JE2 wt vs. JE2 wt hemin on	-0.553	-0.7197 to -0.3863	Yes	****	<0.0001
JE2 wt vs. JE2 *hrtA*::tn	0.6435	0.4768 to 0.8102	Yes	****	<0.0001
JE2 wt vs. JE2 *hrtA*::tn hemin on	-0.4438	-0.6105 to -0.2772	Yes	****	<0.0001
JE2 wt vs. JE2 *hrtB::tn*	0.4057	0.2390 to 0.5723	Yes	****	<0.0001
JE2 wt vs. JE2 *hrtB::tn* hemin on	-0.4433	-0.6100 to -0.2767	Yes	****	<0.0001
JE2 wt hemin on vs. JE2 *hrtA*::tn	1.197	1.030 to 1.363	Yes	****	<0.0001
JE2 wt hemin on vs. JE2 *hrtA*::tn hemin on	0.1092	-0.05750 to 0.2758	No	ns	0.3701
JE2 wt hemin on vs. JE2 *hrtB::tn*	0.9587	0.7920 to 1.125	Yes	****	<0.0001
JE2 wt hemin on vs. JE2 *hrtB::tn* hemin on	0.1097	-0.05700 to 0.2763	No	ns	0.3652
JE2 *hrtA*::tn vs. JE2 *hrtA*::tn hemin on	-1.087	-1.254 to -0.9207	Yes	****	<0.0001
JE2 *hrtA*::tn vs. JE2 *hrtB::tn*	-0.2378	-0.4045 to -0.07116	Yes	**	0.0019
JE2 *hrtA*::tn vs. JE2 *hrtB::tn* hemin on	-1.087	-1.254 to -0.9202	Yes	****	<0.0001
JE2 *hrtA*::tn hemin on vs. JE2 *hrtB::tn*	0.8495	0.6828 to 1.016	Yes	****	<0.0001
JE2 *hrtA*::tn hemin on vs. JE2 *hrtB::tn* hemin on	0.0005	-0.1662 to 0.1672	No	ns	>0.9999
JE2 *hrtB::tn* vs. JE2 *hrtB::tn* hemin on	-0.849	-1.016 to -0.6823	Yes	****	<0.0001

Raw Data for Figure 3.25

JE2wt	JE2 *mspA::Tn*	JE2 NE 42	JE2 NE 627	JE2 NE 866
86.78	20.00	64.74	60.53	62.73
83.21	19.88	53.85	66.67	62.20
87.00	22.55	55.83	70.29	80.61
87.70	28.06	64.62	62.50	84.57
75.00	12.90	60.43	81.63	47.98
75.38	11.36	65.16	69.34	60.43

Statistical Data (ANOVA)

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
JE2wt vs. JE2 *mspA::Tn*	63.39	49.38 to 77.39	Yes	****	<0.0001
JE2wt vs. JE2 NE 42	21.74	7.740 to 35.74	Yes	**	0.001
JE2wt vs. JE2 NE 627	14.02	0.01741 to 28.02	Yes	*	0.0496
JE2wt vs. JE2 NE 866	16.09	2.092 to 30.09	Yes	*	0.0186
JE2 *mspA::Tn* vs. JE2 NE 42	-41.64	-55.65 to -27.64	Yes	****	<0.0001
JE2 *mspA::Tn* vs. JE2 NE 627	-49.37	-63.37 to -35.37	Yes	****	<0.0001
JE2 *mspA::Tn* vs. JE2 NE 866	-47.29	-61.29 to -33.29	Yes	****	<0.0001
JE2 NE 42 vs. JE2 NE 627	-7.723	-21.72 to 6.279	No	ns	0.4991
JE2 NE 42 vs. JE2 NE 866	-5.648	-19.65 to 8.353	No	ns	0.7598
JE2 NE 627 vs. JE2 NE 866	2.075	-11.93 to 16.08	No	ns	0.9921

Raw Data for Figure 3.26

SH1000 wt.	SH1000 *mspA::Tn*	SH1000 NE42	SH1000 NE627	SH1000 NE866
61.07	0.00	61.69	64.42	64.74
62.24	1.41	70.25	70.71	62.45
71.26	3.77	78.98	80.31	75.56
79.49	3.49	77.19	85.27	85.19
76.22	2.92	79.05	94.08	79.81
76.19	1.50	85.59	85.22	86.08

Statistical Data (ANOVA)

