

Probing the activity of FmtA, a novel esterase in
Staphylococcus aureus*, *in vitro* and *in vivo
through mutagenesis

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Abstract

The versatile bacterium *Staphylococcus aureus* (*S. aureus*) resides within the human microbiota, manifesting commensal and pathogenic characteristics. The emergence of methicillin-resistant *S. aureus* (MRSA) underscores the global challenge of antibiotic resistance. FmtA, a protein exhibiting structural similarities and distinct features relative to Penicillin-binding proteins (PBPs) within the Penicillin penicillin-recognizing proteins (PRPs) umbrella, significantly contributes to the *S. aureus* antibiotic resistance mechanism. FmtA acts on Wall Teichoic Acid (WTA), an abundant poly-ribitol polymer in *S. aureus* peptidoglycan, where FmtA removes D-Alanine (D-Ala) from WTA, which is esterified post-synthesis where the D-alanylation level in WTA is an important contributor in *S. aureus* antibiotic resistance and physiology. Previous research addressed the structural intricacies of FmtA and based on the positioning of key amino acid residues, it was proposed that the enzyme's active site has evolved to specifically bind WTA. It was further hypothesized that FmtA has adapted to carry out D-amino esterase activity against WTA, highlighting its specialized functional evolution. In this study, I investigate the role of specific amino acid residues present in the active site of FmtA, focusing on their interactions with WTA using both *in vitro* and *in vivo* approaches. Given the overall negative charge of WTA, derived from its phosphodiester backbone, it is a target for cationic polymers like Branched Polyethyleneimine (BPEI). Previous research has shown that BPEI binding to WTA creates a steric hindrance, disrupting the function of penicillin-binding proteins (PBPs) such as PBP4 in *Staphylococcus aureus* and PBP2a in *Staphylococcus epidermidis*. This disruption impacts key bacterial physiological processes, including cell division, virulence, and peptidoglycan biosynthesis. My findings reveal that BPEI can compete with FmtA for WTA binding, though less effectively, leading to partial inhibition of FmtA's enzymatic activity. Additionally, BPEI binding

affects the physiological activities of *S. aureus*, including biofilm formation, which is typically mediated by FmtA. These results suggest that while BPEI hinders FmtA's interaction with WTA and its subsequent enzymatic function, it does not completely block FmtA activity.

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List of Abbreviations

<i>ΔfntA</i> - <i>fntA</i> deleted strain	PBS- Phosphate-buffered saline
Ala - Alanine	PG- Peptidoglycan
Asn - Asparagine	Phe- Phenylalanine
Asp- Aspartic acid	Pi- Inorganic phosphate
Bp- Base pair	RT- Room temperature
D-Ala- D-Alanine	PRP- Penicillin-recognizing protein
DAA- D-amino amidase	SDS-PAGE- Sodium dodecyl sulphate-
DAP- D, D-amino peptidase	polyacrylamide gel electrophoresis
DSS- Sodium trimethylsilyl propane	Ser- Serine
sulfonate	TCA- Trichloroacetic acid
GlcNAc- N-acetyl glucosamine	TEM- Transmission electron microscopy
Glu- Glutamic acid	TP- Transpeptidase
His- Histidine	TSB- Tryptic soy broth
HMW- High molecular weight	Tyr- Tyrosine
IPTG- Isopropyl P-D-thiogalactopyranoside	TSB- Tryptic soy broth
LTA- Lipoteichoic acid	UDP- Undecaprenyl phosphate
Leu- Leucine	VRSA- Vancomycin-resistant
LMW- Low molecular weight	<i>Staphylococcus aureus</i>
LTA- Lipoteichoic acid	WTA- Wall teichoic acid
Lys- Lysine	
ManNAc- N-acetyl mannose amine	
MurNAc- N-acetyl muramic acid	
MRSA- Methicillin-resistant <i>Staphylococcus</i>	
<i>aureus</i>	
MW- Molecular weight	
nPAGE- Native polyacrylamide gel	
electrophoresis	
NMR- Nuclear magnetic resonance	
PBP- Penicillin-binding proteins	

Chapter One

Introduction

1.1 *Staphylococcus aureus*

Staphylococcus aureus (*S. aureus*) is a Gram-positive, facultative, anaerobic, coccus-shaped bacteria ubiquitously present in the nasal cavity and on the skin of humans (Gulzar & Zehra, 2018). Although it is typically a commensal organism, *S. aureus* is a significant opportunistic pathogen implicated in a spectrum of infections, ranging from superficial skin lesions to severe systemic conditions like pneumonia, endocarditis, and sepsis (Del Giudice, 2020). Its ability to thrive in diverse environmental niches and antibiotic resistance renders it a substantial public health threat (de Lencastre et al., 2007).

The identification of *S. aureus* dates back to the late 19th century when Sir Alexander Ogston first isolated the bacterium from pus in surgical abscesses (Myles & Datta, 2012). He recognized its pathogenicity, which prompted extensive research into its virulence determinants and infection mechanisms. While the introduction of antibiotics in the mid-20th century initially offered effective therapeutic interventions, the advent of antibiotic-resistant strains, such as methicillin-resistant *Staphylococcus aureus* (MRSA), has significantly complicated clinical management and generated the urgent need for continuous research and novel therapeutic strategies (de Lencastre et al., 2007).

S. aureus exhibits a distinct spherical morphology, clustering in characteristic grape-like formations when observed under a microscope (Latif Bhutto et al., 2015). On agar plates, *S. aureus* produces golden-yellow colonies due to the presence of the carotenoid pigment staphyloxanthin, which contributes to its virulence by providing resistance against oxidative stress (Yehia et al., 2022). *S. aureus* also produces virulence factors, such as SpA, FnbpA, FnbpB, ClfA, α and β

hemolysins etc. which enhances its pathogenic potential (Rita Costa et al., 2013). Additional factors contributing to its virulence include clumping factor and protein A (Palmqvist et al., 2005). The clumping factor aids in adherence to fibrinogen, while fibronectin-binding proteins facilitate binding to the host's extracellular matrix (T. J. Foster, 2017). Protein A interacts with the Fc region of immunoglobulins, disrupting opsonization and phagocytosis, thus allowing the bacteria to evade the host's immune response (Ko et al., 2013). Additionally, *S. aureus* secretes various enzymes that further enhance its pathogenicity. For instance, coagulase promotes the formation of fibrin clots, creating a protective barrier against phagocytic cells, while hyaluronidase breaks down hyaluronic acid in connective tissues, aiding in bacterial invasion (Drăgulescu and Codîbă, 2015).

Furthermore, *S. aureus* produces several toxins, including cytotoxins, exfoliative toxins, enterotoxins, and superantigens (Otto, 2014). Cytotoxins, such as alpha-toxin, induce cell membrane disruption and subsequent cell lysis (Bhakdil & Trandum-Jensen, 1991). The exfoliative toxins are implicated in *staphylococcal* scalded skin syndrome (Ladhani, 2001), while enterotoxins are associated with food poisoning. Superantigens, such as toxic shock syndrome toxin-1 (TSST-1), can cause severe systemic effects by hyperactivating the immune system (Krakauer Teresa, 1999).

The infection process of *S. aureus* generally commences with bacterial colonization, followed by tissue invasion and immune evasion (Ryu et al., 2014). Surface adhesins facilitate attachment to host tissues, particularly in the anterior nares, skin, and mucosal surfaces (Berry et al., 2022). Once adhered, the bacterium utilizes various enzymes to degrade tissue barriers, facilitating deeper invasion (Berry et al., 2022). Bloodborne spread can also cause infections throughout the body, affecting multiple organs (Kwiecinski & Horswill, 2020).

A key feature of *S. aureus* pathogenicity is its capacity to form biofilms on both medical devices and host tissues (Idrees et al., 2021). Biofilms are a critical concern in medical device-related infections, as they form protective niches that shield bacteria from antibiotics and the host immune system (Di Domenico et al., 2022). These biofilm-associated infections are particularly challenging to treat, often requiring the removal of the infected device to fully eradicate the bacteria (Khardori & Yassien, 1995).

1.1.1 Clinical manifestations of *S. aureus*

S. aureus infections encompass a wide spectrum, ranging from minor skin and soft tissue infections (SSTIs) to severe systemic diseases (Dryden, 2010). SSTIs caused by *S. aureus* include impetigo, folliculitis, furuncles, and carbuncles, and are often marked by localized erythema, swelling, and suppuration (Silverberg, 2021). Folliculitis, a superficial infection of hair follicles, manifests as small, erythematous, and tender pustules (Jes' et al., 2004). Furuncles, commonly known as boils, are deeper infections of hair follicles characterized by painful, pus-filled nodules, while carbuncles are clusters of interconnected furuncles accompanied by systemic symptoms like fever (Auliya et al., 2024; Jes' et al., 2004). Impetigo, predominantly affecting children, is a highly contagious superficial skin infection presenting as honey-coloured crusted lesions, frequently on the face and extremities (Chauhan et al., 2004).

Abscess formation in *S. aureus* infection is characterized by localized collections of pus within tissues, necessitating surgical drainage and antibiotic therapy (Thomer et al., 2016). Beyond SSTIs, *S. aureus* is a principal cause of osteomyelitis, septic arthritis and pyomyositis (Esposito et al., 2017). Osteomyelitis, an infection of the bone, can be acute or chronic, typically resulting from hematogenous spread or contiguous infection from adjacent tissues, with symptoms including localized bone pain, swelling, erythema, and systemic signs of infection (Ikpeme IA et al., 2018).

Septic arthritis, an infection of the joints, presents with acute onset of joint pain, swelling, erythema, and restricted movement, necessitating prompt diagnosis and treatment to prevent joint destruction (Shirtliff & Mader, 2002). Pyomyositis, a purulent infection of skeletal muscles more common in tropical regions but increasingly reported worldwide, manifests with muscle pain, swelling, and systemic symptoms, often requiring surgical drainage and antibiotics (Chauhan et al., 2004).

Furthermore, *S. aureus* bacteremia can lead to metastatic infections, including endocarditis, which involves heart valve infection and can result in serious complications such as heart failure and embolic events (Horino & Hori, 2020). *S. aureus* infections, such as myocarditis and pericarditis, affecting the heart muscle and pericardium, respectively, manifest with symptoms like chest pain, fever, and signs of cardiac dysfunction (Radovanovic et al., 2022).

1.1.2 Antibiotic resistance demonstrated by *S. aureus*

The emergence of antibiotic-resistant *S. aureus* strains, particularly MRSA, presents a formidable challenge in clinical settings (DeLeo & Chambers, 2009). Resistance mechanisms in *S. aureus* include the production of β -lactamases, modification of antibiotic targets, and the operation of efflux pumps that expel antibiotics from the bacterial cell (Mohanty et al., 2021).

Efflux pumps are transmembrane proteins that actively extrude a wide range of toxic substrates, including antibiotics, from the bacterial cytoplasm, reducing intracellular drug concentrations and rendering treatments less effective (Van Bambeke et al., 2000). These pumps are driven by the proton motive force or ATP hydrolysis, enabling the active transport of antimicrobial agents against their concentration gradient (Van Bambeke et al., 2000).

In *S. aureus*, several efflux pumps have been identified that contribute to resistance against various classes of antibiotics, such as tetracyclines, fluoroquinolones, macrolides, and

aminoglycosides (Santos Costa et al., 2013). The NorA efflux pump, encoded by the *norA* gene, is among the most well-characterized pumps in *S. aureus*. NorA confers resistance primarily to fluoroquinolones, such as ciprofloxacin and norfloxacin, by reducing the intracellular accumulation of these antibiotics (Yu et al., 2022). This decreased accumulation limits the drug's ability to interact with its bacterial target, typically DNA gyrase or topoisomerase IV, essential for bacterial DNA replication and transcription (Yu et al., 2022).

Other efflux pumps, such as NorB, NorC, and MepA, also play significant roles in multidrug resistance. NorB and NorC share functional similarities with NorA, contributing to resistance against fluoroquinolones and other antimicrobials like biocides (Santos Costa et al., 2013). The MepA pump, encoded by the *mepA* gene, is associated with resistance to multiple antibiotics, including tigecycline and trimethoprim. This pump operates through an ATP-binding cassette (ABC) transport system, which uses ATP hydrolysis to expel antibiotics from the bacterial cytoplasm (Huang et al., 2023; Kaatz et al., 2006; Santos Costa et al., 2013).

Efflux pumps in *S. aureus* are often regulated by global transcriptional regulators, such as MgrA, NorR, and ArlRS, which sense environmental signals and modulate pump expression accordingly. *mgrA*, a global virulence regulator, acts as a repressor of the *norA* gene under normal conditions (Hassanzadeh et al., 2020; Truong-Bolduc et al., 2005). However, in the presence of stressors, such as antibiotics, *mgrA* repression is alleviated, leading to the upregulation of *norA* and enhanced efflux activity. This regulatory flexibility allows *S. aureus* to dynamically adjust its efflux pump expression in response to antimicrobial pressure (Andersen et al., 2015).

The contribution of efflux pumps to antibiotic resistance in *S. aureus* is particularly problematic because they confer low-level resistance that when combined with other resistance mechanisms such as target site mutations or enzymatic drug inactivation, results in high-level,

clinically relevant resistance (Dashtbani-Roozbehani & Brown, 2021; T. J. Foster, 2016). For instance, fluoroquinolone resistance often involves both NorA-mediated efflux and mutations in the DNA gyrase or topoisomerase IV genes, which together can produce resistance levels that are difficult to overcome with standard antibiotic dosages (Luis et al., 1999).

β -lactamases are key enzymes produced by *S. aureus* that confer resistance to β -lactam antibiotics, including penicillins, cephalosporins, and carbapenems (Bush & MacIelag, 2010). These antibiotics typically function by inhibiting bacterial cell wall synthesis through the inactivation of PBPs, enzymes involved in the cross-linking of the peptidoglycan layer. However, β -lactamases disrupt this mechanism by binding to the antibiotics where they demonstrate affinity towards the β -lactam ring of the antibiotic, rendering it ineffective (González-Bello et al., 2020).

In *S. aureus*, the primary β -lactamase is encoded by the *blaZ* gene (Nomura et al., 2020). Upon production, β -lactamase is secreted into the periplasmic space, where it binds to the antibiotic before it can interact with its target PBPs (Kaderabkova et al., 2022). The *bla* operon consists of four genes: *blaZ* (encoding β -lactamase), *blaR1* (a sensor-transducer protein), *blaI* (a repressor protein), and *blaR2* (another transducer component). This operon plays a critical role in regulating β -lactamase expression based on environmental antibiotic pressure (Safo et al., 2005).

Studies have shown that the regulatory control of β -lactamase production is highly dynamic and can be influenced by environmental conditions, such as nutrient availability and exposure to sublethal antibiotic concentrations (Dehbashi et al., 2021; Fisher & Mobashery, 2021; Sargantanis & Karim, 1996, 1998). Also, β -lactamase inhibitors, such as clavulanic acid, sulbactam, and tazobactam, have been developed to block β -lactamase activity and restore the efficacy of β -lactam antibiotics (Higgins et al., 2004). These inhibitors work by binding irreversibly to the active site of β -lactamase, preventing the binding with the antibiotic (Higgins et al., 2004). However, *S.*

aureus can evolve resistance to β -lactamase inhibitors by producing inhibitor-resistant β -lactamases or by upregulating alternative resistance mechanisms (Bush & Bradford, 2019).

Additionally, modification of antibiotic targets is a crucial mechanism by which *S. aureus* develops resistance to various antibiotic classes (T. J. Foster, 2017; Pantosti et al., 2007). This process involves alterations to the bacterial target sites that antibiotics bind to, rendering these drugs less effective or entirely ineffective (Pantosti et al., 2007). In *S. aureus*, several prominent mechanisms of target modification contribute to antibiotic resistance, particularly against β -lactams, glycopeptides, and other antibiotics (T. J. Foster, 2017).

In MRSA, resistance to antibiotic pressure is achieved through the acquisition of the *mecA* gene, which encodes PBP2a (Niemeyer et al., 1996). Unlike native PBPs, PBP2a has a low affinity for β -lactam antibiotics, which effectively circumvents the action of β -lactam antibiotics, making MRSA highly resistant to this entire class of drugs. The *mecA* gene is typically carried by the staphylococcal cassette chromosome *mec* (SCC*mec*), a mobile genetic element that facilitates the spread of resistance within and between bacterial populations through horizontal gene transfer (Shore & Coleman, 2013).

Another form of target modification involves alterations to the ribosomal binding sites of antibiotics that inhibit protein synthesis, such as macrolides and lincosamides (Leclercq & Courvalin, 1991). These antibiotics bind to the 50S ribosomal subunit, specifically to the 23S rRNA, and block the elongation of the growing polypeptide chain (Mikłasińska-Majdanik, 2021). Resistance to macrolides, lincosamides, and streptogramin B (collectively known as MLS_B antibiotics) in *S. aureus* is often mediated by methylation of adenine residues in the 23S rRNA, which reduces the binding affinity of these drugs (Leclercq & Courvalin, 1991). This modification is catalyzed by methyltransferases encoded by the *erm* (erythromycin ribosome methylation)

genes. *S. aureus* can also develop resistance to antibiotics that target metabolic pathways, such as trimethoprim, which inhibits dihydrofolate reductase (DHFR), an enzyme essential for folate biosynthesis. Folate is necessary for the synthesis of nucleotides, making DHFR a critical target for antibiotics (T. J. Foster, 2017; Reygaert, 2013).

Resistance to trimethoprim in *S. aureus* arises through mutations in the *dhfrA* gene, which encodes DHFR. These mutations reduce the drug's affinity for the enzyme, allowing folate synthesis to continue despite the presence of the antibiotic (Sato et al., 2022; Vickers et al., 2009). Alternatively, *S. aureus* can acquire plasmid-encoded versions of DHFR that are inherently resistant to trimethoprim, further compounding resistance (Sato et al., 2022).

1.2 Bacterial cell wall:

The cell wall of *S. aureus* is a pivotal element of its pathogenicity and resilience, acting as both a protective barrier and a dynamic structure essential for host tissue adherence and immune system evasion (Foster et al., 2014). Comprised predominantly of peptidoglycan (PG) and teichoic acids the cell wall forms a multilayered matrix that endows structural integrity and safeguards against environmental stresses (Boris A. Dmitriev, 1999; Romaniuk & Cegelski, 2018). Teichoic acids are anionic polymers either covalently attached to the peptidoglycan layer (wall teichoic acids, WTAs) or anchored in the cytoplasmic membrane (lipoteichoic acids, LTAs) (Weidenmaier & Peschel, 2008). These molecules contribute to cell wall integrity, ion homeostasis, and bacterial adhesion to surfaces, playing a key role in colonization and infection (Swoboda et al., 2010). Furthermore, surface proteins anchored to the peptidoglycan layer enhance virulence by mediating interactions with host tissues and evading immune responses (Nikolic & Mudgil, 2023). This well-defined structure encases the *S. aureus* cell, forming its outermost layer and playing a crucial role

in maintaining cell shape, strength, and flexibility essential for growth and morphological stability (Wang et al., 2022).

1.2.1 Peptidoglycan structure and formation

PG, also called murein, is a crucial and distinctive bacterial cell wall component (Garde et al., 2021). It provides structural integrity while protecting it from osmotic pressure and various environmental stresses (Garde et al., 2021). PG's intricate, mesh-like structure is unique to bacteria, rendering it a prime target for antibiotics such as penicillin (Lade & Kim, 2021).

In *S. aureus* PG is a polymer composed of sugars and amino acids, forming a continuous, covalently linked network (Gautam et al., 2011). The glycan portion consists of repeating disaccharide units of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc), connected by β -(1,4)-glycosidic bonds (Gautam et al., 2011). Attached to the MurNAc residue is a penta-peptide chain, typically composed of alternating L- and D-amino acids (Vollmer, Joris, et al., 2008). This peptide chain is vital for the cross-linking that imparts strength and rigidity to the PG network (Vollmer, Joris, et al., 2008).