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
SH1000 wt. vs. SH1000 *mspA::Tn*	68.9	54.70 to 83.09	Yes	****	<0.0001
SH1000 wt. vs. SH1000 NE42	-4.381	-18.58 to 9.816	No	ns	0.8918
SH1000 wt. vs. SH1000 NE627	-8.924	-23.12 to 5.273	No	ns	0.3711
SH1000 wt. vs. SH1000 NE866	-4.557	-18.75 to 9.640	No	ns	0.8774
SH1000 *mspA::Tn* vs. SH1000 NE42	-73.28	-87.47 to -59.08	Yes	****	<0.0001
SH1000 *mspA::Tn* vs. SH1000 NE627	-77.82	-92.02 to -63.62	Yes	****	<0.0001
SH1000 *mspA::Tn* vs. SH1000 NE866	-73.45	-87.65 to -59.26	Yes	****	<0.0001
SH1000 NE42 vs. SH1000 NE627	-4.543	-18.74 to 9.654	No	ns	0.8786
SH1000 NE42 vs. SH1000 NE866	-0.176	-14.37 to 14.02	No	ns	>0.9999
SH1000 NE627 vs. SH1000 NE866	4.367	-9.830 to 18.56	No	ns	0.8929

Raw Data for Figure 3.28

	JE2 WT					
0	0.98	0.94	0.96	0.96	0.95	0.97
0.0586	1.00	0.99	0.95	0.95	0.97	1.00
	JE2 *mspA::tn*					
0	0.93	0.81	0.88	0.88	0.93	0.92
0.0586	0.95	0.82	0.93	0.91	0.92	0.91
	JE2 *NE42::tn*					
0	0.95	0.85	0.93	0.98	0.96	0.95
0.0586	0.98	0.88	1.00	0.97	0.96	0.97
	JE2 *NE627::tn*					
0	0.96	0.87	0.93	0.90	0.95	0.92
0.0586	0.97	1.01	0.95	0.93	0.94	0.98
	JE2 *NE866::tn*					
0	0.98	0.85	0.93	0.90	0.93	0.94
0.0586	1.03	0.94	0.97	0.93	0.93	0.93

Statistical Data (ANOVA)

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
JE2 WT vs. JE2 *mspA::tn*	0.07	0.006123 to 0.1339	Yes	*	0.0267
JE2 WT vs. JE2*NE42::tn*	0.0235	-0.04038 to 0.08738	No	ns	0.8147
JE2 WT vs. JE2*NE627::tn*	0.03967	-0.02421 to 0.1035	No	ns	0.3829
JE2 WT vs. JE2 *NE866::tn*	0.03817	-0.02571 to 0.1020	No	ns	0.4207
JE2 *mspA::tn* vs. JE2*NE42::tn*	-0.0465	-0.1104 to 0.01738	No	ns	0.236
JE2 *mspA::tn* vs. JE2*NE627::tn*	-0.03033	-0.09421 to 0.03354	No	ns	0.6366
JE2 *mspA::tn* vs. JE2 *NE866::tn*	-0.03183	-0.09571 to 0.03204	No	ns	0.5943
JE2*NE42::tn* vs. JE2*NE627::tn*	0.01617	-0.04771 to 0.08004	No	ns	0.944
JE2*NE42::tn* vs. JE2 *NE866::tn*	0.01467	-0.04921 to 0.07854	No	ns	0.9602
JE2*NE627::tn* vs. JE2 *NE866::tn*	-0.0015	-0.06538 to 0.06238	No	ns	>0.9999

Raw Data for Figure 3.29

	SH1000 WT			
0	0.79	0.76	0.76	0.76
0.1172	0.70	0.74	0.72	0.68
	SH1000 *mspA::tn*			
0	0.65	0.65	0.66	0.63
0.1172	0.51	0.46	0.60	0.48
	SH1000 *NE42::tn*			
0	0.98	0.98	0.93	0.99
0.1172	0.90	0.87	0.85	0.87
	SH1000 *NE627::tn*			
0	0.97	0.94	0.92	0.95
0.1172	0.82	0.77	0.73	0.75
	SH1000 *NE866::tn*			
0	0.95	0.96	0.96	1.01
0.1172	0.79	0.82	0.60	0.76

Statistical Data (ANOVA)

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
SH1000 WT vs. SH1000 *mspA*::tn	0.1203	0.07548 to 0.1650	Yes	****	<0.0001
SH1000 WT vs. SH1000NE42::tn	-0.2013	-0.2460 to -0.1565	Yes	****	<0.0001
SH1000 WT vs. SH1000NE627::tn	-0.1768	-0.2215 to -0.1320	Yes	****	<0.0001
SH1000 WT vs. SH1000 NE866::tn	-0.2033	-0.2480 to -0.1585	Yes	****	<0.0001
SH1000 *mspA*::tn vs. SH1000NE42::tn	-0.3215	-0.3663 to -0.2767	Yes	****	<0.0001
SH1000 *mspA*::tn vs. SH1000NE627::tn	-0.297	-0.3418 to -0.2522	Yes	****	<0.0001
SH1000 *mspA*::tn vs. SH1000 NE866::tn	-0.3235	-0.3683 to -0.2787	Yes	****	<0.0001
SH1000NE42::tn vs. SH1000NE627::tn	0.0245	-0.02027 to 0.06927	No	ns	0.4683
SH1000NE42::tn vs. SH1000 NE866::tn	-0.002	-0.04677 to 0.04277	No	ns	>0.9999
SH1000NE627::tn vs. SH1000 NE866::tn	-0.0265	-0.07127 to 0.01827	No	ns	0.3945

Raw Data for Figure 3.30

	JE2wt	JE2wt hemin on	JE2 mspA::tn	JE2 mspA::tn hemin on	NE42	NE42 hemin on	NE627	NE627 hemin on	NE866	NE866 hemin on
Zero	1.013	1.026	0.903	0.927	0.995	1.003	1.029	1.008	0.974	1.028
	1.01	1.017	0.873	0.936	0.993	0.998	0.99	1.025	1.053	1.009
	0.983	0.996	0.897	0.882	0.978	1.01	0.997	1.002	1.011	1.008
	1.002	1.013	0.891	0.915	0.9887	1.0037	1.005333	1.01167	1.012667	1.015
40	0.169	0.589	0.17	0.44	0.169	0.321	0.178	0.446	0.163	0.607
	0.157	0.639	0.163	0.407	0.157	0.288	0.164	0.647	0.163	0.753

Statistical Data (ANOVA) Figure 3.30

Statistical Data (Zero)

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
JE2wt vs. JE2wt hemin on	-0.011	-0.06872 to 0.04672	No	ns	0.9994
JE2wt vs. JE2mspA::tn	0.111	0.05328 to 0.1687	Yes	****	<0.0001
JE2wt vs. JE2mspA::tn hemin on	0.087	0.02928 to 0.1447	Yes	**	0.001
JE2wt vs. NE42	0.01333	-0.04439 to 0.07106	No	ns	0.9973
JE2wt vs. NE42 hemin on	-0.00167	-0.05939 to 0.05606	No	ns	>0.9999
JE2wt vs. NE627	-0.00333	-0.06106 to 0.05439	No	ns	>0.9999
JE2wt vs. NE627 hemin on	-0.00967	-0.06739 to 0.04806	No	ns	0.9998
JE2wt vs. NE866	-0.01067	-0.06839 to 0.04706	No	ns	0.9995
JE2wt vs. NE866 hemin on	-0.013	-0.07072 to 0.04472	No	ns	0.9978
JE2wt hemin on vs. JE2mspA::tn	0.122	0.06428 to 0.1797	Yes	****	<0.0001
JE2wt hemin on vs. JE2mspA::tn hemin on	0.098	0.04028 to 0.1557	Yes	***	0.0002
JE2wt hemin on vs. NE42	0.02433	-0.03339 to 0.08206	No	ns	0.8799
JE2wt hemin on vs. NE42 hemin on	0.009333	-0.04839 to 0.06706	No	ns	0.9998
JE2wt hemin on vs. NE627	0.007667	-0.05006 to 0.06539	No	ns	>0.9999
JE2wt hemin on vs. NE627 hemin on	0.001333	-0.05639 to 0.05906	No	ns	>0.9999
JE2wt hemin on vs. NE866	0.000333	-0.05739 to 0.05806	No	ns	>0.9999
JE2wt hemin on vs. NE866 hemin on	-0.002	-0.05972 to 0.05572	No	ns	>0.9999
JE2mspA::tn vs. JE2mspA::tn hemin on	-0.024	-0.08172 to 0.03372	No	ns	0.8879
JE2mspA::tn vs. NE42	-0.09767	-0.1554 to -0.03994	Yes	***	0.0003
JE2mspA::tn vs. NE42 hemin on	-0.1127	-0.1704 to -0.05494	Yes	****	<0.0001
JE2mspA::tn vs. NE627	-0.1143	-0.1721 to -0.05661	Yes	****	<0.0001
JE2mspA::tn vs. NE627 hemin on	-0.1207	-0.1784 to -0.06294	Yes	****	<0.0001
JE2mspA::tn vs. NE866	-0.1217	-0.1794 to -0.06394	Yes	****	<0.0001
JE2mspA::tn vs. NE866 hemin on	-0.124	-0.1817 to -0.06628	Yes	****	<0.0001
JE2mspA::tn hemin on vs. NE42	-0.07367	-0.1314 to -0.01594	Yes	**	0.0063
JE2mspA::tn hemin on vs. NE42 hemin on	-0.08867	-0.1464 to -0.03094	Yes	***	0.0008
JE2mspA::tn hemin on vs. NE627	-0.09033	-0.1481 to -0.03261	Yes	***	0.0007
JE2mspA::tn hemin on vs. NE627 hemin on	-0.09667	-0.1544 to -0.03894	Yes	***	0.0003
JE2mspA::tn hemin on vs. NE866	-0.09767	-0.1554 to -0.03994	Yes	***	0.0003
JE2mspA::tn hemin on vs. NE866 hemin on	-0.1	-0.1577 to -0.04228	Yes	***	0.0002
NE42 vs. NE42 hemin on	-0.015	-0.07272 to 0.04272	No	ns	0.9937
NE42 vs. NE627	-0.01667	-0.07439 to 0.04106	No	ns	0.987
NE42 vs. NE627 hemin on	-0.023	-0.08072 to 0.03472	No	ns	0.9098
NE42 vs. NE866	-0.024	-0.08172 to 0.03372	No	ns	0.8879
NE42 vs. NE866 hemin on	-0.02633	-0.08406 to 0.03139	No	ns	0.8261
NE42 hemin on vs. NE627	-0.00167	-0.05939 to 0.05606	No	ns	>0.9999
NE42 hemin on vs. NE627 hemin on	-0.008	-0.06572 to 0.04972	No	ns	>0.9999
NE42 hemin on vs. NE866	-0.009	-0.06672 to 0.04872	No	ns	0.9999
NE42 hemin on vs. NE866 hemin on	-0.01133	-0.06906 to 0.04639	No	ns	0.9992
NE627 vs. NE627 hemin on	-0.00633	-0.06406 to 0.05139	No	ns	>0.9999
NE627 vs. NE866	-0.00733	-0.06506 to 0.05039	No	ns	>0.9999
NE627 vs. NE866 hemin on	-0.00967	-0.06739 to 0.04806	No	ns	0.9998
NE627 hemin on vs. NE866	-0.001	-0.05872 to 0.05672	No	ns	>0.9999
NE627 hemin on vs. NE866 hemin on	-0.00333	-0.06106 to 0.05439	No	ns	>0.9999
NE866 vs. NE866 hemin on	-0.00233	-0.06006 to 0.05539	No	ns	>0.9999