The peptide sequence attached to MurNAc includes L-alanine, D-glutamic acid, meso-diaminopimelic acid (DAP) or L-lysine, and two D-alanine (T. A. Figueiredo et al., 2012). In Gram-positive bacteria, cross-linking generally occurs through a pentaglycine inter-bridge between the L-lysine residue to the first D-Alanine of the adjacent peptide chains, while in Gram-negative bacteria, it typically involves direct cross-linking between the DAP residues (Fig. 1.2.1.1) (Lebar et al., 2013; Rimal et al., 2023).

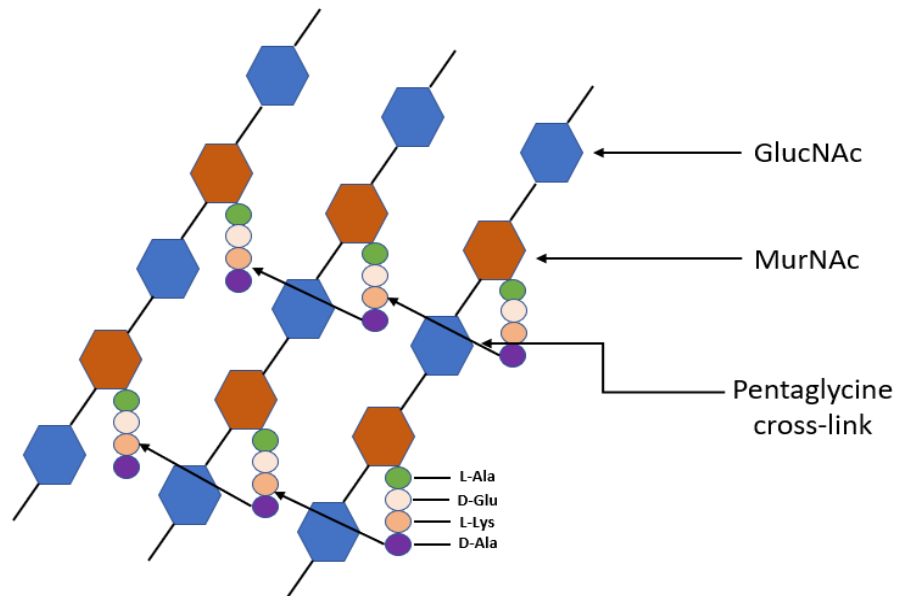


Figure 1.2.1.1. Schematic representation of the mesh-like peptidoglycan network structure showing stem peptides attached to the MurNAc repeating units interconnected by pentaglycine peptide bridges forming a ‘4-3’ crosslink.

In *S. aureus* the biosynthesis of PG is a complex, multistage process comprising several critical steps: the formation of precursor units in the cytoplasm, their transport across the cytoplasmic membrane, and the polymerization and cross-linking of these units to form the mature PG structure (Gautam et al., 2011). Initially, in the cytoplasm, the biosynthesis of PG begins with the formation of UDP-N-acetylglucosamine (UDP-GlcNAc) from fructose-6-phosphate (Barreteau et al., 2008). UDP-GlcNAc serves as a precursor for forming UDP-N-acetylmuramyl-pentapeptide, involving the sequential addition of amino acids to form a pentapeptide chain attached to the MurNAc residue, resulting in the PG precursor, UDP-MurNAc-pentapeptide (Barreteau et al., 2008).

The subsequent stage involves transferring the UDP-MurNAc-pentapeptide to a lipid carrier, undecaprenyl phosphate (bactoprenol), embedded in the cytoplasmic membrane (Matsumoto et al., 2016). This lipid-linked precursor, known as lipid I, is then modified by adding

UDP-GlcNAc to form lipid II, a key intermediate in PG biosynthesis (Matsumoto et al., 2016). Lipid II is translocated across the cytoplasmic membrane to the outer leaflet by the flippase enzyme MurJ or similar proteins (Reed et al., 2015). Once translocated, the disaccharide-pentapeptide units are polymerized into nascent PG chains by the glycosyltransferase activity of penicillin-binding proteins (PBPs) or other transglycosylases (Reed et al., 2015). The final stage involves the cross-linking of the PG chains, catalyzed by the transpeptidase activity of PBPs (Kuroda et al., 2003). This cross-linking is crucial for the mechanical strength of the PG network and is the target of β -lactam antibiotics, which inhibit the transpeptidase activity, leading to cell lysis (Fig. 1.2.1.2) (Kuroda et al., 2003).

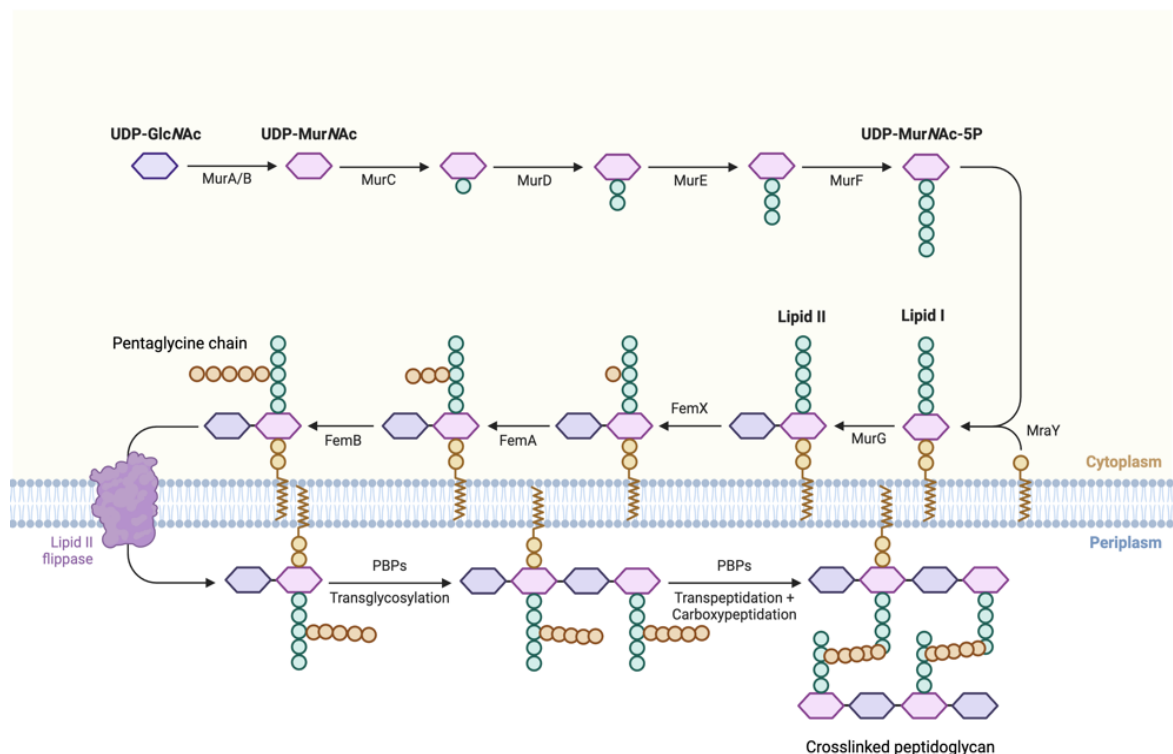


Figure 1.2.1.2. Peptidoglycan biosynthesis in *S. aureus* (Barreteau et al., 2008 and Lade & Kim, 2021).

The synthesis of PG is tightly regulated and coordinated with cell growth and division to maintain cell shape and integrity. Key regulatory proteins such as MreB, FtsZ, and the elongasome and divisome complexes play vital roles in orchestrating the spatial and temporal aspects of PG synthesis (Laddomada et al., 2016). MreB, an actin-like cytoskeletal protein, directs PG synthesis along the lateral walls of rod-shaped bacteria, ensuring uniform cell wall expansion. FtsZ, a tubulin-like protein, assembles into a ring at the future site of cell division and recruits other proteins to form the divisome complex, which coordinates PG synthesis at the division site (Laddomada et al., 2016).

PG is a key target for antibiotics, particularly those in the β -lactam and glycopeptide classes (Sauvage & Terrak, 2016). These antibiotics disrupt peptidoglycan biosynthesis by inhibiting specific enzymes in the pathway, leading to cell wall weakening and bacterial death (Wang et al., 2022). However, *S. aureus* has developed several mechanisms to resist these antibiotics, with peptidoglycan playing a central role in the resistance process (Pantosti et al., 2007). Primarily β -lactam antibiotics, such as penicillin, methicillin, and cephalosporins, target PBPs and inhibit their transpeptidase activity (Poole, 2004). By blocking the cross-linking of peptidoglycan strands, β -lactams prevent the formation of a stable cell wall, leading to bacterial lysis. However, *S. aureus* has developed several strategies to evade this mechanism of action, as previously discussed, including the production of β -lactamases and the expression of PBP2a (Poole, 2004).

Additionally, glycopeptide antibiotics, such as vancomycin, target the D-Ala-D-Ala dipeptide in the peptidoglycan precursor, blocking transglycosylation and transpeptidation. This prevents the incorporation of lipid II into the growing peptidoglycan layer, leading to a weakened cell wall (Kahne et al., 2005). At the time of its initial discovery, *S. aureus* was highly susceptible

to vancomycin, but the emergence of vancomycin-intermediate *Staphylococcus aureus* (VISA) and vancomycin-resistant *Staphylococcus aureus* (VRSA) strains has reduced its efficacy (Howden et al., 2010).

VISA strains exhibit increased cell wall thickness, which acts as a barrier to vancomycin penetration (Howden et al., 2010). This thickened peptidoglycan layer traps vancomycin molecules, reducing their ability to reach their target (Van Bambeke et al., 2008). Although VISA strains do not acquire the *vanA* or *vanB* genes that confer high-level resistance in enterococci, their altered cell wall architecture enables them to survive vancomycin treatment (Howden et al., 2010). In contrast, VRSA strains acquire the *vanA* gene, which modifies the D-Ala-D-Ala dipeptide to D-Ala-D-Lac, preventing vancomycin binding and conferring high-level resistance (Périchon & Courvalin, 2009).

Moreover, in addition to producing alternative PBPs and thickened cell walls, *S. aureus* can modify its peptidoglycan structure to enhance resistance (Wang et al., 2022). These modifications can also impact the immune recognition of *S. aureus*, helping the bacterium evade host defenses and persist during infection (Wang et al., 2022).

1.2.2 Penicillin-binding proteins and their role in peptidoglycan synthesis

PBPs are the primary targets for β -lactam antibiotics and play a crucial role in bacterial PG (PG) synthesis and modification (Macheboeuf et al., 2006). These proteins were initially named for their ability to bind penicillin, though they are sensitive to a broad spectrum of β -lactam antibiotics (Kelly & Kuzin, 1995). PBPs are characterized as enzymes involved in PG biosynthesis, exhibiting a wide range of molecular weights and functional diversity. They are implicated in several critical processes, including antibiotic resistance and cell division

(Macheboeuf et al., 2006). The number, size, and β -lactam affinity of PBPs can vary significantly between different bacterial species (Rhazi et al., 2003).

The identification of PBPs across different bacterial species has been facilitated by assigning specific numbers to each PBP based on their migration in SDS-PAGE gels (Sauvage et al., 2008). Although the assigned numbers may vary between species, PBPs with similar functions often exhibit comparable behaviours. For instance, PBP2 in *S. aureus*, a class A PBP, functions similarly to PBP1a in *Escherichia coli* (Fonzeé et al., 1999). Studies have shown that *E. coli* PBPs, such as PBP2 and PBP3, have functions analogous to PBP3 and PBP1 in *S. aureus*, respectively (Sauvage et al., 2008).

Researchers have long prioritized understanding the biosynthesis of PG, with particular attention to the specific roles of PBPs in this process. The activity of certain PBPs increases during cell division, prompting the assignment of distinct functions based on their roles (Goffin & Ghuysen, 2002). PBPs are categorized by their molecular mass into high molecular mass (HMM) and low molecular mass (LMM) PBPs. HMM-PBPs are further classified based on their catalytic capabilities, including transglycosylation (GT) and transpeptidation (TP) activities (Macheboeuf et al., 2006).

Class A PBPs exhibit both transglycosylase and transpeptidase activities. As transglycosylases, they polymerize N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) units to form the PG backbone (Goffin & Ghuysen, 2002). Additionally, as transpeptidases, these PBPs cross-link adjacent stem peptides, creating a mesh-like structure (Goffin & Ghuysen, 2002). Conversely, class B PBPs possess only transpeptidase activity (Macheboeuf et al., 2006).

The biosynthesis of PG involves various PBPs and Lipid II. The lipid carrier undecaprenyl phosphate (C55-P) transports Lipid II across the membrane (Manat et al., 2014). This lipid carrier undergoes conversion to undecaprenyl pyrophosphate during transport (Manat et al., 2014). Subsequently, undecaprenyl pyrophosphate is recycled back into the cell membrane for hydrolysis and reuse (Jukič et al., 2022).

After the transglycosylation step, the transpeptidation reaction takes place. Transpeptidation enzymes specifically recognize the terminal D-alanine-D-alanine sequence of the pentapeptide stem attached to MurNAc (Morlot et al., 2004). These enzymes catalyze the formation of a peptide bridge by linking the carbonyl group of the terminal D-alanine of one chain to the amino group of the third amino acid in a neighbouring chain, forming a '4-3' crosslink (Morlot et al., 2004). In addition to crosslinking, transpeptidases also possess carboxypeptidase activity, which enables them to remove the terminal D-Ala residue from the peptidoglycan precursor. This removal is a critical step that initiates the crosslinking process, ultimately reinforcing the bacterial cell wall (Fig. 1.2.2) (Izaki et al., 1968; Kozarich And & Strominger, 1978).

The function of PBPs and their acceptor molecules vary with molecular mass and species. For instance, in transpeptidation reactions catalyzed by HMM-PBPs, the acceptor residue is glycine in *S. aureus* and lysine in *S. pneumoniae* (Macheboeuf et al., 2006). LMM-PBPs, on the other hand, catalyze carboxypeptidation reactions by hydrolyzing the terminal D-Ala of the pentapeptide chain in PG, with water as the acceptor molecule (Morlot et al., 2004). Thus, LMM-PBPs play a role in the regulation of PG structure.

In *S. aureus*, several PBPs have distinct roles in peptidoglycan biosynthesis and antibiotic resistance. The four major PBPs found in *S. aureus* are PBP1, PBP2, PBP3, and PBP4, each

contributing differently to cell wall assembly (da Costa et al., 2018). PBP1 is a high-molecular-weight PBP with both transglycosylase and transpeptidase activities (Wacnik et al., 2022). It is responsible for the polymerization of glycan chains and the cross-linking of peptide side chains during cell wall synthesis. PBP1 is essential for bacterial growth and viability, and its inactivation by β -lactam antibiotics results in a compromised cell wall, leading to bacterial lysis (Wacnik et al., 2022). PBP2 is another high-molecular-weight PBP with dual transglycosylase and transpeptidase activities. In addition to its role in peptidoglycan synthesis, PBP2 is the primary target of methicillin and other β -lactam antibiotics (Llarrull et al., 2009). However, in MRSA strains, this function is taken over by PBP2a, with a low affinity for β -lactams, which allows the bacterium to survive despite the presence of these antibiotics (Llarrull et al., 2009).

Additionally, PBP3 is involved in the final stages of cell division and plays a critical role in septal peptidoglycan synthesis. It is a key player in the formation of the division septum, where new cell wall material is synthesized to separate daughter cells during bacterial replication (Egan et al., 2020). Although PBP3 is a target of β -lactam antibiotics, its role in resistance is less pronounced compared to PBP2a (Egan et al., 2020). Finally, PBP4 is an LMW PBP that primarily functions as a transpeptidase (Wang et al., 2022). It is involved in the maturation and remodelling of peptidoglycan, particularly in the formation of highly cross-linked peptidoglycan in the stationary phase of growth (Egan et al., 2020). While PBP4 is not essential for bacterial survival, it contributes to the structural integrity of the cell wall and may play a role in resistance by enhancing the stability of the peptidoglycan layer (Memmi et al., 2008).

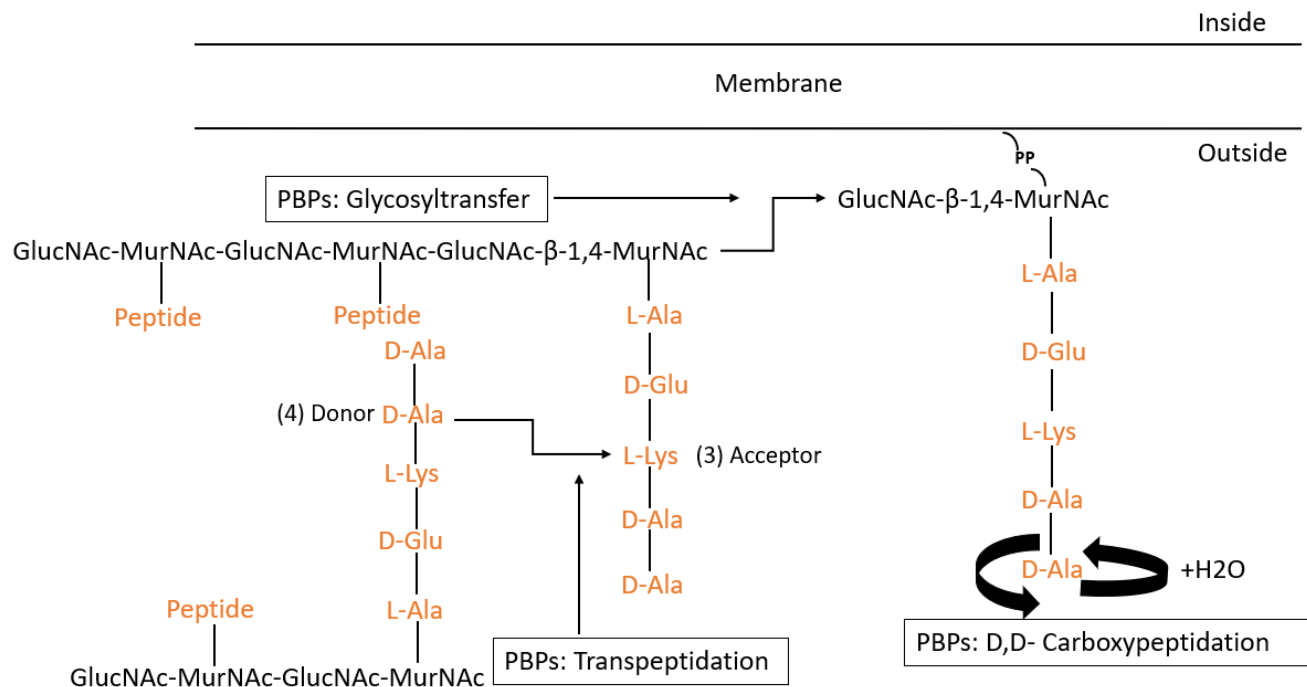


Figure 1.2.2. Schematic representation of the various functions performed by PBPs, aiding in the polymerization and crosslinking of the bacterial peptidoglycan (Macheboeuf et al., 2006)

Research demonstrates that PBPs are crucial participants in bacterial cell division and elongation, requiring precise localization at the cell division site (Errington et al., 2003; S. J. Foster & Popham, 2001; Giesbrecht et al., 1998). This involvement was substantiated through immunoprecipitation and immunofluorescence studies conducted on various organisms, including *E. coli*, *Bacillus subtilis*, *Caulobacter crescentus*, and *S. aureus* (S. J. Foster & Popham, 2001). The findings revealed the essential roles of at least one class A and one class B PBP in conjunction with other molecules such as lytic transglycosylases and PG scaffolding proteins (Sauvage & Terrak, 2016; Straume et al., 2021). Specifically, class B PBPs were implicated in PG elongation and integration into the cell wall, predominantly localized at the division site (Scheffers & Pinho, 2005; Tinajero-Trejo et al., 2022; Wacnik et al., 2022).