Statistical Data (40)

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
JE2wt vs. JE2wt hemin on	-0.3143	-0.6730 to 0.04433	No	ns	0.1174
JE2wt vs. JE2mspA::tn	-0.00333	-0.3620 to 0.3553	No	ns	>0.9999
JE2wt vs. JE2mspA::tn hemin on	-0.174	-0.5327 to 0.1847	No	ns	0.7738
JE2wt vs. NE42	0.002	-0.3567 to 0.3607	No	ns	>0.9999
JE2wt vs. NE42 hemin on	-0.09433	-0.4530 to 0.2643	No	ns	0.9932
JE2wt vs. NE627	-0.00833	-0.3670 to 0.3503	No	ns	>0.9999
JE2wt vs. NE627 hemin on	-0.2933	-0.6520 to 0.06533	No	ns	0.1708
JE2wt vs. NE866	-0.00067	-0.3593 to 0.3580	No	ns	>0.9999
JE2wt vs. NE866 hemin on	-0.426	-0.7847 to -0.06734	Yes	*	0.0124
JE2wt hemin on vs. JE2mspA::tn	0.311	-0.04766 to 0.6697	No	ns	0.1248
JE2wt hemin on vs. JE2mspA::tn hemin on	0.1403	-0.2183 to 0.4990	No	ns	0.9181
JE2wt hemin on vs. NE42	0.3163	-0.04233 to 0.6750	No	ns	0.1132
JE2wt hemin on vs. NE42 hemin on	0.22	-0.1387 to 0.5787	No	ns	0.5037
JE2wt hemin on vs. NE627	0.306	-0.05266 to 0.6647	No	ns	0.1366
JE2wt hemin on vs. NE627 hemin on	0.021	-0.3377 to 0.3797	No	ns	>0.9999
JE2wt hemin on vs. NE866	0.3137	-0.04499 to 0.6723	No	ns	0.1188
JE2wt hemin on vs. NE866 hemin on	-0.1117	-0.4703 to 0.2470	No	ns	0.9787
JE2mspA::tn vs. JE2mspA::tn hemin on	-0.1707	-0.5293 to 0.1880	No	ns	0.7912
JE2mspA::tn vs. NE42	0.005333	-0.3533 to 0.3640	No	ns	>0.9999
JE2mspA::tn vs. NE42 hemin on	-0.091	-0.4497 to 0.2677	No	ns	0.9947
JE2mspA::tn vs. NE627	-0.005	-0.3637 to 0.3537	No	ns	>0.9999
JE2mspA::tn vs. NE627 hemin on	-0.29	-0.6487 to 0.06866	No	ns	0.1809
JE2mspA::tn vs. NE866	0.002667	-0.3560 to 0.3613	No	ns	>0.9999
JE2mspA::tn vs. NE866 hemin on	-0.4227	-0.7813 to -0.06401	Yes	*	0.0133
JE2mspA::tn hemin on vs. NE42	0.176	-0.1827 to 0.5347	No	ns	0.7631
JE2mspA::tn hemin on vs. NE42 hemin on	0.07967	-0.2790 to 0.4383	No	ns	0.998
JE2mspA::tn hemin on vs. NE627	0.1657	-0.1930 to 0.5243	No	ns	0.8163
JE2mspA::tn hemin on vs. NE627 hemin on	-0.1193	-0.4780 to 0.2393	No	ns	0.9677
JE2mspA::tn hemin on vs. NE866	0.1733	-0.1853 to 0.5320	No	ns	0.7773
JE2mspA::tn hemin on vs. NE866 hemin on	-0.252	-0.6107 to 0.1067	No	ns	0.3306
NE42 vs. NE42 hemin on	-0.09633	-0.4550 to 0.2623	No	ns	0.9921
NE42 vs. NE627	-0.01033	-0.3690 to 0.3483	No	ns	>0.9999
NE42 vs. NE627 hemin on	-0.2953	-0.6540 to 0.06333	No	ns	0.165
NE42 vs. NE866	-0.00267	-0.3613 to 0.3560	No	ns	>0.9999
NE42 vs. NE866 hemin on	-0.428	-0.7867 to -0.06934	Yes	*	0.0118
NE42 hemin on vs. NE627	0.086	-0.2727 to 0.4447	No	ns	0.9965
NE42 hemin on vs. NE627 hemin on	-0.199	-0.5577 to 0.1597	No	ns	0.63
NE42 hemin on vs. NE866	0.09367	-0.2650 to 0.4523	No	ns	0.9935
NE42 hemin on vs. NE866 hemin on	-0.3317	-0.6903 to 0.02699	No	ns	0.0848
NE627 vs. NE627 hemin on	-0.285	-0.6437 to 0.07366	No	ns	0.1969
NE627 vs. NE866	0.007667	-0.3510 to 0.3663	No	ns	>0.9999
NE627 vs. NE866 hemin on	-0.4177	-0.7763 to -0.05901	Yes	*	0.0148
NE627 hemin on vs. NE866	0.2927	-0.06599 to 0.6513	No	ns	0.1728
NE627 hemin on vs. NE866 hemin on	-0.1327	-0.4913 to 0.2260	No	ns	0.9398
NE866 vs. NE866 hemin on	-0.4253	-0.7840 to -0.06667	Yes	*	0.0125

Raw Data for Figure 3.31

	SH1000wt	SH1000wt hemin on	SH1000 mspA::tn	SH1000 mspA::tn hemin on	SH1000 NE42	SH1000 NE42 hemin on	SH1000 NE627	SH1000 NE627 hemin on	SH1000 NE866	SH1000N E866 hemin on
Zero	0.667	0.695	0.644	0.533	0.975	1.021	0.997	1.004	**0.995**	**1.034**
	0.656	0.678	0.672	0.511	1.001	1.026	1.013	0.984	**1.004**	**1.005**
	0.7	0.723	0.674	0.573	1	1.036	1.041	1.037	**1.04**	**1.048**
	0.6	0.661	0.668	0.498	0.99	1.017	0.992	0.986	**0.989**	**1.019**
	0.665	0.66	0.652	0.51	0.993	1.008	0.986	1	**0.998**	**1.025**
	0.658	0.645	0.631	0.563	0.989	1.001	0.965	0.978	**0.933**	**0.992**
	0.657666667	0.677	0.656833333	0.531333333	0.991333333	1.018166667	0.999	0.998166667	**0.993166667**	**1.0205**
40	0.374	0.647	0.163	0.218	0.173	0.582	0.293	0.608	**0.33**	**0.822**
	0.476	0.65	0.159	0.249	0.282	0.692	0.41	0.623	**0.427**	**0.79**
	0.181	0.552	0.171	0.17	0.158	0.433	0.254	0.595	**0.224**	**0.763**
	0.377	0.701	0.206	0.191	0.263	0.4	0.331	0.617	**0.395**	**0.794**
	0.496	0.652	0.169	0.183	0.303	0.464	0.353	0.67	**0.445**	**0.826**

Statistical Data (Zero)