1.2.3 Wall teichoic acid

Amongst all the essential components of a Gram-positive bacterial cell wall, teichoic acids (TAs) are one of them (Brown et al., 2013). LTA and WTA are collectively known as teichoic acids, where the former is a polymer of glycerol-3-phosphate (poly GroP) attached to the cell membrane and the latter is found to be attached to the PG of the bacterial cell wall (Reichmann et al., 2013). WTAs are composed primarily of ribitol phosphate (poly RboP) repeats, which are linked together by phosphodiester bonds (Wu et al., 2021) (Fig. 1.2.3.1). Functionally, WTAs are associated with different roles, from cell shape maintenance, pathogenesis, morphogenesis, antibiotic resistance, biofilm formation, and autolysin activity to a probable role in bacterial PG biosynthesis regulation (Formstone et al., 2008).

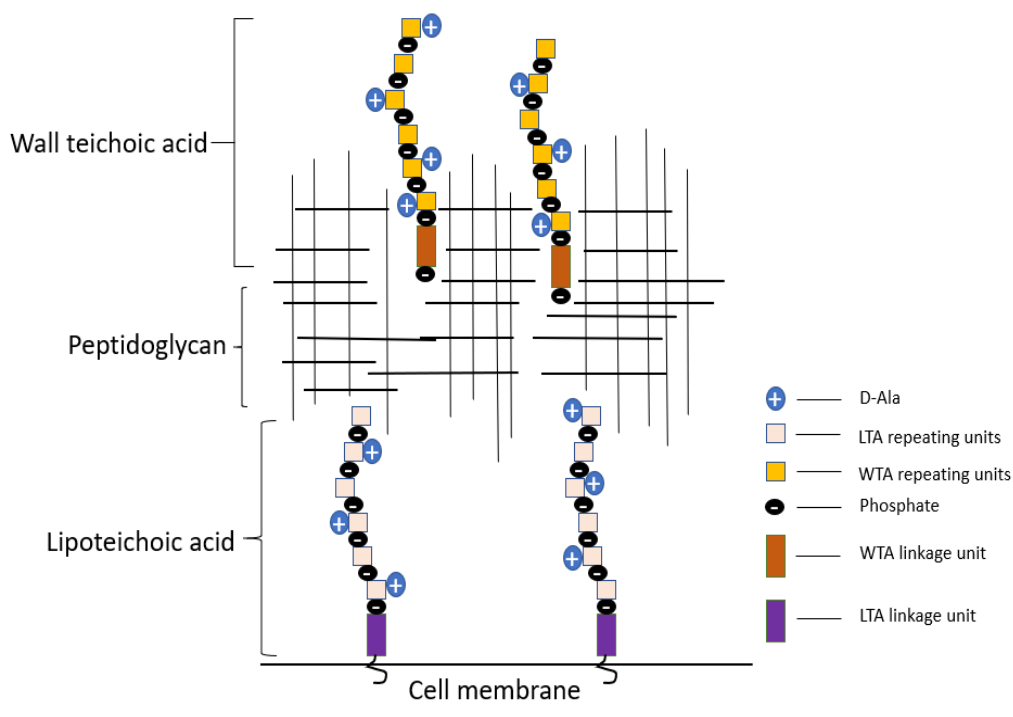


Figure 1.2.3.1. A schematic representation of Wall teichoic acid (WTA), Lipoteichoic acid (LTA) and peptidoglycan within a Gram-positive bacterial cell wall. The WTA is anchored to the peptidoglycan while LTA is attached to the cell membrane by their linkage units. (Brown et al., 2013)

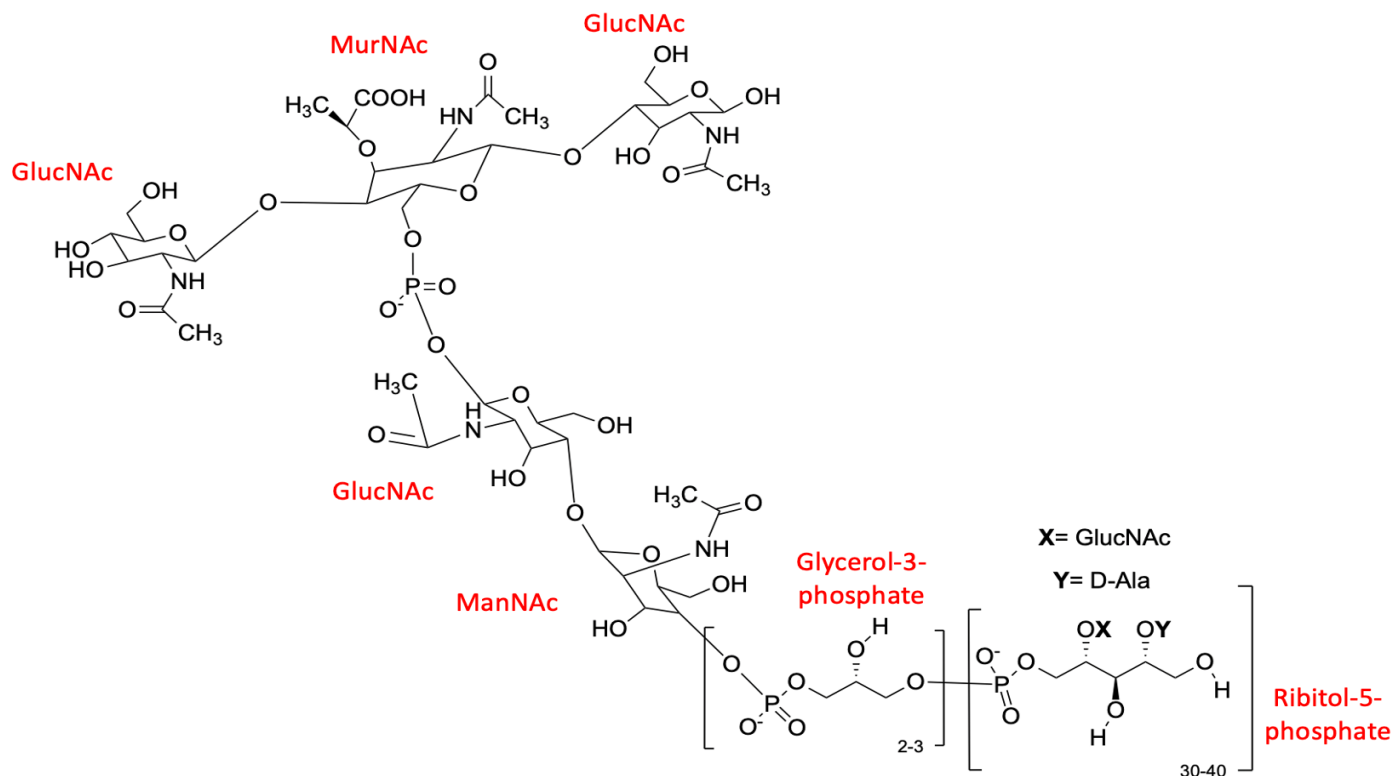


Figure 1.2.3.2. A schematic representation of the chemical structure of WTA (Ali et al., 2021)

Structurally, WTAs are RboP polymers bound to the bacterial PG covalently with the help of a conserved linkage unit containing disaccharides β -1,4-N-acetylglucosamine (GlucNAc), N-acetylmannosamin (ManNAc) and one to three molecules of GroP (Yokoyama et al., 1986). A possibility remains that the chemical structure of the backbone chain of WTA may vary, but the linkage unit remains highly conserved. As a key function, the linkage unit serves as the connector between the WTA and the MurNAc of the bacterial PG, which is present in abundance (Fig. 1.2.3.2) (Brown et al., 2013). The genes responsible for WTA synthesis were first discovered in the bacterial strain *Bacillus subtilis* 168 and were named *tag* genes due to their involvement in polymerizing GroP units (Bhavsar et al., 2005). Based on sequence homology, similar genes were identified in *S. aureus* (Ginsberg et al., 2006). In *S. aureus*, the genes were named *tar* genes based on their role in RboP polymerization (Ginsberg et al., 2006). In *S. aureus*, the synthesis of WTAs

takes place on the cytoplasmic region of the cell membrane, and this sequential mechanism requires the involvement of a carrier molecule called undecaprenyl-phosphate (C55-P) (Qian et al., 2006). The proposed mechanism of WTA biosynthesis requires the enzymes TarO, TarA and TarB (TagOAB in *B. subtilis*) to work in harmony where the first GlucNAc molecule is transferred from a UDP-activated sugar molecule to the C55-P carrier molecule by the enzyme TarO (Xia et al., 2010). The intermediate product is known as lipid- α , which is then converted to lipid- β by the addition of a ManNAc, which is made available to lipid- α by a UDP-ManNAc donor molecule where the reaction is mediated by the TarA enzyme (Fig.1.2.3.3) (Schertzer & Brown, 2003). Then the complex lipid- β is further modified when one GroP molecule is attached to it. (Lazarevic et al., 2002). This step in the process is catalyzed by the enzyme TarB which utilizes a CDP-glycerol substrate as a source of GroP, produced by the enzyme TarD (Ginsberg et al., 2006; Zhang et al., 2006). In a later step, two more glycerol is added to the complex in the presence of the enzyme TarF, and finally, 30-40 repeating units of RboP are attached to the complex, with RboP being sourced from the precursor CDP-ribitol. This process occurs in the presence of the TarL/TarK (Fig.1.2.3.3) (Qian et al., 2006).

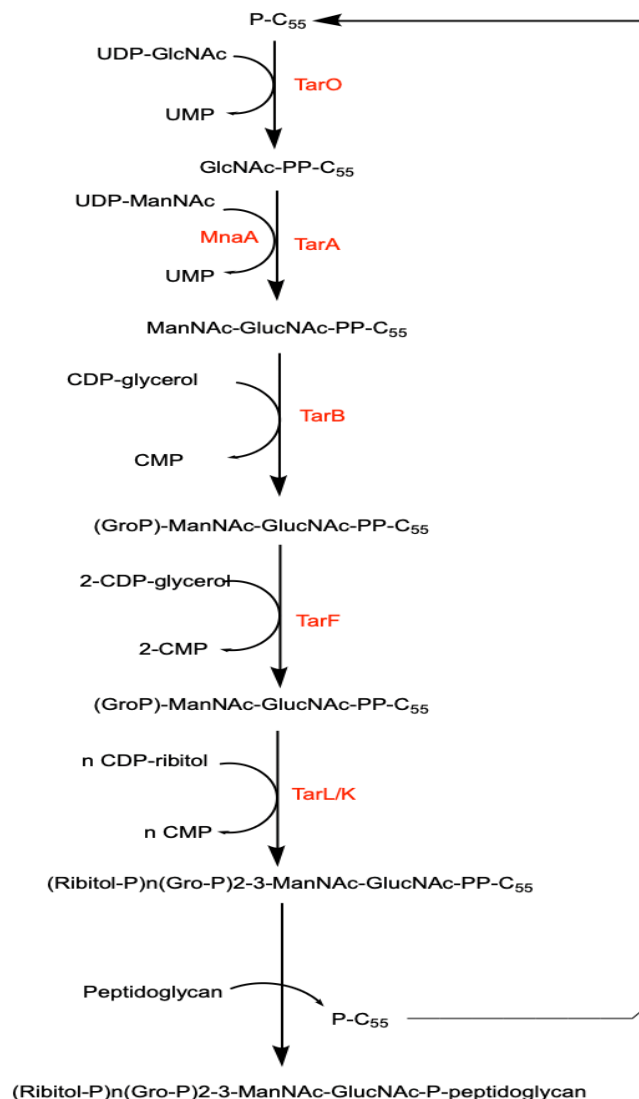


Figure 1.2.3.3. Schematic representation of the steps involved in *S. aureus* WTA biosynthesis where a TarO catalysed GlcNAc attaches to the MurNAc repeating units of the bacterial peptidoglycan. The reaction progresses by adding a ManNAc (catalyzed by TarA) to the already available GlcNAc molecule followed by the addition of $n = 1-2$ glycerol-3-phosphate molecule catalyzed by TarB and TarF enzyme and finally 30-40 ribitol-5-phosphate molecule catalyzed by TarL/K. (Adapted from Brown et al., 2013 and Navarre & Schneewind, 1999)

In *S. aureus*, the WTA biosynthesis process is initiated by the enzyme TarO. Evidence of the proposed pathway was provided by deleting the gene *tarO* from the *S. aureus* SA113 strain which was eventually found to lack WTA (Weidenmaier et al., 2004). In *S. aureus*, TarF specifically appends two glycerol phosphate (GroP) units to the linker molecule, playing a critical

role in cell WTA assembly (Ginsberg et al., 2006) and further two additional gene products, TarK and TarL, were identified. TarK appears to function as a RboP primase - an essential enzyme in DNA replication (Ginsberg et al., 2006). Primase synthesizes short RNA primers, typically 10-12 nucleotides long, that serve as starting points for DNA polymerases to initiate new strand synthesis (Guilliam et al., 2015). This step initiates the polymerization of ribitol phosphate chains for WTA synthesis, adding one to two RboPs to the backbone of WTA, after which the process is controlled by the polymerase TarL. The purpose of this enzyme was determined to catalyze the reaction of attaching multiple RboP units to form the complete WTA polymer (D'Elia et al., 2006; Lazarevic et al., 2002; Qian et al., 2006).

The proposed pathway generated a few questions regarding the distinct purpose of the identified gene products TarK and TarL due to their nearly similar sequence homology. Qian et al. (2006) by means to answer these questions performed genomic analysis and proposed that in *S. aureus* there may not be a distinct primase or polymerase, but both the TarK and TarL might act as a polymerase depending on the strain. Questions were also raised regarding the essentiality of the gene products involved in WTA biosynthesis and their relation to cell viability, as it was previously demonstrated that viable cells do exist without WTA where *tarO* is tampered with (Weidenmaier et al., 2004). To answer these questions Brown et al. (2013) performed *in vitro* reconstitution experiments and identified that the genes *tarA*, *tarB*, *tarL*, *tarF*, positioned downstream to *tarO* are essential in producing polyGroP or polyRboP WTAs. Further, they highlighted the essentiality of the enzymes TarD, TarI, TarJ as well which function to supply the CDP-glycerol or CDP-ribitol substrates for complete WTA biosynthesis (Badurina et al., 2003; Pereira & Brown, 2004).

PolyRboP residues in WTA are post-synthetically modified by the attachment of external D-alanine molecules (Neuhaus & Baddiley, 2003), where Lipoteichoic acids (LTAs) serve as the primary source of D-Ala for WTA (Wu et al., 2021). This modification plays a key role in modulating the biological activity of WTA (Fischer et al., 1981). The role of WTA in regulating autolytic activity within the cell wall is directly associated with its levels of D-alanylation. D-alanine residues, when lost, can be repeatedly incorporated into WTA, and this modification is crucial for controlling autolysin activity and maintaining cell wall integrity (Koprivnjak et al., 2006). A few experiments aimed at demonstrating the purpose of D-alanylation of WTAs found that WTA devoid of D-alanine shows susceptibility towards different cationic antimicrobial peptides including nisin and defensin (Lambert et al., 1975). Also, a faster killing of the bacterial cells by phagocytic cells and neutrophils was demonstrated when post-synthesis D-alanine modification was absent (Collins et al., 2002). Functionally, *S. aureus* strains lacking D-alanine modification tend to show disruption of colonization glass or polystyrene surfaces and a reduced adherence to nasal epithelial cells was also observed (Kristian et al., 2005). A similar result was documented in other Gram-positive organisms as well (Peschel et al., 1999; Poyart et al., 2003).

Further investigation into the D-alanylation process of WTA revealed the involvement of the conserved *dltABCD* operon in *B. subtilis* 168. In *S. aureus*, however, this gene cluster comprises five genes, specifically *dltXABCD*, which regulate D-alanylation (Koch et al., 1985; Neuhaus & Baddiley, 2003). Further analysis to determine the essentiality of the genes shows only the proteins encoded by the *dltABCD* operon were required to modify WTA in *B. subtilis* (Koprivnjak et al., 2006). The primary proteins encoded by the *dlt* operon are DltA, DltB, DltC and DltD amongst which only the proteins DltA and DltC have been assigned to a specific function (Koprivnjak et al., 2006; Perego et al., 1995). The DltA acts as a D-alanine-D-alanyl carrier protein

ligase where the DltC molecule has been tagged as a carrier protein for D-Ala (Fischer, 1994). Functions of the DltB protein remain elusive like DltD although the former was found to be a multi-domain spanning protein (Heatont & Neuhaus, 1994) while DltD was determined to be fixed to the membrane by a hydrophobic sequence (Heatont & Neuhaus, 1994). Numerous researchers have experimented to determine the specific functions of these proteins. To shed light on the process to date, two models have been proposed by two different groups where the first model is known as the Fischer model. Fischer and group hypothesized that the process of D-alanine modification is mediated by a lipid-linked undecaprenyl phosphate (C55-P) where the said process is initiated by DltA by ligating D-alanines to DltC (Fischer, 1994). DltB was then proposed to mediate the transfer of D-Ala through the membrane where the undecaprenyl phosphate acts as a carrier molecule (Perego et al., 1995). Finally, the process of D-alanylation of LTA is catalysed by the enzyme DltD which typically takes place outside of the cell membrane (Fig.1.2.3.4) (Perego et al., 1995). The only argument with their proposed model was that there is no experimental proof regarding the involvement of the carrier C55-P molecule. Based on the experiments done by Debabov et al., on *Lactobacillus rhamnosus* Neuhaus and Baddeley proposed a second model highlighting the mechanism of D-alanylation of WTA. According to their model, DltD catalyzes the D-Ala transfer between DltA and DltC by acting as a platform to bring them together where experimentally it was proven that in the presence of DltD, the reaction increases by two folds (Debabov et al., 1996). Unlike the Fischer model, this pathway does not agree on the involvement of the C55-P carrier molecule but proposes the DltC to be the carrier itself. The model recommends that the DltC molecule travels through the membrane by a channel formed by DltB to supply D-Alas to LTA (Fig.1.2.3.4) (Neuhaus & Baddiley, 2003).