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
SH1000wt vs. SH1000wt hemin on	-0.01933	-0.06643 to 0.02776	No	ns	0.9338
SH1000wt vs. SH1000mspA*::tn*	0.000833	-0.04626 to 0.04793	No	ns	>0.9999
SH1000wt vs. SH1000mspA*::tn* hemin on	0.1263	0.07924 to 0.1734	Yes	****	<0.0001
SH1000wt vs. SH1000NE42	-0.3337	-0.3808 to -0.2866	Yes	****	<0.0001
SH1000wt vs. SH1000NE42 hemin on	-0.3605	-0.4076 to -0.3134	Yes	****	<0.0001
SH1000wt vs. SH1000NE627	-0.3413	-0.3884 to -0.2942	Yes	****	<0.0001
SH1000wt vs. SH1000NE627 hemin on	-0.3405	-0.3876 to -0.2934	Yes	****	<0.0001
SH1000wt vs. SH1000NE866	-0.3355	-0.3826 to -0.2884	Yes	****	<0.0001
SH1000wt vs. SH1000NE866 hemin on	-0.3628	-0.4099 to -0.3157	Yes	****	<0.0001
SH1000wt hemin on vs. SH1000mspA*::tn*	0.02017	-0.02693 to 0.06726	No	ns	0.9159
SH1000wt hemin on vs. SH1000mspA*::tn* hemin on	0.1457	0.09857 to 0.1928	Yes	****	<0.0001
SH1000wt hemin on vs. SH1000NE42	-0.3143	-0.3614 to -0.2672	Yes	****	<0.0001
SH1000wt hemin on vs. SH1000NE42 hemin on	-0.3412	-0.3883 to -0.2941	Yes	****	<0.0001
SH1000wt hemin on vs. SH1000NE627	-0.322	-0.3691 to -0.2749	Yes	****	<0.0001
SH1000wt hemin on vs. SH1000NE627 hemin on	-0.3212	-0.3683 to -0.2741	Yes	****	<0.0001
SH1000wt hemin on vs. SH1000NE866	-0.3162	-0.3633 to -0.2691	Yes	****	<0.0001
SH1000wt hemin on vs. SH1000NE866 hemin on	-0.3435	-0.3906 to -0.2964	Yes	****	<0.0001
SH1000mspA*::tn* vs. SH1000mspA*::tn* hemin on	0.1255	0.07841 to 0.1726	Yes	****	<0.0001
SH1000mspA*::tn* vs. SH1000NE42	-0.3345	-0.3816 to -0.2874	Yes	****	<0.0001
SH1000mspA*::tn* vs. SH1000NE42 hemin on	-0.3613	-0.4084 to -0.3142	Yes	****	<0.0001
SH1000mspA*::tn* vs. SH1000NE627	-0.3422	-0.3893 to -0.2951	Yes	****	<0.0001
SH1000mspA*::tn* vs. SH1000NE627 hemin on	-0.3413	-0.3884 to -0.2942	Yes	****	<0.0001
SH1000mspA*::tn* vs. SH1000NE866	-0.3363	-0.3834 to -0.2892	Yes	****	<0.0001
SH1000mspA*::tn* vs. SH1000NE866 hemin on	-0.3637	-0.4108 to -0.3166	Yes	****	<0.0001
SH1000mspA*::tn* hemin on vs. SH1000NE42	-0.46	-0.5071 to -0.4129	Yes	****	<0.0001
SH1000mspA*::tn* hemin on vs. SH1000NE42 hemin on	-0.4868	-0.5339 to -0.4397	Yes	****	<0.0001
SH1000mspA*::tn* hemin on vs. SH1000NE627	-0.4677	-0.5148 to -0.4206	Yes	****	<0.0001
SH1000mspA*::tn* hemin on vs. SH1000NE627 hemin on	-0.4668	-0.5139 to -0.4197	Yes	****	<0.0001
SH1000mspA*::tn* hemin on vs. SH1000NE866	-0.4618	-0.5089 to -0.4147	Yes	****	<0.0001
SH1000mspA*::tn* hemin on vs. SH1000NE866 hemin on	-0.4892	-0.5363 to -0.4421	Yes	****	<0.0001
SH1000NE42 vs. SH1000NE42 hemin on	-0.02683	-0.07393 to 0.02026	No	ns	0.6779
SH1000NE42 vs. SH1000NE627	-0.00767	-0.05476 to 0.03943	No	ns	>0.9999
SH1000NE42 vs. SH1000NE627 hemin on	-0.00683	-0.05393 to 0.04026	No	ns	>0.9999

SH1000NE42 vs. SH1000NE866	-0.00183	-0.04893 to 0.04526	No	ns	>0.9999
SH1000NE42 vs. SH1000NE866 hemin on	-0.02917	-0.07626 to 0.01793	No	ns	0.5694
SH1000NE42 hemin on vs. SH1000NE627	0.01917	-0.02793 to 0.06626	No	ns	0.937
SH1000NE42 hemin on vs. SH1000NE627 hemin on	0.02	-0.02709 to 0.06709	No	ns	0.9197
SH1000NE42 hemin on vs. SH1000NE866	0.025	-0.02209 to 0.07209	No	ns	0.7575
SH1000NE42 hemin on vs. SH1000NE866 hemin on	-0.00233	-0.04943 to 0.04476	No	ns	>0.9999
SH1000NE627 vs. SH1000NE627 hemin on	0.000833	-0.04626 to 0.04793	No	ns	>0.9999
SH1000NE627 vs. SH1000NE866	0.005833	-0.04126 to 0.05293	No	ns	>0.9999
SH1000NE627 vs. SH1000NE866 hemin on	-0.0215	-0.06859 to 0.02559	No	ns	0.8813
SH1000NE627 hemin on vs. SH1000NE866	0.005	-0.04209 to 0.05209	No	ns	>0.9999
SH1000NE627 hemin on vs. SH1000NE866 hemin on	-0.02233	-0.06943 to 0.02476	No	ns	0.8559
SH1000NE866 vs. SH1000NE866 hemin on	-0.02733	-0.07443 to 0.01976	No	ns	0.6551

Statistical Data (40)

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
SH1000wt vs. SH1000wt hemin on	-0.2677	-0.4018 to -0.1336	Yes	****	<0.0001
SH1000wt vs. SH1000mspA::*tn*	0.2018	0.06775 to 0.3359	Yes	***	0.0003
SH1000wt vs. SH1000mspA::*tn* hemin on	0.167	0.03291 to 0.3011	Yes	**	0.005
SH1000wt vs. SH1000NE42	0.125	-0.009085 to 0.2591	No	ns	0.0869
SH1000wt vs. SH1000NE42 hemin on	-0.1375	-0.2716 to -0.003415	Yes	*	0.0402
SH1000wt vs. SH1000NE627	0.034	-0.1001 to 0.1681	No	ns	0.9975
SH1000wt vs. SH1000NE627 hemin on	-0.2593	-0.3934 to -0.1252	Yes	****	<0.0001
SH1000wt vs. SH1000NE866	-0.01583	-0.1499 to 0.1183	No	ns	>0.9999
SH1000wt vs. SH1000NE866 hemin on	-0.4127	-0.5468 to -0.2786	Yes	****	<0.0001
SH1000wt hemin on vs. SH1000mspA::*tn*	0.4695	0.3354 to 0.6036	Yes	****	<0.0001
SH1000wt hemin on vs. SH1000mspA::*tn* hemin on	0.4347	0.3006 to 0.5688	Yes	****	<0.0001
SH1000wt hemin on vs. SH1000NE42	0.3927	0.2586 to 0.5268	Yes	****	<0.0001
SH1000wt hemin on vs. SH1000NE42 hemin on	0.1302	-0.003919 to 0.2643	No	ns	0.0638
SH1000wt hemin on vs. SH1000NE627	0.3017	0.1676 to 0.4358	Yes	****	<0.0001
SH1000wt hemin on vs. SH1000NE627 hemin on	0.008333	-0.1258 to 0.1424	No	ns	>0.9999
SH1000wt hemin on vs. SH1000NE866	0.2518	0.1177 to 0.3859	Yes	****	<0.0001
SH1000wt hemin on vs. SH1000NE866 hemin on	-0.145	-0.2791 to -0.01091	Yes	*	0.0245
SH1000mspA::*tn* vs. SH1000mspA::*tn* hemin on	-0.03483	-0.1689 to 0.09925	No	ns	0.997
SH1000mspA::*tn* vs. SH1000NE42	-0.07683	-0.2109 to 0.05725	No	ns	0.671
SH1000mspA::*tn* vs. SH1000NE42 hemin on	-0.3393	-0.4734 to -0.2052	Yes	****	<0.0001
SH1000mspA::*tn* vs. SH1000NE627	-0.1678	-0.3019 to -0.03375	Yes	**	0.0047
SH1000mspA::*tn* vs. SH1000NE627 hemin on	-0.4612	-0.5953 to -0.3271	Yes	****	<0.0001
SH1000mspA::*tn* vs. SH1000NE866	-0.2177	-0.3518 to -0.08358	Yes	****	<0.0001
SH1000mspA::*tn* vs. SH1000NE866 hemin on	-0.6145	-0.7486 to -0.4804	Yes	****	<0.0001
SH1000mspA::*tn* hemin on vs. SH1000NE42	-0.042	-0.1761 to 0.09209	No	ns	0.9883
SH1000mspA::*tn* hemin on vs. SH1000NE42 hemin on	-0.3045	-0.4386 to -0.1704	Yes	****	<0.0001
SH1000mspA::*tn* hemin on vs. SH1000NE627	-0.133	-0.2671 to 0.001085	No	ns	0.0535
SH1000mspA::*tn* hemin on vs. SH1000NE627 hemin on	-0.4263	-0.5604 to -0.2922	Yes	****	<0.0001
SH1000mspA::*tn* hemin on vs. SH1000NE866	-0.1828	-0.3169 to -0.04875	Yes	**	0.0015
SH1000mspA::*tn* hemin on vs. SH1000NE866 hemin on	-0.5797	-0.7138 to -0.4456	Yes	****	<0.0001