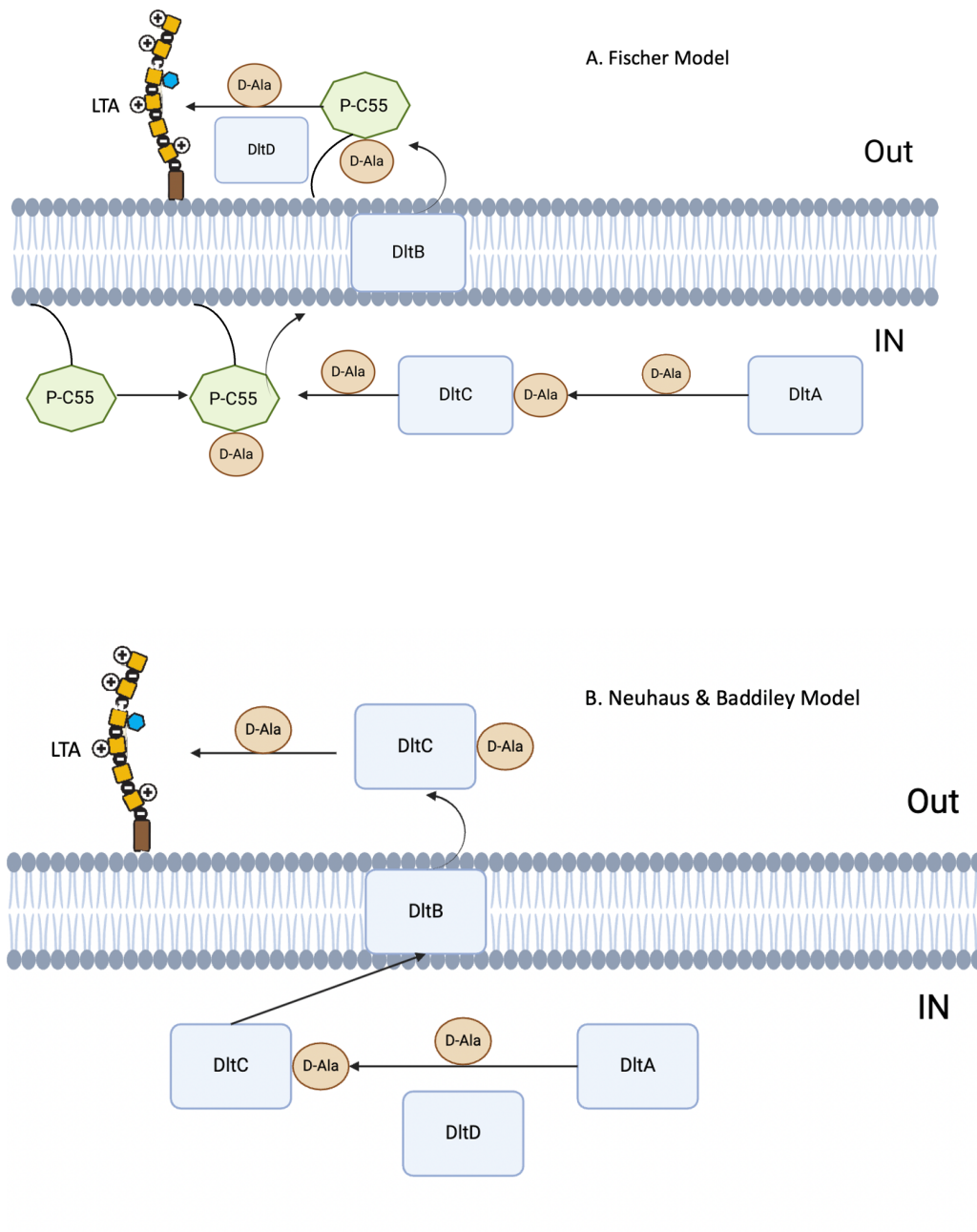


Figure 1.2.3.4. Schematic representation of the two different models proposed regarding the process of D-alanine modification post-teichoic acid synthesis. A) Fisher model, B) Neuhaus and Baddiley model. (Adapted and modified from (Fischer, 1994; Neuhaus & Baddiley, 2003; Wörmann et al., 2011))

Soon after the discovery of WTA by (J Armstrong et al., 1955) attention was drawn towards it due to its wide range of functions but to date, its biosynthesis process is not well understood. By investigation, it was determined that the genes participating in WTA biosyntheses such as *tarO*,

tarAHGBXD, *tarI'J'KFIJL*, *mnaA* or the genes responsible for D-Ala modification namely *dltXABCD*, *tarM*, *tarS*, *tarp* and the genes involved in N-acetyl glycosylation are distributed throughout the genome (Winstel, Sanchez-Carballo, et al., 2014). The answer to the question of how all these genes work in sync despite being well distributed to produce WTA is still elusive. It was then identified that between species, the chain length of WTA varies along with the amount present (Swoboda et al., 2010). Only a limited number of experiments have provided insight into the underlying mechanisms (Swoboda et al., 2010). As previously mentioned, the chain length of WTA varies depending on whether the RboP polymerase TarK or TarL is involved in its synthesis. Experimentally it was determined that WTAs that are produced in the presence of TarK (K-WTA) are nearly 50% shorter than the WTAs found in the presence of TarL (L-WTA) (Swoboda et al., 2010). Regulation of K/L-WTA was found to be under the influence of *agr* quorum-sensing system. It was demonstrated that under low *agr* activity both K-WTA and L-WTA are produced. Although at a higher *agr* activity, it was found that L-WTA production is dominant over K-WTA (Meredith et al., 2008). Additionally, a study focused on strain-specific *agr* activity highlights its role in WTA chain length determination and significance in virulence. According to the study, a higher *agr* activity is observed in a virulent strain where the WTA chain length was influenced by TarL (Wanner et al., 2017). The involvement of a *tarH* gene was also determined from its higher expression in virulent strains which codes for a TarG/H transporter. The transporter is proposed to carry WTA precursors from the cytoplasm to the extracellular space (Wanner et al., 2017). From multiple investigations, it was postulated that the process of transport consists of a rate-limiting step (Brown et al., 2013; Campbell et al., 2012; Swoboda et al., 2009). A repressor *rot* was identified to be influencing the promoter of *tarH* by binding directly to it reducing expression (Wanner et al., 2017).

During the 1960s, it was experimentally proven that WTAs are non-essential in *in vitro* cell survival (Swoboda et al., 2009). This study influenced future investigators where their primary focus remained on identifying the pattern of dispensability of the genes involved in WTA synthesis (Aly et al., 1980; Bracha et al., 1974). These studies highlighted the presence of several other genetically uncharacterized WTA null variants (Weidenmaier et al., 2004). The first gene involved in WTA synthesis *tarO* was deleted and its influence was characterized by a group of researchers led by Peschel. It was reported in their study that although the $\Delta tarO$ strain remains viable, its functions are highly impaired such as their ability to bind to epithelial or endothelial tissues (Weidenmaier et al., 2004). The significance of this research was enhanced by the identification of the role of WTA in host infection and pathogenesis. Such assumptions were made as $\Delta tarO$ strains were unable to colonise nasal passage due to adhesion impairment which is a key function of a virulent strain (Weidenmaier et al., 2004). In another research, led by Brown and his co-workers (2013), found that similar to *tarO*, *tarA* can also be deleted. Their research highlighted the phenotypical similarity found between the *tarO* and the *tarA* deleted strains (Brown et al., 2013). Interestingly, they found that the genes downstream to *tarO*, *tarA* i.e. *tarF* and *tarL* can not be deleted if initially *tarO* or *tarA* is not deleted (Sewell & Brown, 2014). Such downstream genes were termed as “conditionally essential” and further investigation on this topic exposed the later stage genes responsible for WTA biosynthesis such as *tarI*, *tarJ*, *tarD* to be more involved in cell viability than the initial ones (D’Elia et al., 2006). In a separate study by Bhavsar et al. in 2005, it was demonstrated that the deletion of the *tagD* gene resulted in impaired growth and increased temperature sensitivity.

WTAs are thought to serve as scaffolds that organize and direct PBPs to specific locations within the cell wall (Brown et al., 2013; Winstel, et al., 2014). For instance, WTAs contribute to

the localization of PBP2a in *S. aureus*, particularly in the septum during cell division (Qamar & Golemi-Kotra, 2012). The mis-localization of PBPs due to WTA disruption can lead to defective cell wall synthesis, abnormal cell morphology, and a weakened cell wall structure, leading to a reduction in β -lactam resistance (Fisher & Mobashery, 2021). This, in turn, can affect bacterial survival (Fisher & Mobashery, 2021). The negative charge of WTAs also influences the interaction between PBPs and other cell wall synthesis machinery (Brown et al., 2013), where WTAs interact electrostatically with cationic components, including PBPs, which influence their spatial distribution and activity (Caudill et al., 2020). For example, alterations in WTA D-alanylation (which reduces the negative charge) can impair the binding of PBPs to the cell wall, affecting peptidoglycan synthesis and weakening the cell wall structure (Winstel, et al., 2014). This can lead to increased susceptibility to antibiotics, especially those targeting cell wall synthesis (Winstel, et al., 2014).

AtlA is the primary autolysin in *S. aureus*, responsible for cleaving peptidoglycan during cell division and promoting autolysis under certain conditions (Biswas et al., 2006). AtlA degrades the bacterial cell wall, facilitating cell separation during division and playing a role in the regulation of cell wall turnover (Biswas et al., 2006). Proper localization and regulation of autolysins are essential for maintaining cell wall integrity and avoiding uncontrolled autolysis, which would be lethal for the bacterium (Wang et al., 2022). WTAs play an important role in the localization of AtlA to specific regions of the bacterial cell, particularly the septum, where cell division occurs (Poupel et al., 2018). By guiding the precise placement of AtlA, WTAs ensure that cell wall degradation occurs in a controlled manner during cell division (Poupel et al., 2018). Disruption of WTA biosynthesis or structure can delocalize AtlA, leading to impaired cell division or excessive autolysis (Bibek et al., 2019).

The regulation of autolysis is closely linked to antibiotic susceptibility (Antignac et al., 2007). Many antibiotics, such as β -lactams, not only inhibit peptidoglycan synthesis but also induce autolysis by stimulating autolysins like AtlA (Antignac et al., 2007). Proper localization of AtlA by WTAs is crucial for balancing the effects of autolysis during antibiotic treatment (Bibek et al., 2019). Disruption of WTAs can lead to either enhanced susceptibility to antibiotics by promoting cell lysis or, conversely, contribute to resistance by reducing the bacterium's ability to undergo autolysis (Bibek et al., 2019). WTAs also modulate the activity of autolysins through their charge properties. The anionic nature of WTAs helps regulate the binding of autolysins to the cell wall (Wu et al., 2021). Alterations in WTA D-alanylation or glycosylation can affect how tightly autolysins like AtlA bind to peptidoglycan, influencing their activity (Wu et al., 2021). In MRSA strains, where autolysin regulation is critical for survival under antibiotic pressure, WTAs play a key role in balancing autolytic activity to prevent uncontrolled cell wall degradation (Bibek et al., 2019).

Beyond PBPs and AtlA, WTAs are also involved in the spatial regulation of various other enzymes critical for cell wall maintenance and antibiotic resistance. These include enzymes involved in peptidoglycan remodelling, cell wall hydrolysis, and resistance mechanisms (Wang et al., 2022). Enzymes involved in the early stages of peptidoglycan precursor synthesis, such as MurA and MurB, may also be influenced by the presence and composition of WTAs (Bibek et al., 2019). Irregular WTAs within the cell wall could affect the availability of precursors and the efficiency of peptidoglycan synthesis, particularly during cell division or in response to antibiotic stress (Wang et al., 2022). *S. aureus* also expresses a range of cell wall-modifying enzymes, including N-acetylglucosaminidases and endopeptidases, which are essential for maintaining the dynamic nature of the bacterial cell wall (Wang et al., 2022). These enzymes allow the bacterium

to remodel its cell wall in response to environmental changes, including antibiotic exposure. WTAs play a role in the proper localization and regulation of these enzymes, ensuring that cell wall remodelling occurs in a controlled manner to prevent cell death from excessive wall degradation (Wang et al., 2022).

In conclusion, it can be stated that, although the precise function of WTA in bacterial physiology remains elusive, all the studies focusing on WTA and its functions highlight their significant role in the virulence and host-invasive nature of a Gram-positive bacterium. It is also suggested that WTA might take over the role of an outer membrane which in general Gram-positive organisms lack and be involved in mediating extracellular interactions and controlling the membrane permeability (Swoboda et al., 2010). Other functions like controlling the activity of PG degrading enzymes such as N-acetylmuramidases, N-acetylmuramyl-L-alanine amidases, etc. (Atilano et al., 2010; Schlag et al., 2010) and controlling the cell shape and diameter (Rice & Bayles, 2008) were also assigned to WTA. Most importantly their roles in evading killing by antimicrobial peptides and biofilm formation (T. Kohler et al., 2009; O’Gara, 2007) are of high significance as these directly link to its influence on the pathogenicity of the strain.

1.3 Biofilms formed by *S. aureus*

One of *S. aureus*’s key virulence factors is its ability to form biofilms, a complex aggregation of microorganisms growing on a surface, embedded within a self-produced matrix of extracellular polymeric substances (EPS) (Liu et al., 2021). These biofilms pose significant clinical challenges due to their increased resistance to antibiotics and the host immune system, contributing to persistent and chronic infections (A. Singh et al., 2022). In *S. aureus*, biofilms are a critical factor in its pathogenicity and persistence (A. M. S. Figueiredo et al., 2017). Biofilms can form on

various surfaces, including medical devices, tissues, and host cells, contributing to a range of infections (A. Singh et al., 2022).

The biofilm matrix, composed of extracellular DNA (eDNA), proteins, polysaccharides, and lipids, provides both structural stability and a protective environment for bacteria (Campoccia et al., 2021). This complex architecture includes microcolonies, which are clusters of cells encased within the matrix and form the fundamental structural units of the biofilm (Schilcher & Horswill, 2020). Interspersed among these microcolonies are water channels that facilitate the transport of nutrients and waste, allowing the biofilm to maintain metabolic activity (Davey & O'toole, 2000). Additionally, surface adherence is crucial for biofilm development, often mediated by adhesins and surface proteins, enabling the initial attachment to a surface (Santore, 2022).

Biofilms exhibit several distinctive properties. Firstly, biofilm-associated bacteria demonstrate significantly increased resistance to antibiotics compared to their planktonic (free-living) counterparts (Lopes et al., 2021). This resistance arises from multiple factors, including limited antibiotic penetration through the matrix, altered microenvironments within the biofilm, and the presence of persister cells (Uruén et al., 2020). Secondly, biofilms can evade the host immune system, leading to chronic and recurrent infections (Arciola et al., 2018). The biofilm matrix facilitates this evasion by acting as a barrier to immune cells and antibodies (Hanke & Kielian, 2012).

The formation of *S aureus* biofilms is a dynamic and multi-step process involving initial attachment, microcolony formation, maturation, and dispersion, each regulated by a complex interplay of genetic and environmental factors (Alotaibi, 2021). Initially, biofilm formation begins with the attachment of planktonic bacteria to a surface (Alotaibi, 2021). This initial adherence is mediated by surface proteins and adhesins, such as microbial surface components recognizing

adhesive matrix molecules (MSCRAMMs) (T. J. Foster et al., 2014). Factors influencing this stage include surface characteristics like hydrophobicity, roughness, and chemical composition, as well as environmental conditions such as nutrient availability, pH, temperature, and the presence of shear forces (Krsmanovic et al., 2021). Once attached, the bacteria begin to proliferate and form microcolonies (Schilcher & Horswill, 2020). This stage involves cell-cell adhesion through cell surface proteins and intercellular adhesins, the production of extracellular polymeric substances (EPS) forming the scaffold of the biofilm matrix, and quorum sensing, where communication between bacterial cells through signalling molecules (e.g., autoinducing peptides) coordinates gene expression related to biofilm formation (Lopes et al., 2021). As the biofilm matures, it develops into a three-dimensional structure with distinct microenvironments. Key aspects of this stage include matrix development, where the EPS matrix becomes more complex and robust, incorporating eDNA, proteins, and lipids; the formation of nutrient gradients within the biofilm, leading to metabolic heterogeneity among the bacteria; and differentiation, where bacteria differentiate into distinct phenotypic states, including persister cells that are highly resistant to antibiotics (Sauer et al., 2022; Uruén et al., 2020). The final stage of biofilm development is dispersion, where bacteria are released from the biofilm to colonize new sites. This process can be triggered by environmental cues, nutrient depletion, or the activation of dispersal genes. Dispersion plays a crucial role in the spread of infections and biofilm recalcitrance (Guilhen et al., 2017).

The ability of *S. aureus* to form biofilms has profound implications for both clinical settings and public health (Idrees et al., 2021). The significance of these biofilms lies in their impact on infection persistence, treatment challenges, and their association with medical device-related infections (Di Domenico et al., 2022). Biofilm-associated infections are often chronic and difficult to eradicate, primarily due to biofilms' contribution to antibiotic resistance through multiple

mechanisms, including physical barriers, metabolic dormancy, efflux pumps, and genetic exchange (Lebeaux et al., 2014). Infections associated with medical devices, such as catheters, prosthetic joints, and cardiovascular implants, are particularly concerning (Di Domenico et al., 2022). These infections are challenging to treat and often necessitate device removal (Di Domenico et al., 2022). Key factors contributing to these challenges include surface colonization, where medical devices provide a surface for initial bacterial attachment and biofilm formation; resistance to sterilization, as biofilms can withstand standard sterilization procedures, making device-related infections difficult to prevent; and persistent infections, which often continue despite aggressive antibiotic therapy, requiring device removal and replacement (Di Domenico et al., 2022; Lebeaux et al., 2014). Furthermore, biofilms enable *S. aureus* to evade the host immune system through various mechanisms (Ricciardi et al., 2018). The biofilm matrix protects bacteria from phagocytosis and the action of antibodies, biofilm-associated bacteria can modulate host immune responses, leading to chronic inflammation and tissue damage, and *S. aureus* can invade and survive within host cells, using them as a reservoir for persistent infection (Gil et al., 2014). These capabilities make biofilm-associated *staph* infections particularly resilient and difficult to manage (Gil et al., 2014).

Teichoic acids, particularly WTAs, play a crucial role in the initial adhesion of *S. aureus* cells to surfaces, especially abiotic ones such as medical implants (Berry et al., 2022). The negative charge of teichoic acids allows *S. aureus* to interact with positively charged or hydrophobic surfaces (Jeong et al., 2023). WTAs can reduce the repulsive electrostatic forces between the negatively charged bacterial cell wall and the surface, thereby facilitating adhesion (Jeong et al., 2023). The decoration of WTAs with D-alanine residues, which modulates their charge, further fine-tunes this interaction, allowing the bacterium to adhere to different surfaces under various

environmental conditions (Berry et al., 2022). Several surface-associated proteins, including MSCRAMMs, also interact with teichoic acids to promote initial adhesion (T. J. Foster, 2017). For instance, fibronectin-binding proteins (FnBPs) and clumping factors (ClfA and ClfB) have been shown to interact with teichoic acids, facilitating the attachment of *S. aureus* to host tissues and medical devices (Palmqvist et al., 2005). WTAs act as scaffolds that display these adhesion molecules on the bacterial surface, thus enhancing the bacterium's ability to adhere to both biotic and abiotic surfaces (T. J. Foster, 2016). WTAs and LTAs are also crucial for mediating the interactions between cells during the aggregation process of biofilm formation following the adhesion phase (Vergara-Irigaray et al., 2008). Moreover, eDNA plays an essential role in stabilizing biofilms, and WTAs contribute to this process by binding to eDNA and anchoring it within the biofilm matrix (Schilcher & Horswill, 2020). This interaction enhances the structural integrity of the biofilm and provides an additional layer of protection against antibiotics and immune system attacks. The role of WTAs in eDNA binding is particularly important in biofilms formed on medical devices, where eDNA is abundant and contributes significantly to the robustness of the biofilm (Schilcher & Horswill, 2020).

AtlA plays a dual role in biofilm formation, facilitating both cell separation during division and promoting intercellular adhesion (Peng et al., 2023). During biofilm development, AtlA mediates adhesion between cells by binding to WTAs on neighbouring cells, with WTAs acting as a substrate for this process (Tomlinson et al., 2021). This interaction enables the aggregation of cells into microcolonies (Tomlinson et al., 2021). The dual functionality of AtlA, supported by WTAs, underscores the critical role of teichoic acids in regulating both cell division and biofilm formation (Schilcher & Horswill, 2020). Additionally, the *piaA* gene encodes a protein involved in WTA biosynthesis, and mutations in this gene result in defects in biofilm formation (S. Kohler

et al., 2016). This suggests that WTAs not only contribute to initial adhesion but are also essential for maintaining the structural integrity of biofilms (Schilcher & Horswill, 2020). Proper WTA synthesis is required for the production of a robust biofilm matrix, which protects *S. aureus* from environmental stresses and antibiotics (Tomlinson et al., 2021).

WTAs also interact with polysaccharides, such as PNAG, to create a highly structured matrix. PNAG is synthesized by the products of the *ica* operon, and its production is critical for the maturation and stability of biofilms (O’Gara, 2007). WTAs can bind to PNAG, helping to retain this polysaccharide within the biofilm matrix and enhancing the cohesion between bacterial cells (Vergara-Irigaray et al., 2008). This interaction is particularly important in biofilm-associated infections, where the matrix serves as a physical barrier against antibiotics and the host immune response (Vergara-Irigaray et al., 2008). Also, several two-component systems (TCSs), such as WalKR and GraSR, are involved in the regulation of WTA synthesis and modification in response to environmental cues (Keinhörster et al., 2019). These TCSs sense changes in the environment and modulate the expression of genes involved in WTA biosynthesis. For instance, WalKR has been shown to regulate the expression of *tagA*, which encodes a key enzyme in WTA biosynthesis (Dubrac et al., 2007). Through this regulation, *S. aureus* can adjust its WTA composition to promote biofilm formation under different environmental conditions (Dubrac et al., 2007). WTA D-alanylation and glycosylation which influence their charge and binding properties play a critical role in determining the structural properties of the biofilm matrix (Bucher et al., 2015). For example, D-alanylation reduces the negative charge of WTAs, which may affect the binding of cationic antimicrobial peptides (CAMPs) and antibiotics (Wu et al., 2021). This modification can also influence the interaction between WTAs and other components of the biofilm matrix, such as eDNA and polysaccharides, thereby affecting biofilm stability and resilience (Wu et al., 2021).