SH1000NE42 vs. SH1000NE42 hemin on	-0.2625	-0.3966 to -0.1284	Yes	****	<0.0001
SH1000NE42 vs. SH1000NE627	-0.091	-0.2251 to 0.04309	No	ns	0.4404
SH1000NE42 vs. SH1000NE627 hemin on	-0.3843	-0.5184 to -0.2502	Yes	****	<0.0001
SH1000NE42 vs. SH1000NE866	-0.1408	-0.2749 to -0.006748	Yes	*	0.0323
SH1000NE42 vs. SH1000NE866 hemin on	-0.5377	-0.6718 to -0.4036	Yes	****	<0.0001
SH1000NE42 hemin on vs. SH1000NE627	0.1715	0.03741 to 0.3056	Yes	**	0.0036
SH1000NE42 hemin on vs. SH1000NE627 hemin on	-0.1218	-0.2559 to 0.01225	No	ns	0.1042
SH1000NE42 hemin on vs. SH1000NE866	0.1217	-0.01242 to 0.2558	No	ns	0.1052
SH1000NE42 hemin on vs. SH1000NE866 hemin on	-0.2752	-0.4093 to -0.1411	Yes	****	<0.0001
SH1000NE627 vs. SH1000NE627 hemin on	-0.2933	-0.4274 to -0.1592	Yes	****	<0.0001
SH1000NE627 vs. SH1000NE866	-0.04983	-0.1839 to 0.08425	No	ns	0.9637
SH1000NE627 vs. SH1000NE866 hemin on	-0.4467	-0.5808 to -0.3126	Yes	****	<0.0001
SH1000NE627 hemin on vs. SH1000NE866	0.2435	0.1094 to 0.3776	Yes	****	<0.0001
SH1000NE627 hemin on vs. SH1000NE866 hemin on	-0.1533	-0.2874 to -0.01925	Yes	*	0.0137
SH1000NE866 vs. SH1000NE866 hemin on	-0.3968	-0.5309 to -0.2627	Yes	****	<0.0001

Raw Data for Figure 4.6

PBP1	PBP2	PBP3	PBP4	PBP2a
0.45	3.00	2.15	0.50	0.11
0.43	2.69	2.16	0.35	0.98
0.50	3.48	2.73	0.23	1.09

Statistical Data (ANOVA)

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
PBP1 vs. PBP2	-2.593	-3.504 to -1.681	Yes	****	<0.0001
PBP1 vs. PBP3	-1.885	-2.797 to -0.9739	Yes	***	0.0004
PBP1 vs. PBP4	0.09757	-0.8140 to 1.009	No	ns	0.9961
PBP1 vs. PBP2a	-0.2643	-1.176 to 0.6473	No	ns	0.8692
PBP2 vs. PBP3	0.7072	-0.2044 to 1.619	No	ns	0.154
PBP2 vs. PBP4	2.69	1.779 to 3.602	Yes	****	<0.0001
PBP2 vs. PBP2a	2.328	1.417 to 3.240	Yes	****	<0.0001
PBP3 vs. PBP4	1.983	1.071 to 2.895	Yes	***	0.0002
PBP3 vs. PBP2a	1.621	0.7096 to 2.533	Yes	**	0.0012
PBP4 vs. PBP2a	-0.3619	-1.273 to 0.5497	No	ns	0.6937

Raw Data for Figure 4.7

JE2				JE2 *mspA::tn*				JE2 *mspA::tn* (*pmspA*)			
3820	3906	3683	2970	4592	4411	5597	4057	2674	3793	3869	3615

Statistical Data (ANOVA)

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
JE2 vs. *JE2 mspA::tn*	-1070	-2165 to 25.92	No	ns	0.0554
JE2 vs. JE2 *mspA::tn*	107	-988.4 to 1202	No	ns	0.96
JE2 mspA::tn vs. JE2 *mspA::tn*	1177	81.08 to 2272	Yes	*	0.0362

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