1.4 An introduction to FmtA

Active site serine enzymes, often referred to as Penicillin-Recognizing Proteins (PRPs), that encompass both PBPs and β -lactamases, are characterized by a conserved serine residue in their active sites (Peitsaro et al., 2008). This serine is integral to the catalytic mechanism, executing a nucleophilic attack on the β -lactam ring of antibiotics, resulting in the formation of an acyl-enzyme intermediate (Fink, 1985). The precise location and chemical milieu of this serine are important for enzymatic activity, with other conserved residues such as lysine playing crucial roles in proton transfer and transition state stabilization (Peitsaro et al., 2008).

The recognition of penicillin by these enzymes is predicated on their ability to interact with the antibiotic's β -lactam ring and thiazolidine ring (González-Durruthy et al., 2021). Structural studies have demonstrated that the active site accommodates the β -lactam ring, aligning it for nucleophilic attack by the serine residue (Stojanoski et al., 2016). Key residues in the binding pocket, such as tyrosine and asparagine, engage the β -lactam ring through hydrogen bonding and van der Waals forces, stabilizing the antibiotic in a reactive conformation (Doucet & Pelletier, 2007).

The discovery of penicillin recognizing active site serine protein, FmtA dates back to 1997 when Komatsuzawa et al. first identified the protein. Subsequent studies have elucidated its significant role in antibiotic resistance. MRSA strains with a deleted *fmtA* gene ($\Delta fmtA$) exhibit increased sensitivity to detergents and β -lactam antibiotics, highlighting the importance of FmtA in the resistance mechanisms of these bacteria (Komatsuzawa et al., 1997). The deletion of *fmtA* also results in reduced methicillin resistance, further underscoring its role in counteracting the effects of this antibiotic. These observations suggest that FmtA is a critical component in the

defence arsenal of *S. aureus*, enabling it to withstand the onslaught of antibiotic treatments (Komatsuzawa et al., 1999).

Further research has shown that the expression of *fmtA* is upregulated in response to cell wall inhibitors such as oxacillin and methicillin (Bernal et al., 2010; McAleese et al., 2006; McCallum et al., 2006; Sengupta et al., 2012; R. G. Sobral et al., 2007; Utaida et al., 2003). This upregulation is part of a broader stress response mechanism that allows *S. aureus* to adapt to and survive in hostile environments where antibiotics are present. The inclusion of *fmtA* in the core cell wall stimulon—a set of genes activated in response to cell wall stress—highlights its pivotal role in maintaining cell wall integrity and functionality under stress conditions (Chen et al., 2016; McAleese et al., 2006; Y. Zhao et al., 2012)

The functional importance of FmtA extends beyond its role in antibiotic resistance. FmtA is integral to the biosynthesis and maintenance of the bacterial cell wall, particularly through the de-alanylation of WTAs (Rahman et al., 2016). The de-alanylation process carried out by FmtA involves the removal of D-alanine residues from WTAs (Qamar & Golemi-Kotra, 2012; Rahman et al., 2016). This enzymatic activity ensures that the cell wall remains structurally sound and capable of adapting to various environmental stresses (Qamar & Golemi-Kotra, 2012).

The ability of FmtA to modify WTAs and thereby maintain cell wall integrity is closely linked to its role in antibiotic resistance (Qamar et al., 2012). The binding of FmtA to WTA is determined by the shape and charge distribution of its active site, which are tailored to facilitate this interaction (Dalal et al., 2019). Structural studies have provided insights into these binding interactions, revealing how the protein's active site is configured to engage with WTAs effectively (Dalal et al., 2019). Given the critical role of FmtA in antibiotic resistance and remodelling of the cell wall, it

presents an attractive target for the development of novel antibacterial agents. Inhibitors designed to block FmtA's activity by competing with WTA binding could potentially disrupt the bacterium's ability to maintain its cell wall and resist antibiotics. Such inhibitors could be an invaluable addition to the arsenal of treatments against MRSA and other antibiotic-resistant strains of *S. aureus*.

1.5 Goals of the project

The goal of my thesis is to investigate the unique role of FmtA's PRP-like core structure in the removal of D-Ala from WTA, through mutagenesis. I hypothesize that the conserved catalytic residues of FmtA have been specifically adapted to catalyze this removal and that the shape of the FmtA active site has been modified to effectively bind WTA. To investigate these hypotheses, this research focuses on mutating key amino acids located in strategic positions predicted by Dalal et al., (2019) to be involved in WTA catalysis or binding and comparing the wild type with these variants.

The strategy includes both *in vitro* (Chapter 2) and *in vivo* (Chapter 3) approaches. The *in vitro* experiments involve generating the proposed mutations on the pET24a(+) vector harbouring the *fmtA*^{Δ27} gene and conducting comparative studies between the mutant and wild-type proteins. Detailed experimental procedures for these studies are outlined in each chapter.

In addition, a series of *in vivo* experiments are performed to investigate the role of *fmtA* mutations in *S. aureus* growth and morphology. In these experiments, *S. aureus* Δ*fmtA* mutant complemented with variants of *fmtA* are used, generated through mutagenesis of the pMK4::*fmtA* plasmid (constructed by a former lab member). The *in vivo* experiments include electron microscopy and cell-based assays such as biofilm formation, as described by Qamar et al. (2012).

Finally, in Chapter 4 of this thesis, I examine the specificity and affinity of the FmtA protein towards WTA by evaluating the effects of Branched Polyethylenimine (BPEI), a branched amine known to bind WTA electrostatically. This analysis involves introducing BPEI into the FmtA-WTA interaction to better understand the binding dynamics and potential competitive effects of BPEI on the interaction between FmtA and WTA.

This comprehensive approach aims to provide a deeper understanding of how FmtA's structure and catalytic mechanisms have evolved to interact with WTA, contributing to the broader knowledge of bacterial cell wall biosynthesis and its potential as a target for antimicrobial strategies.

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Chapter Two

Probing the activity of FmtA^{Δ27} through *in vitro* experimentation

2.1 Introduction

2.1.1 An Introduction to FmtA and Penicillin-Recognizing Proteins

Since it was discovered, Methicillin-resistant *Staphylococcus aureus* (MRSA), appropriately dubbed the "Superbug," has earned its reputation by developing resistance to numerous antibiotics (Washer & Joffe, 2006). FmtA, a novel esterase in *S. aureus*, removes the D-Ala from teichoic acid where teichoic acids play a significant role in antibiotic resistance amongst other physiological activities (Dalal et al., 2021). Structurally, FmtA closely resembles penicillin-recognizing proteins (PRPs), a broad category of proteins with diverse functions (Dalal et al., 2022). PRPs are characterized by a core active site situated between an all α -helical domain and an α/β domain, where serine acts as the nucleophile (Dalal et al., 2019). Although PRPs typically feature a cavity-shaped active site, this feature is absent in FmtA. Despite these structural resemblances, FmtA's unique esterase activity sets it apart from other PRPs (Dalal et al., 2019) (Fig 2.1.1). Since the discovery of FmtA by Komatsuzawa et al. in 1997, it has been observed that MRSA strains with a deleted *fmtA* gene ($\Delta fmtA$) exhibit increased sensitivity to detergents and β -lactams, which are widely used antibiotics for treating bacterial infections (Komatsuzawa et al., 1997). Additionally, these $\Delta fmtA$ strains show reduced methicillin resistance, highlighting the importance of FmtA in antibiotic resistance mechanisms in MRSA (Ayala et al., 2018; Komatsuzawa et al., 1997).

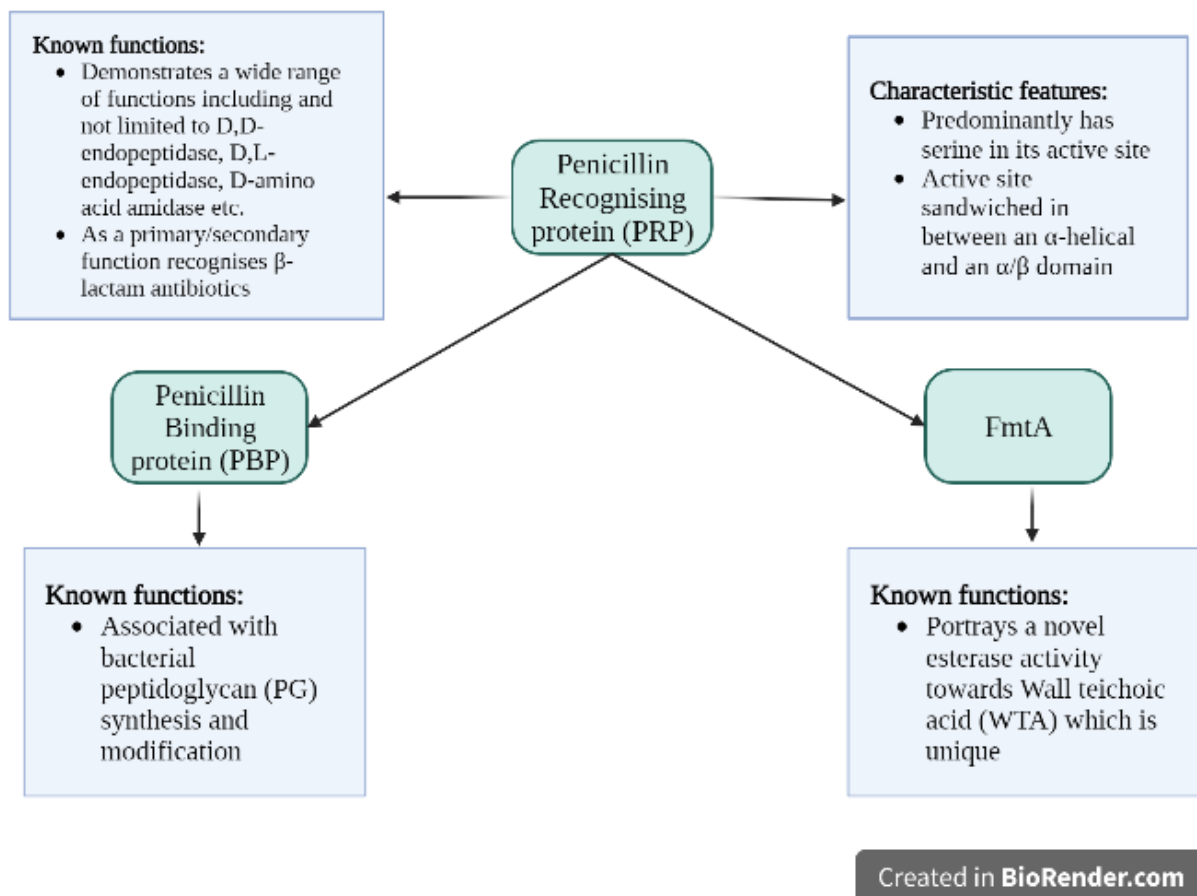


Figure 2.1.1. Flowchart representing the Penicillin Recognizing Protein (PRP) umbrella and FmtA's position in this family of proteins.

2.1.2 Established structural and functional aspects of FmtA

Dalal et al. (2019) conducted a structural analysis on the FmtA protein demonstrating that FmtA retains the core structure common to PRPs with some differences. Unlike typical PRPs, FmtA^{Δ27} lacks the characteristic cavity-shaped active site; instead, its active site is fully exposed and shallow. This feature is thought to have evolved to accommodate larger molecules like teichoic acids (Dalal et al., 2019) (Fig. 2.1.2.1). Due to FmtA's lack of typical PBP or β-lactamase activity, structural studies were employed to understand the reasons behind this absence of activity.

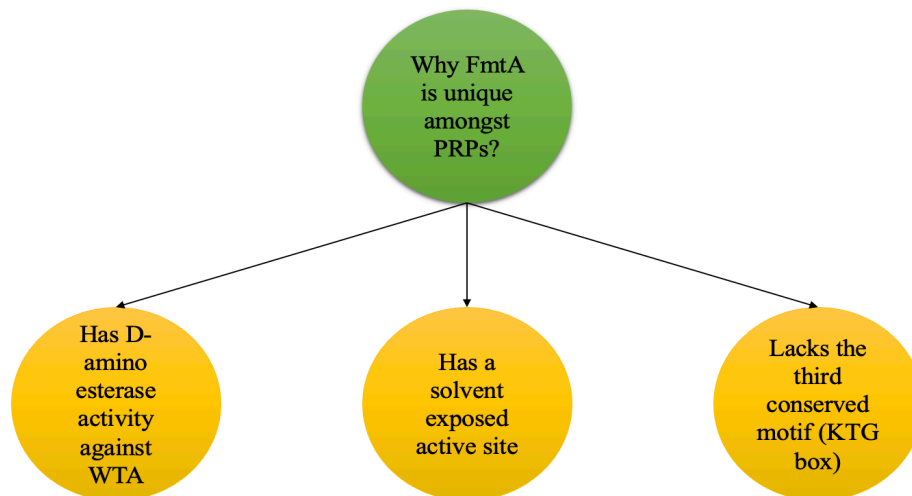


Figure 2.1.2.1. A flowchart describing the unique aspects of FmtA amongst PRPs.

FmtA exists as a monomer in the solution, where the X-ray crystallography analysis revealed FmtA consisting of 14 α -helices and 15 β -strands (Fig. 2.1.2.2) (Dalal et al., 2019).

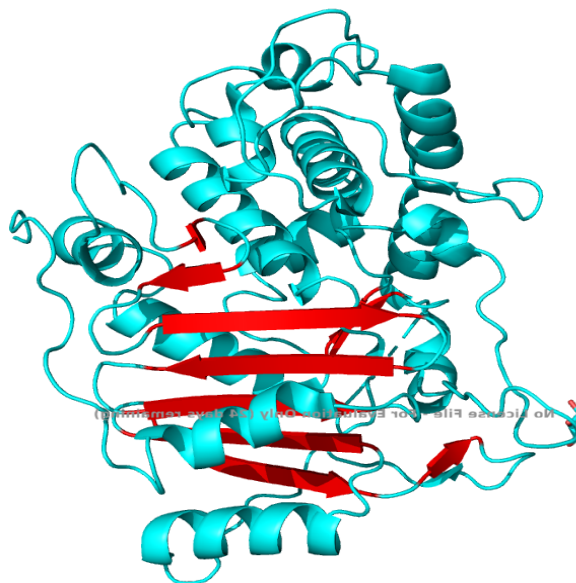


Figure 2.1.2.2. Illustrations represent the 3-D structure of FmtA.

A typical PRP contains three conserved motifs—SXXK, Y(S)XN, and the KTG box—and exhibits a two-domain structural fold, with its active site positioned between an all α -helical domain and an α/β domain (Dalal et al., 2019). FmtA shares a similar structural arrangement, with its active site located within two of these three conserved motifs (SXXK and Y(S)XN), while the

KTG box is absent. A unique characteristic of FmtA was found to be its active site, a U-shaped shallow entrance where it was assumed that the residues Tyr282 and Phe346 (located at the opposite side of the base of the active site) contribute towards this shape (Fig. 2.1.2.3) (Dalal et al., 2019).

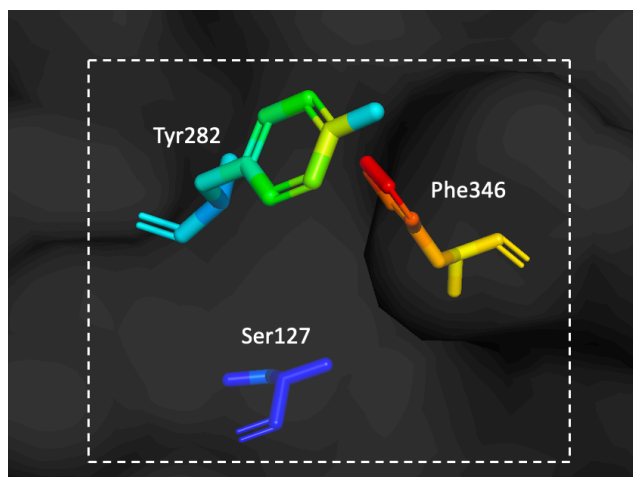


Figure 2.1.2.3. A cartoon representation of the U-shaped active site entrance of FmtA showing the active site nucleophile Ser127 (outlined by a dashed rectangle) flanked by two key active site residues, Tyr282 and Phe346.

The amino acid residues located within the β 13 strand (Fig 2.1.2.4) of PRPs are assumed to contribute to the substrate specificity of the proteins (Bompard-Gilles et al., 2000; Dalal et al., 2019; Okazaki et al., 2007) (Fig. 2.1.2.4). The FmtA structure was compared by Dalal et al. (2019) with those of D, D-amino peptidase (DAP) and D-amino amidase (DAA) (both PRPs). A glycine residue at the second position in the KTG box of DAP enables the binding of longer side chain substrates, while a leucine residue in the corresponding position in DAA reduces space, impacting the conformation of the binding site and facilitating binding to shorter side chain substrates (Dalal et al., 2019). In FmtA, this position is occupied by an asparagine (Asn343), and it was predicted to contribute towards WTA binding (Fig. 2.1.2.4) (Fig. 2.1.2.6) (Dalal et al., 2019).

The active site nucleophile Ser127 of FmtA (Qamar & Golemi-Kotra, 2012) is found surrounded by a few hydrophobic residues, which include the residues Tyr282 and Phe346

flanking both sides of the FmtA active site entrance (Dalal et al., 2019) (Fig. 2.1.2.3 and Fig. 2.1.2.4). Different catalytic roles have been assigned to the lysine at position 130 (in FmtA) (Fig. 2.1.2.3) based on the known functions of its analogous molecules in other PRPs. For example, in Class C β -lactamases, the residue equivalent to Lys130 was proposed to stabilize the enzyme's catalytic reaction by lowering the pKa of the Tyrosine residue (Tyr211) present in the Y(S)XN motif (Oefner et al., 1983). In FmtA the third position of this motif is occupied by an Aspartic acid (Asp213) (Dalal et al., 2019; Fan et al., 2007) (Fig. 2.1.2.5 and Fig. 2.1.2.6).

Instead of a KTG box (prevalent in PRPs) structurally, FmtA has a sequence of amino acid residues, namely 342-LNG (LNG box) (Dalal et al., 2019). Unlike other PRPs, in FmtA the LNG box is considered not to participate in catalysis but to contribute towards the shape of the active site where the side chain of the residue Leu342 was found to face down towards the plane of the β 13 strand (Fig. 2.1.2.4 and Fig. 2.1.2.6) (Dalal et al., 2019). Such positioning of the Leu342 residue in FmtA creates a space next to the Tyr211 residue which is occupied by a water molecule (Z505) and was predicted by molecular docking to be accommodating the D-Ala of teichoic acid (Fig. 2.1.2.4 and Fig. 2.1.2.6) (Dalal et al., 2019).

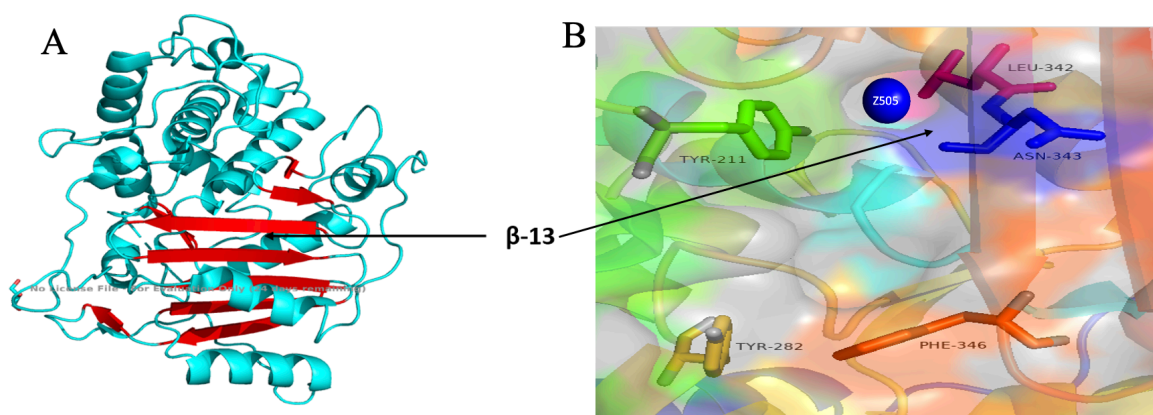


Figure 2.1.2.4. Illustrations representing the FmtA active site where A: The β -13 sheet is highlighted in the FmtA active site domain and B: Represents the key amino acid residues probably involved in the binding of WTA as a substrate are depicted in different coloured stick figures.

Tyr211 in FmtA was observed to be within the hydrogen-bonding distance of the active site nucleophile Ser127 and two water molecules, Z505 and Z516. Z505 is also within a hydrogen-bonding distance of Ser127 (Dalal et al., 2019). In some PRPs, an equivalent residue to Tyr211 has been proposed to act as an activator for the nucleophile Ser (Kelly & Kuzin, 1995). However, in FmtA, Tyr211 is believed to function as an anchor, positioning WTA within the active site (Dalal et al., 2019). Based on the structural analysis a prediction was made that the Tyr211 residue might be serving to position the carbonyl moiety of D-Ala for hydrolysis (Dalal et al., 2019) (Fig. 2.1.2.5).

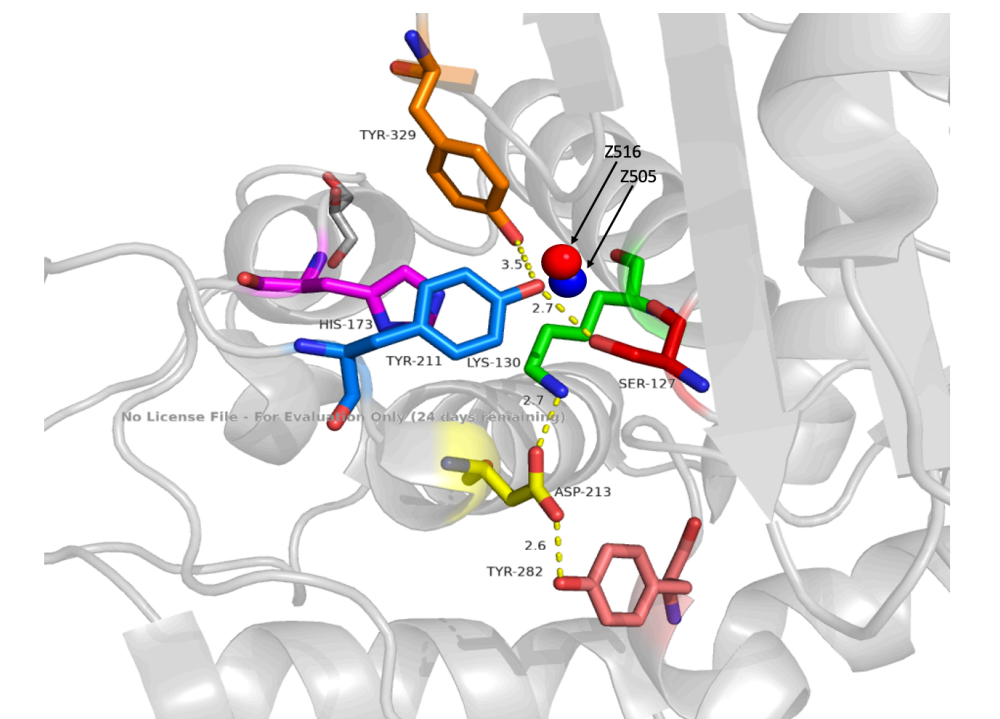


Figure 2.1.2.5. Illustrations representing the FmtA active site where the key amino acid residues probably involved in the catalytic activity of FmtA are depicted in different coloured stick figures.

The Lys130 residue in FmtA was considered to activate the Ser127, as it was found within the hydrogen-bonding distance of Ser127 as well as Asp213 (Dalal et al., 2019). The Asp213 was also found to be within the hydrogen-bonding distance of Tyr282, where it was predicted that a

hydrogen bonding network present within Lys130, Asp213, and Tyr282 can stabilize the deprotonated Lys130 while it acts as a general base (Fig. 2.1.2.5 and Fig. 2.1.2.6) (Dalal et al., 2019).

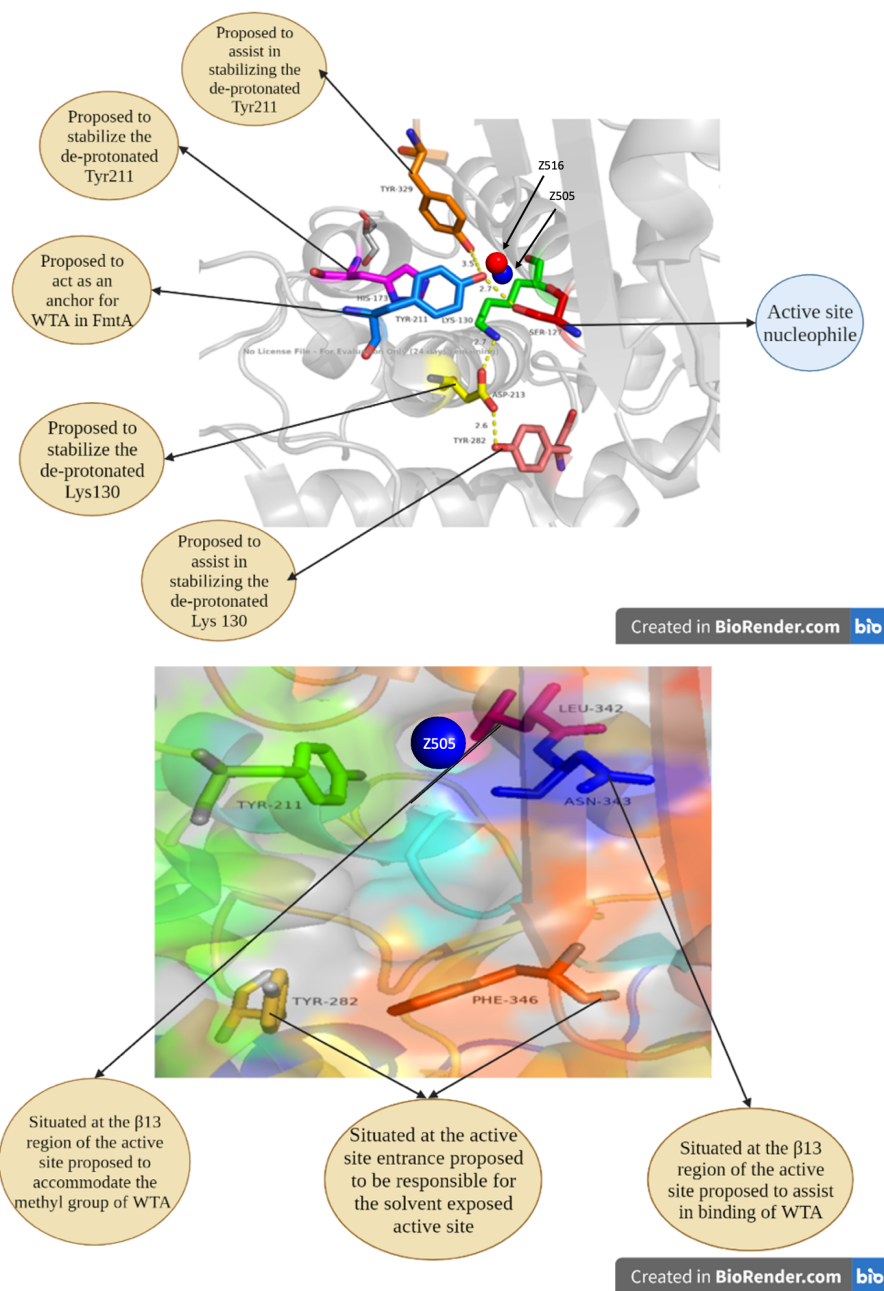
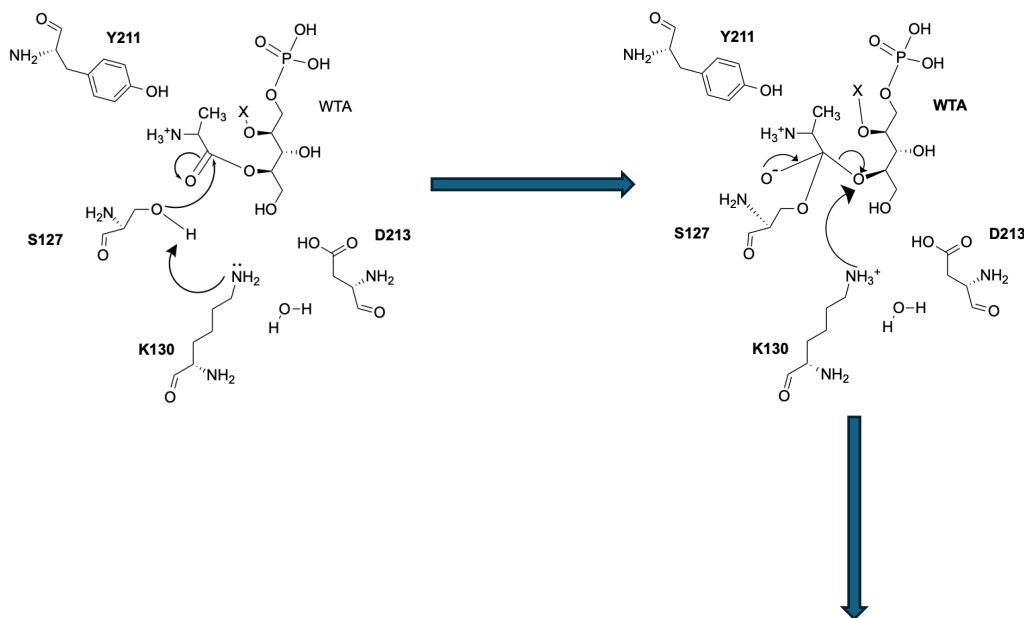


Figure 2.1.2.6. Schematic representation of the inter-relationships and the proposed roles of the key amino acids identified in the FmtA active site core.

FmtA catalyzes the ester bond present between D-Ala and the backbone of the WTA which is classified as the D-amino esterase activity of FmtA (Rahman et al., 2016). The D-amino esterase activity of FmtA is presumed to play a significant role in *S. aureus* virulence and its routine physiological functions as they depend on the D-alanylation levels of WTA.

FmtA catalyzes WTA in an active site nucleophile-based enzymatic reaction, where FmtA's active site serves as a region for substrate binding. A nucleophile (Serine127 in FmtA) present in this active site is capable of donating electrons to form a new covalent bond with the substrate (WTA) (Rahman et al., 2016).

The enzymatic process begins with FmtA binding to its substrate, positioning it within the active site adjacent to the nucleophilic group. This alignment facilitates hydrolysis reaction by enabling the formation of a transient covalent bond between the enzyme and the substrate. Consequently, an enzyme-substrate complex (FmtA-WTA) is formed. The enzyme's active site residue interacts with the transition state to stabilize it (Fig 2.1.2.7) (Dalal et al., 2022).



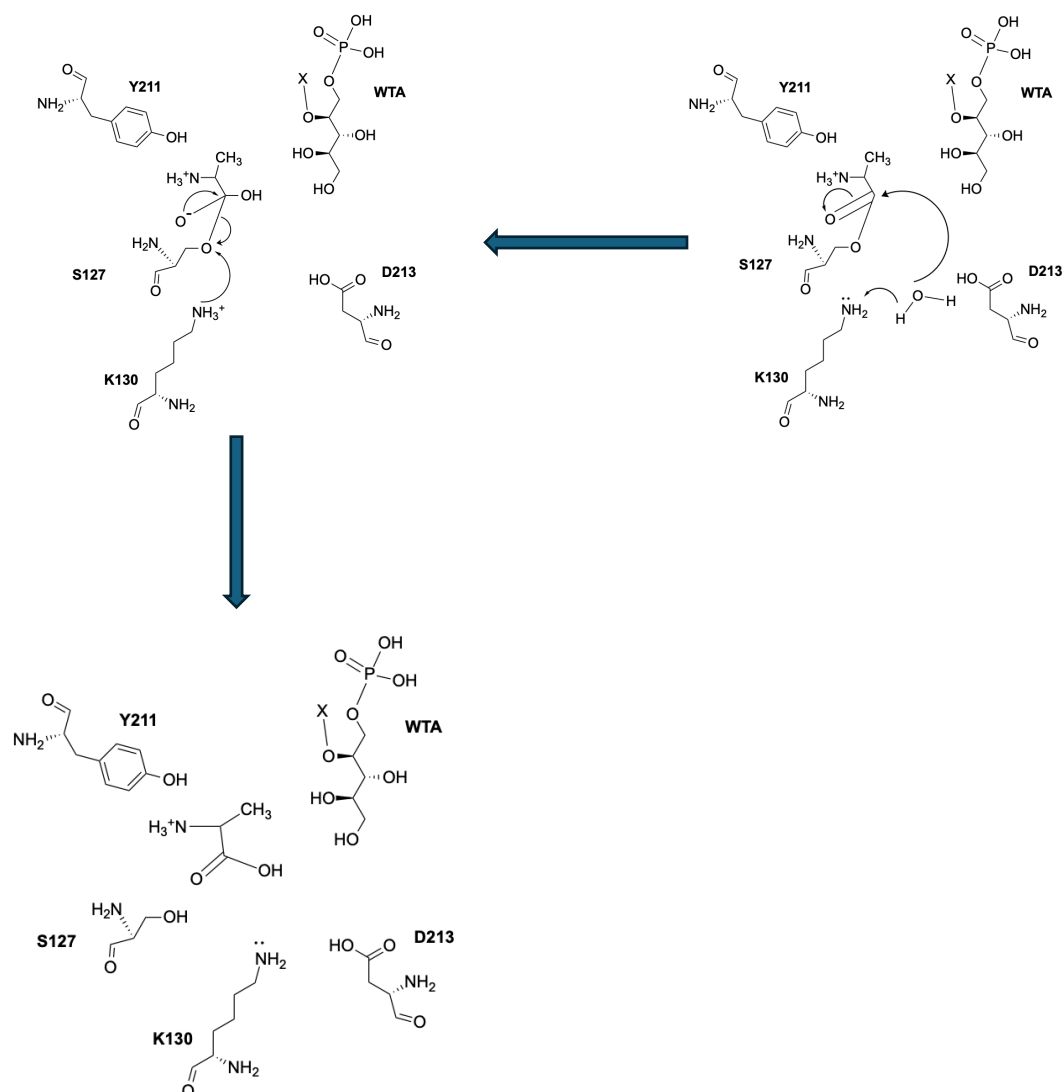


Figure 2.1.2.7. An illustration of the proposed nucleophilic reaction between FmtA-WTA, where Ser127 is the active site nucleophile and K130 is the general base. In this process, S127 attacks the carbonyl carbon of WTA when K130 facilitates the removal of a proton from S127 where an acyl-enzyme complex forms. A nearby water molecule then donates a proton to the complex and the K130 residue, releasing D-Ala. Here X represents N-acetyl glucosamine (Dalal et al. 2022))

2.1.3 Chapter 2 Objectives

In this chapter, using an *in vitro* approach through site-directed mutagenesis, I aim to elucidate how adaptations in the conserved catalytic residues of FmtA contribute to its esterase activity toward WTA or enable it to recognize WTA as a substrate through *in vitro* experimentation. Here I divided the variants into two groups: one focusing on the adaptations in

FmtA's conserved catalytic residues that resulted in esterase activity towards WTA, and the other investigating the modifications that allow FmtA to recognize WTA as a substrate.

2.2 Materials and Methods

2.2.1 Materials and Chemicals

The growth media used for protein purification was sourced from Fisher Scientific, while the media for WTA isolation was obtained from Sigma-Aldrich. Primers and enzymes were obtained from Sigma-Aldrich (Canada), and Bocillin FL penicillin, sodium salt was purchased from Invitrogen.

2.2.2 Construction of the *fmtA*^{Δ27} mutant variants

The pET24a(+):*fmtA*^{Δ27} construct was previously prepared in our laboratory (Fan et al., 2007). Using this construct as the template, the specified variants were generated (as listed in Table 2.2.2) utilizing the QuikChange™ Site-Directed Mutagenesis method (by Stratagene).

The process was initiated by introducing one of the target mutations to create a single mutant. Subsequently, the mutated construct was used as a template to introduce the second mutation. Following PCR amplification (The PCR parameters are as follows where all the variants were generated following the same protocol: initial denaturation at 95°C for 30 seconds, followed by 16 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 1 minute, and extension at 68°C for 10 minutes. This is followed by a final extension at 68°C for 10 minutes, with a final hold at 4°C), the resulting products were subjected to digestion with Dpn1, a restriction endonuclease employed to eliminate the original methylated template DNA (Fig. 2.2.2).

Subsequently, *E. coli* DH5α cells were transformed with the digested products, and the preparation of the correct construct by mutagenesis was confirmed through DNA sequencing (The Center for Applied Genomics at the Hospital for Sick Kids in Toronto, Canada). Considering the

sequencing results, the verified correct constructs were introduced into *E. coli* BL21(DE3) for the subsequent phases of protein expression and purification.

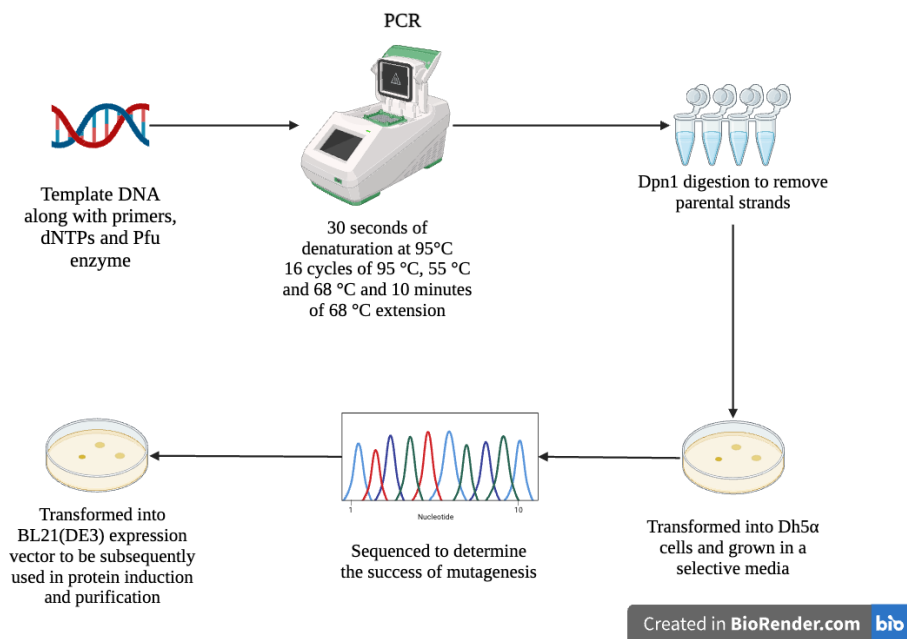


Figure 2.2.2. Illustration depicting the parameters adopted for the site-directed mutagenesis experiment

Table 2.2.2. The table shows the mutations generated with the pET24a(+):*fmtA*^{Δ27} constructs along with their designed primers.

Protein	Primers
FmtA-Y211F	Dir- 5'GAAATACAAAAAGCACATG T TTAACGATGGGAATTATTTAG 3' Rev- 5' CTAAATAATTCCCATCGTT A ACATGTGCTTTTTGTATTTC 3'
FmtA-D213N	Dir- 5' CAAAAAGCACATGTATAAC A ATGGGAATTATTTAG 3' Rev-5' CTAAATAATTCCC A T T GTTATAACATGTGCTTTTTG 3'
FmtA-Y282F	Dir-5' CCTAATATTTTGGACCA A T T CTATGGTGCAGG 3' Rev-5' CCTGCACCATAG A ATTGGTCCAAAATATTAGG 3'
FmtA-Y329F	Dir-5' GAAACAGTATCCAGATGA A T T TCGATATGGTTTC 3' Rev-5' GAAACCATATCG A A T TCATCTGGATACTGTTTC 3'
FmtA-H173A	Dir-5' CCCATTGAAAGATTTAATGTT G CTCAAAGTGG 3' Rev-5' CCACTTTG A GCCAACATTAAATCTTTCAATGGG 3'
FmtA-Y282A	Dir-5' ATTTTGGACCA A GCCTATGGTGCAGG 3' Rev-5' CCTGCACCATAG G CTTGGTCCAAAAT 3'
FmtA-N343A	Dir-5' TGAATAGACTT G CCGGGGGATTCTT 3' Rev-5' AAGAATCCCC G GCAAGTCTATTCA 3'
FmtA-N343G	Dir-5' ATAGACTT G GCGGGGGATTCTTTGG 3' Rev-5' CCAAAGAATCCCC G CAAGTCTAT 3'
FmtA-L342H	Dir-5' CCAACATTGAATAGAC A TAACGGGGG 3' Rev-5' CCCCCGTT A TGTCTATTCAATGTTGG 3'
FmtA-Y211A	Dir-5' GCACATG G CTAACGATGGGAA 3' Rev-5' ATTCCCATCGTT A GCCATGTG 3'
FmtA-D213A	Dir-5' GCACATGTATAAC G CTGGGAATTATTTAGTACT 3' Rev-5' GCAAGTACTAAATAATTCCC A GCGTTATACATG 3'
FmtA-F346A	Dir-5' TTAACGGGGG A GCCTTTGGACAAG 3' Rev-5' CTTGTCCAA A G G CTCCCCCGTTAA 3'

2.2.3 Expressing and purifying wild-type FmtA^{Δ27} and its variants

The protein expression and purification processes for both wild-type FmtA^{Δ27} and its variants were executed according to the protocol published by Fan et al. (2007). In summary, an overnight seed culture was initiated in 5 ml of LB broth, which was supplemented with 30 µg/ml of kanamycin. This culture was then inoculated with a scratch of the BL21(DE3) cell glycerol stock containing pET24a(+):*fmtA*^{Δ27} or its respective mutant variants. Subsequently, it was incubated at 37 °C and agitated at 200 rpm for 16 hours.

5 ml of the overnight seed culture was transferred into 1L of terrific broth media, supplemented with 0.4 M D-Sorbitol, 2.5 mM β-betaine, and 30 µg/ml kanamycin. The inoculated medium was grown at 37 °C and agitated at 120 rpm until the optical density at 600 nm (O.D.₆₀₀) reached a range of 0.6 to 0.7.

At this point, the culture was induced with 0.1 M Isopropyl β-D-thiogalactopyranoside (IPTG) to initiate protein expression, followed by incubation at 25 °C and agitation at 120 rpm for an additional 16 hours. Subsequently, the cells were harvested by centrifugation at 6300 rpm for 20 minutes at 4 °C, and the cell pellets were preserved at -80 °C if the purification process was not undertaken immediately.

For the purification procedure, the harvested cell pellets were resuspended into 50 mM sodium phosphate buffer, pH 7.2 and all the necessary steps were carried out at 4 °C. The resuspended cells were then sonicated (16 cycles, 1 min on and 1 min off; 30% power and 65% pulse) to liberate the protein and then subsequently centrifuged at 13000 rpm, 4 °C for 1 hour, to separate the supernatant from the cell pellets. The supernatant was loaded into a sp-sepharose column (CV 100 ml) which was pre-equilibrated with 50 mM sodium phosphate buffer pH 7.2. The protein FmtA^{Δ27} or its variants were eluted at 70-80% of a liner gradient of 0-1 M sodium

chloride in 50 mM sodium phosphate buffer, pH 7.2. The collected fractions of the protein were combined and concentrated to a volume of 1 ml and were loaded onto a size exclusion column (HiPrep 26/60 Sephacryl, 320 ml) which was also pre-equilibrated with 50 mM sodium phosphate buffer, pH 7.2. The collected fractions were finally concentrated using a centrifugal concentrator Ultracel-10 K (Amicon, Milipore). Protein homogeneity was assessed by 12.5% SDS-PAGE gel.

For the purification process, the harvested cell pellets were resuspended in a 50 mM sodium phosphate buffer at pH 7.2, with all subsequent procedures conducted at a constant temperature of 4 °C. The resuspended cells were then subjected to sonication (16 cycles of 1 minute on and 1 minute off, 30% power, and 65% pulse) to lyse the cells and effectively release the proteins. Subsequently, the lysed cells were centrifuged at 13,000 rpm, 4 °C for 1 hour, facilitating the separation of the supernatant from the cell pellet.

The resulting supernatant was introduced into a Sp-Sephacryl column (with a column volume of 100 ml) that had been pre-equilibrated with a 50 mM sodium phosphate buffer at pH 7.2. The elution of the FmtA^{Δ27} protein or its respective variants was achieved by utilizing a linear gradient of 0 to 1 M sodium chloride in a 50 mM sodium phosphate buffer at pH 7.2, specifically recovering the proteins between the 70-80% elution range.

The fractions containing the proteins were pooled and subsequently concentrated to a final volume of 1 mL, after which they were loaded onto a size exclusion column (HiPrep 26/60 Sephacryl with a column volume of 320 ml), which had been pre-equilibrated with a 50 mM sodium phosphate buffer at pH 7.2 (Fig. 2.2.3).

Finally, the collected fractions were further concentrated using an Ultracel-10 K centrifugal concentrator (Amicon, Millipore). To assess the homogeneity of the protein, a 12.5% SDS-PAGE gel was employed and was stained with Coomassie Blue.

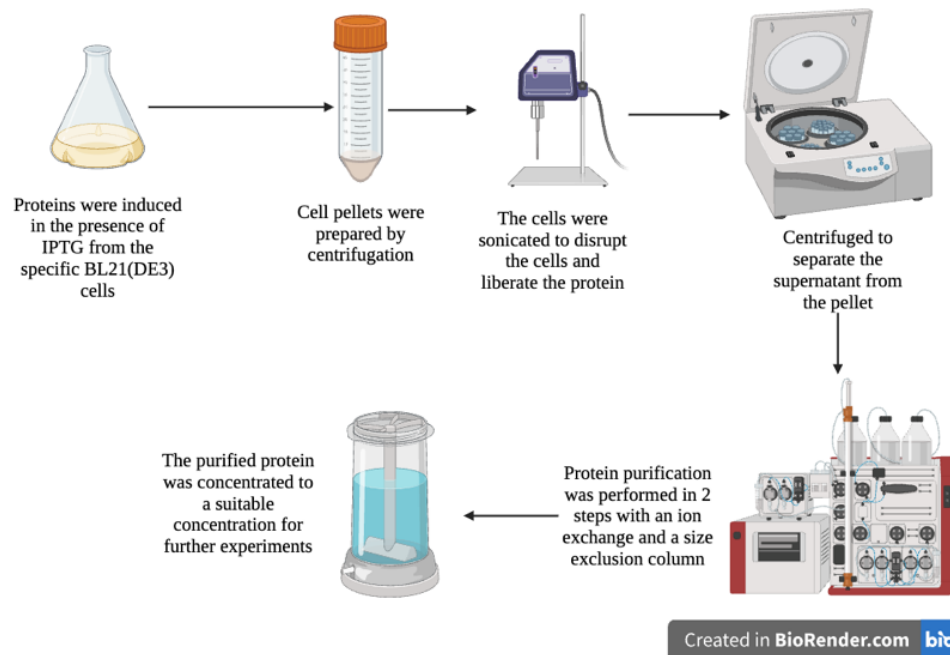


Figure 2.2.3. A brief schematic representation of the protein purification process.

2.2.4 Isolation of Wall Teichoic Acid

WTA was isolated from *S. aureus* RN4220 strain as described by Fridman et al. (2013). Briefly, a 1 L Tryptic Soy Broth (TSB) culture was inoculated with an overnight RN4220 cell culture and grown until it reached an O.D.₆₀₀ of 1.2 at 37 °C. The cells were then harvested through centrifugation (6000 x g for 15 min at room temperature) and suspended in 10 mL of 2 M NaCl. Then subsequent sonication (1 sec ON, 9 sec OFF, 2 min total, 35% amplitude) was performed at room temperature to facilitate cell disruption. Cell wall fragments were obtained through centrifugation (14000 x g; 5 min at 4 °C), and the resulting pellets were suspended in phosphate-buffered saline (PBS) (which contains 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄) and recovered by centrifugation (14000 x g; 5 min at 4 °C). The pellets underwent incubation with 20 mL of 4% w/v sodium dodecyl sulphate (SDS) at 37 °C for 4 hours. Cell walls were then recovered by centrifugation (14000 x g; 5 min at 25 °C) and subjected to three washes with water (10 mL each time).

To eliminate non-covalently bound components, the obtained pellets underwent incubation at 37 °C with 4 mL trypsin (0.2 mg/mL), RNase (0.1 mg/mL), and DNase (0.1 mg/mL) in 50 mM Tris-HCl, pH 7.0, 5 mM MgCl₂ overnight with stirring. Following recovery by centrifugation (14000 x g; 5 min at 4 °C), the cell wall was subjected to washes with 1 M Tris-HCl (pH 7.0), then with 1 M Tris-HCl + 1 M NaCl (pH 7.0), and again with 1 M Tris-HCl (pH 7.0). The washed cell wall was recovered by centrifugation (14000 x g; 5 min at 4 °C) and underwent three additional washes by resuspending in water (5 mL each time) with centrifugation steps (14000 x g; 5 min at 4°C).

To release WTA from the peptidoglycan, the pellets were incubated in 12% w/v aqueous trichloroacetic acid (TCA) at 4 °C for 48 hours. Subsequently, peptidoglycan removal was achieved through centrifugation (5300 x g; 45 min; 4 °C), and WTA precipitation from the supernatant occurred in 95% ice-cold ethanol, held for 5 days at 4 °C. After securing the WTA pellets by centrifugation (5000 x g; 30 min; 4 °C), two additional washes with 95% ethanol (5 mL each, 5000 x g; 30 min; 4 °C) were performed. Following the final ethanol wash, the solvent was lyophilized to collect purified WTA and was stored at -20°C (Fig 2.2.4).

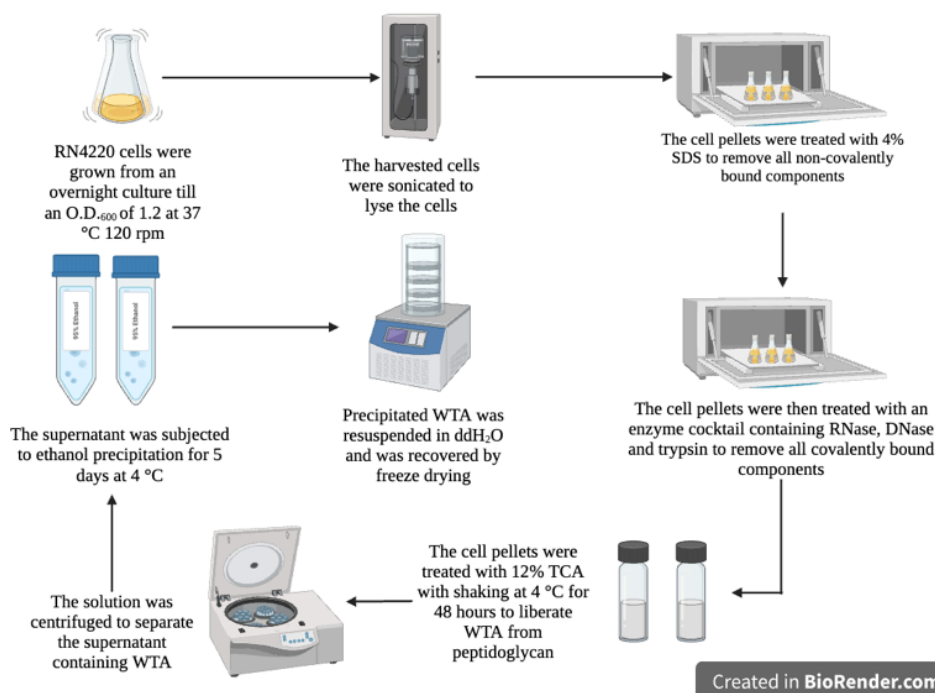


Figure 2.2.4. A brief schematic representation of the WTA isolation process

2.2.5 Circular Dichroism (C.D.) Spectroscopy Studies

UV circular dichroism (CD) spectra (200-260 nm) of FmtA^{Δ27} and its variants were measured at 1 nm intervals with a scan rate of 50 nm/min (response time: 32 seconds, sensitivity: 100 mdeg) using a Jasco J-810 spectrophotometer. The purpose of this experiment was to compare the secondary structures of the variants with the wild-type protein to assess their structural similarity.

The samples, at a concentration of 10 μM, were prepared in 50 mM sodium phosphate buffer, pH 7.2. The cuvette used had a 1.0-mm light path length and all the experiments were performed at 22 °C. To ensure data accuracy and reliability, an average of three scans was obtained for each sample, and the resulting spectra were adjusted for interference from the buffer signal (Fig 2.2.5).

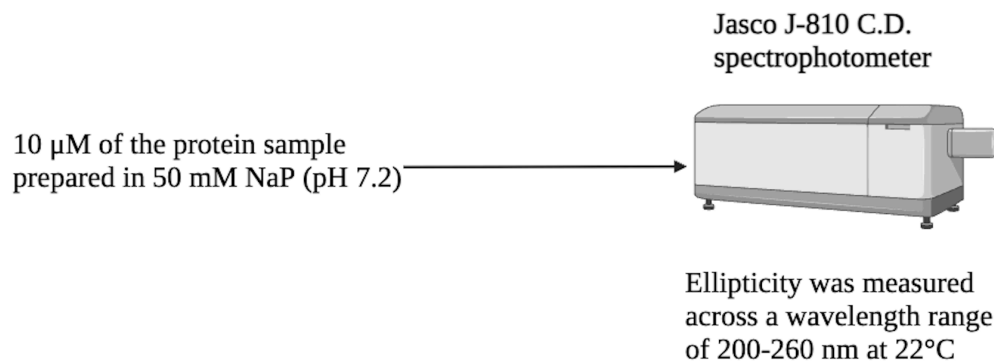
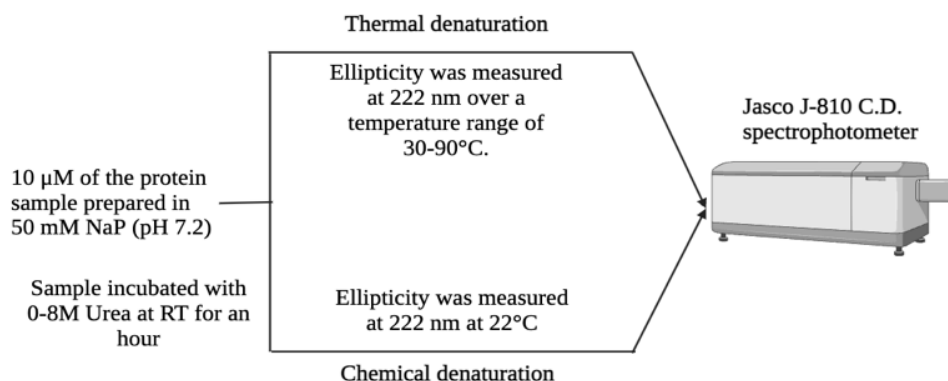


Figure 2.2.5. Illustration representing the experimental parameters adapted for the C.D. spectroscopy experiment to investigate the possible changes in the secondary structure of the protein due to the mutations.

2.2.6 Thermal and Chemical denaturation of FmtA^{Δ27} and its variants

Thermal and chemical denaturation experiments were conducted by measuring the ellipticity of protein solutions at 222 nm. For thermal denaturation, measurements were taken across a temperature range of 30-90°C, with data collected at each degree. The response time for each measurement was 32 seconds, with a sensitivity of 100 m.deg, a delay time of 1 second, and a temperature slope of 1°C/min. For chemical denaturation, the experiments involved varying urea concentrations from 0 to 8 M, with readings taken at each 1 M increment. The response time for each measurement was 32 seconds, with a sensitivity of 100 m.deg (standard). The samples were prepared with a protein concentration of 10 μ M in 50 mM sodium phosphate buffer, pH 7.2. For the chemical denaturation experiment, the protein samples were incubated with the specified urea concentrations for 1 hour at room temperature before recording the spectra (Fig 2.2.6). The purpose of conducting a thermal and chemical denaturation study was to assess the stability and folding properties of the variants compared to the wild type.



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Figure 2.2.6. Illustration representing the experimental parameters adapted for the thermal and chemical denaturation experiment to investigate the possible changes in the thermal and chemical stability of the protein due to the mutations.

2.2.7 Concentration-dependent binding studies of FmtA ^{Δ 27} variants with Bocillin

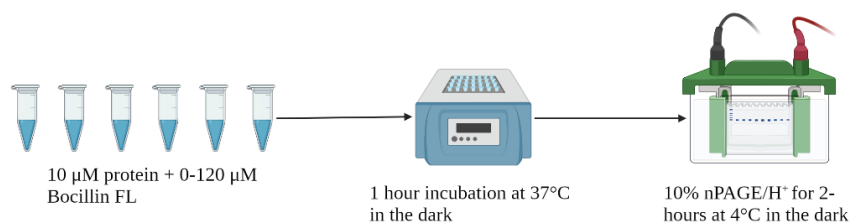
10 μ M of FmtA ^{Δ 27} and its variants were subjected to incubation with varying concentrations of Bocillin FL, which is a fluorescently labelled derivative of penicillin G. These concentrations spanned between 0 to 120 μ M, all prepared in a 50 mM sodium phosphate buffer at pH 7.2. Reaction mixtures of 15 μ l, were incubated for an hour at 37 °C, away from light. To analyze the interaction between the proteins and Bocillin FL, 10% acidic-native polyacrylamide gel electrophoresis (nPAGE/H⁺) was employed. Subsequent imaging of the gel was performed using the Typhoon FLA 9500 fluorescent scanner with the Alexa Fluor™ 488 channel, employing an excitation wavelength of 495 nm and an emission wavelength of 519 nm. The fluorescence of the bands was then quantified using NIH ImageJ V1.53 software.

This experiment incorporated BlaR^S—the penicillin-binding domain of the transmembrane signal-transducing protein BlaR—as a positive control. This inclusion stems from BlaR’s

established behaviour: As Golemi-Kotra et al. (2003) noted, it is known to undergo a 100% modification when in the presence of Bocillin FL. The control 15 μ l reaction mixture was composed of 4 μ M BlaR^S, which was incubated with 120 μ M Bocillin FL for an hour at 37 °C in the dark.

It's also worth noting that unbound Bocillin FL does not enter the gel under acidic native conditions. This absence is attributed to its negative charge, for which it is repelled by the negatively charged cathode in the nPAGE/H⁺ process (Fig 2.2.7).

This assay aims to investigate the impact of the mutations on the binding affinity of the FmtA ^{Δ 27} protein to Bocillin FL., as the strategic mutations target the active site of the protein, with some mutations potentially rendering the protein structurally similar to PBPs.



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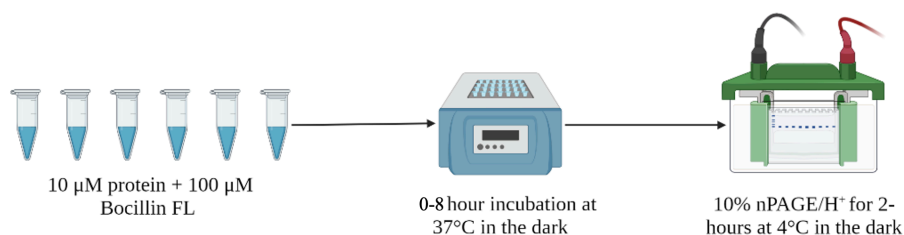
Figure 2.2.7. Illustration representing the experimental parameters adapted to examine the concentration-dependent binding between FmtA ^{Δ 27}, its variants and Bocillin FL using acidic native gel (n/PAGE/H⁺) electrophoresis. FmtA ^{Δ 27} and its variants were incubated with varying concentrations of Bocillin FL (ranging between 0-120 μ M) for one hour at 37°C in the dark before electrophoresis.

2.2.8 Time course-dependent interaction of FmtA ^{Δ 27} variants with Bocillin

Informed by the insights from the concentration-dependent binding of Bocillin FL with FmtA ^{Δ 27} and its variants, a time course-dependent experiment was devised to further investigate

this interaction. 15 μL reaction mixtures comprising 10 μM of FmtA $^{\Delta 27}$ and its variants were incubated with 100 μM of Bocillin FL over varying periods, ranging from 0 to 24 hours, at a temperature of 37°C in the absence of light. These experiments were conducted in a 50 mM sodium phosphate buffer at pH 7.2.

Following the incubation, the reaction mixtures were analyzed by 10% nPAGE/H $^+$ gel. The resulting fluorescent gel images were captured using a Typhoon FLA 9500 fluorescent scanner and subsequently, the fluorescent bands obtained were quantified using NIH ImageJ V1.53 software. A parallel 15 μL reaction mixture was prepared as a positive control, composed of 4 μM of BlaR S incubated with 120 μM of Bocillin FL for one hour at 37 °C, also conducted in the absence of light. This control experiment establishes a 100% binding level, serving as an internal control to quantify the specificity of the protein-Bocillin FL interaction (Fig 2.2.8). This study was conducted to evaluate how the interaction between FmtA $^{\Delta 27}$ and Bocillin FL progressed over time.



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Figure 2.2.8. Illustration representing the experimental parameters adapted to examine the time course-dependent binding between FmtA $^{\Delta 27}$, its variants and Bocillin FL using acidic native gel (n/PAGE/H $^+$) electrophoresis. FmtA $^{\Delta 27}$ and its variants were incubated with 100 μM Bocillin FL for different periods (0-8 hours) at 37 °C in the dark before electrophoresis.

2.2.9 Binding studies of FmtA^{Δ27} variants with WTA

Established by Qamar & Golemi-Kotra, (2012) and further substantiated by Rahman et al. (2016), the difference in binding between FmtA^{Δ27} and its variants towards WTA serves as a focal point of this experiment. To discern the impact of the said mutations on this binding, an established protocol was employed, developed by Rahman et al. (2016).

15 µl reaction mixtures, comprising 10 µM FmtA^{Δ27} protein along with its variants, were subjected to incubation with an increasing concentration of WTA (ranging from 0-1.3 mg/ml (0-67 µM)). The incubation was carried out at 37°C for one hour, all prepared in 50 mM sodium phosphate buffer at pH 7.2. Subsequently, the samples were run in a 10% nPAGE/H⁺ gel at 4°C.

Following electrophoresis, the gels underwent Coomassie staining to visualize the protein bands. The Coomassie-stained bands were then quantified using ImageJ V1.53 software, enabling measurements of the extent of binding. Notably, FmtA^{Δ27}, being positively charged, is visible in a nPAGE/H⁺ gel. However, upon binding with WTA, the overall negative charge of WTA neutralizes the positive charge of FmtA^{Δ27}, which prevents the complex from entering the gel (only the unbound FmtA protein travels toward the anode in this experimental setting) when a complete binding between FmtA^{Δ27} and WTA is achieved (Fig 2.2.9).

The primary objective of this investigation is to quantify the percentage binding between the proteins and WTA, thus establishing a comparative analysis between the wild type and the variants. As a control, a parallel 10 µM protein sample without WTA was run concurrently, serving as a baseline reference for assessing the impact of WTA binding to the protein bands.

This enzymatic function is crucial for the physiology of *S. aureus* as the level of D-alanylation in WTA influences various physiological processes (Bertsche et al., 2013; Tabuchi et

al., 2010). These processes include and are not limited to adhesion, virulence, defense against antimicrobial peptides, biofilm formation etc. highlighting the critical role of FmtA^{Δ27} in the bacterial lifecycle and pathogenicity (Rahman et al., 2016).

The molecular weight of WTA was calculated considering there is 40 Ribitol-5-phosphate, 41 N-acetylglucosamine (40 units in the backbone and one in the linker), 1 N-acetylmannosamin, 10 D-Alanine units and 2 units of Glycerol-3-phosphate. So, the Molecular Weight (M.W.) of WTA was calculated as:

$$\begin{aligned}
 & 40x (\text{M.W. Ribitol-5-phosphate}) + 41x (\text{M.W. N-acetylglucosamine}) + 1x (\text{M.W. N-acetylmannosamin}) + 10x \\
 & (\text{M.W. D-Alanine}) + 2x (\text{M.W. Glycerol-3-phosphate}) \\
 & = 40x 232.13 + 41x 221.2 + 1x 221.2 + 10x 89.09 + 2x 172.1 \\
 & = 9285.2 + 9069.2 + 221.2 + 890.9 + 344.2 \\
 & = 19,810.7 \text{ g/mol}
 \end{aligned}$$

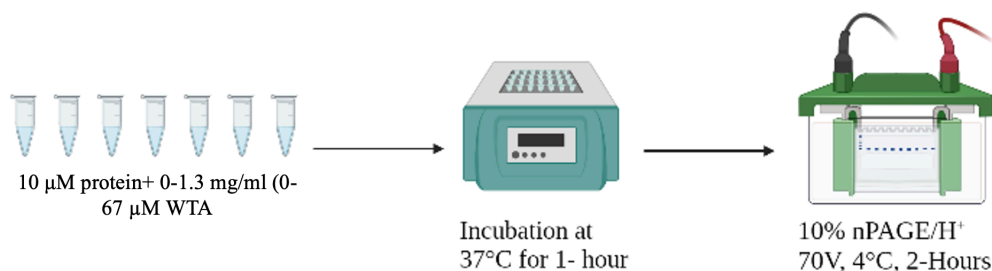


Figure 2.2.9 Illustration representing the experimental parameters adapted to examine the binding between FmtA^{Δ27}, its variants and WTA using acidic native gel (n/PAGE/H⁺) electrophoresis. 10 μM FmtA^{Δ27} and its variants were incubated with varying concentrations of WTA (ranging between 0-1.3 μg/ml (0-67 μM)) for one hour at 37°C before electrophoresis.

2.2.10 Fluorescent-based enzyme-coupled assay

2.2.10.1 Fluorescent-based enzyme-coupled assay with FmtA^{Δ27} variants

To investigate the enzymatic activity of FmtA^{Δ27} and its variants, an experimental setup was devised in the context of D-Ala release from WTA. The experiment involves an enzymatic cascade where FmtA^{Δ27} releases D-Ala from WTA where the released D-Ala reacts with D-Amino acid oxidase (DAO) in the presence of Flavin adenine dinucleotide (FAD). This reaction yields Pyruvate and Hydrogen peroxide (H₂O₂). Subsequently, in the presence of Horseradish peroxidase (HRP), H₂O₂ engages with Amplex Red, generating the quantifiable fluorescent product Resorufin. Originally, the method was described by Gutheil et al. (2000) and then modified by Rahman et al. (2016).

The experimental design involved 100 µl reaction mixtures, comprising 10 µM FmtA^{Δ27} and its variants, which were incubated with increasing concentrations of WTA (ranging from 0-15 µM or 0-0.3 mg/ml) at a temperature of 37°C for 2 hours. This initial incubation period allowed for the interaction between the proteins and WTA to take place.

Following this incubation period, the reaction mixtures were subjected to a second incubation in triplets in a 96-well flat bottom plate. The working solution, containing DAO, FAD, HRP, and Amplex Red, was introduced to each reaction mixture and incubated for an additional hour in the dark at room temperature. This step is conducted in darkness, as the working solution is light-sensitive.

After the incubation period with the working solution, the fluorescent readings were recorded using a Synergy H4 Hybrid plate reader. The emission wavelength was set at 563 nm, while the extinction wavelength was maintained at 584 nm (Fig. 2.2.10.1).

This experiment allows for the quantification of the fluorescent Resorufin, serving as a reliable indicator of the enzymatic activity of the protein. By comparing the enzymatic profiles of the mutant proteins with the wild-type FmtA^{Δ27}, this approach provides insights into the variations in enzymatic activity resulting from different mutations, contributing to a comprehensive understanding of the implications of these genetic alterations.

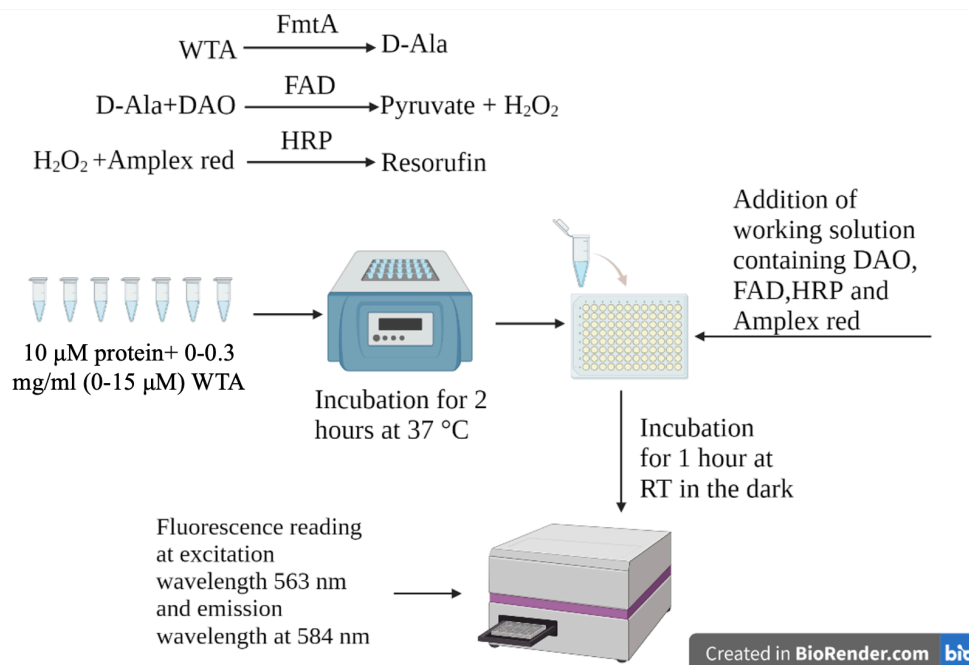


Figure 2.2.10.1. Illustration representing the experimental parameters adapted for the fluorescence-based enzyme coupled assay (FBEC) experiment performed with FmtA^{Δ27} and its variants incubated with different concentrations of (0-15 μM/0-0.3 mg/ml) WTA for 2-hours at 37 °C before coupling with the working solution composed of DAO, FAD, HRP and Amplex red.

2.2.10.2 D-Alanine standard curve

A D-Ala standard curve was prepared to quantify the amount of D-Ala generated in the fluorescent-based enzyme-coupled assay assessing the enzymatic activity of FmtA^{Δ27} and its variants against WTA. This involved creating different concentrations of D-Ala ranging from 0 to 100 pM in 100 μl of reaction mixtures. The detection reagent was formulated in 50 mM Tris-HCl buffer (pH 8.5) containing Amplex red, Horseradish peroxidase (HRP), 2.5 μg/ml Flavin adenine

dinucleotide (FAD), and 0.05 µg/ml D-Amino acid oxidase (DAO), as established by previous studies (Gutheil et al., 2000; Qamar & Golemi-Kotra, 2012).

The assay was conducted in a 96-well plate by combining the reaction mixture and detection reagent in a 1:1 ratio, resulting in a final volume of 40 µl. This experiment was performed in triplicates, and the final reaction mixture was then incubated for an hour in the dark to allow for fluorescence development at room temperature. Subsequently, the fluorescence intensity was measured using a Synergy Hybrid H4 plate reader at excitation and emission wavelengths of 563 nm and 584 nm, respectively, at room temperature. Finally, the fluorescence intensity values were plotted against the corresponding D-Ala concentrations to generate the standard curve.

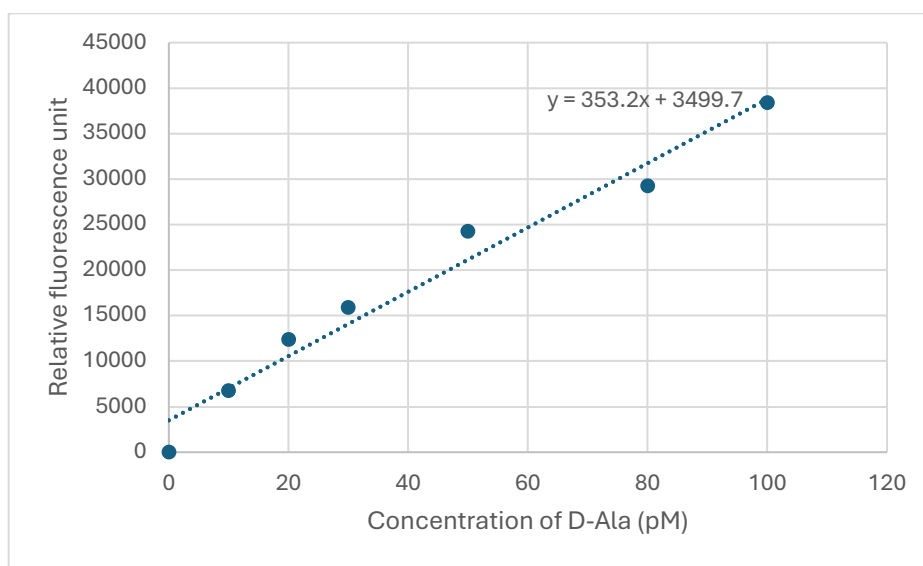


Figure 2.2.10.2. Graph representing the RFU vs D-Ala concentration standard curve

2.2.11 Study of enzyme kinetics of FmtA^{Δ27} and its variants by NMR

For the kinetic experiment, a solution of 130 µM (2.5 mg/ml) of WTA (final volume being 300 µl), isolated from RN4220, was prepared in 50 mM sodium phosphate buffer (pH 7.2) and 10% D₂O with 10 µM of FmtA^{Δ27} and its subsequent variants in a 3 mm NMR tube. The purpose of this study

was to determine and compare the kinetic rates of the variants with the wild-type protein, to assess the impact of mutagenesis on the protein's *in vitro* activity (Fig. 2.2.11).

The enzymatic release of D-Ala from WTA was monitored over 10 hours using 700 MHz ^1H -NMR at 25°C where the spectra were collected on a Bruker AV III 700 NMR spectrometer equipped with a 5 mm, liquid nitrogen-cooled H/C/N Prodigy probe. The 1D spectra were acquired using the standard “zgesgp” excitation sculpting pulse program, using 32,768 data points, a spectral width of 16 ppm, with a 7-second relaxation delay and 64 scans. The kinetic data was acquired using a Bruker automation program, which facilitates the acquisition of sequential data sets with identical parameters. The resulting data were analyzed with Topspin 4.3.0 and Dynamic Center 2.8.4 software (Bruker Ltd.). The function used to calculate the rate constant of the appearance of free D-Ala was $f(t) = I_0 * e^{(-kt)}$ where I_0 represents the initial value, k is the rate constant, and t is the time.

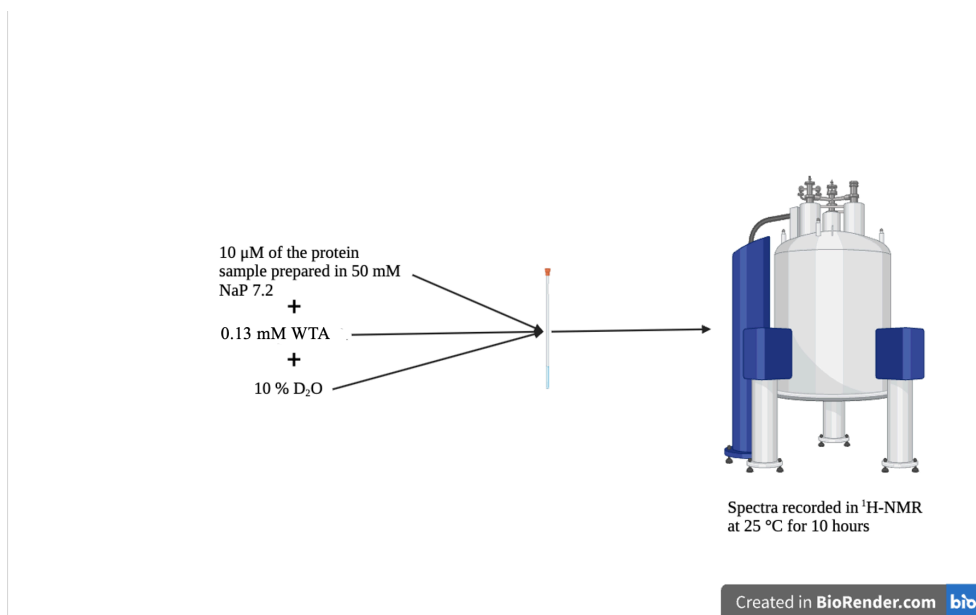


Figure 2.2.11. Illustration representing the experimental parameters adapted for the kinetic experiment designed with FmtA $^{\Delta 27}$ and its variants against WTA analyzed by ^1H -NMR where the chemical shift of ^1H is measured for the bound and free D-Ala

2.3 Results

2.3.1 Expression and purification of the FmtA^{Δ27} and its mutant proteins

The FmtA^{Δ27} was constructed and purified according to the protocol of Fan et al. (2007). Briefly, the construct lacks the first 27 amino acids to remove the signal peptide. This protocol typically yielded approximately 6 ml of 50 μM protein (95-98% pure) per litre of cell culture and the concentration of the purified mutant proteins was determined by UV-spectrophotometer using an extinction coefficient of $\epsilon_{280} = 56,160 \text{ M}^{-1}\text{cm}^{-1}$. In an SDS-PAGE gel run, the FmtA^{Δ27} protein appears as a monomer and a dimer (Fig. 2.3.1).

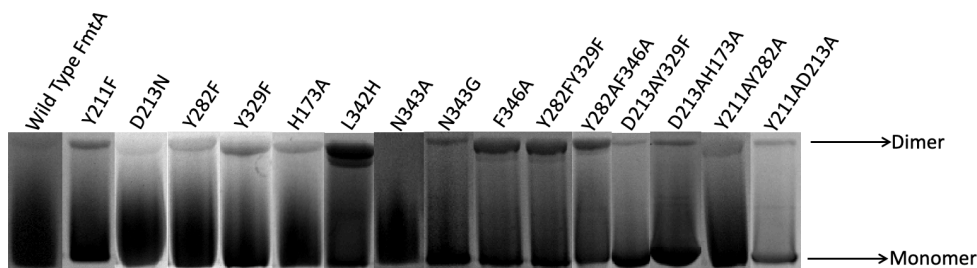


Figure 2.3.1. 12.5% SDS-PAGE analysis of purified FmtA^{Δ27} and mutant proteins, visualized using Coomassie blue staining on an SDS-polyacrylamide gel.

2.3.2 Analyzing the effects of the mutations on the conformation of the protein

The UV-CD spectra were analyzed for the wild type as well as for the variants, and the resultant graphs (Fig. 2.3.2) are comparable to the previously established data (Qamar & Golemi-Kotra, 2012), which suggests that the mutation did not affect the secondary structure of the protein.

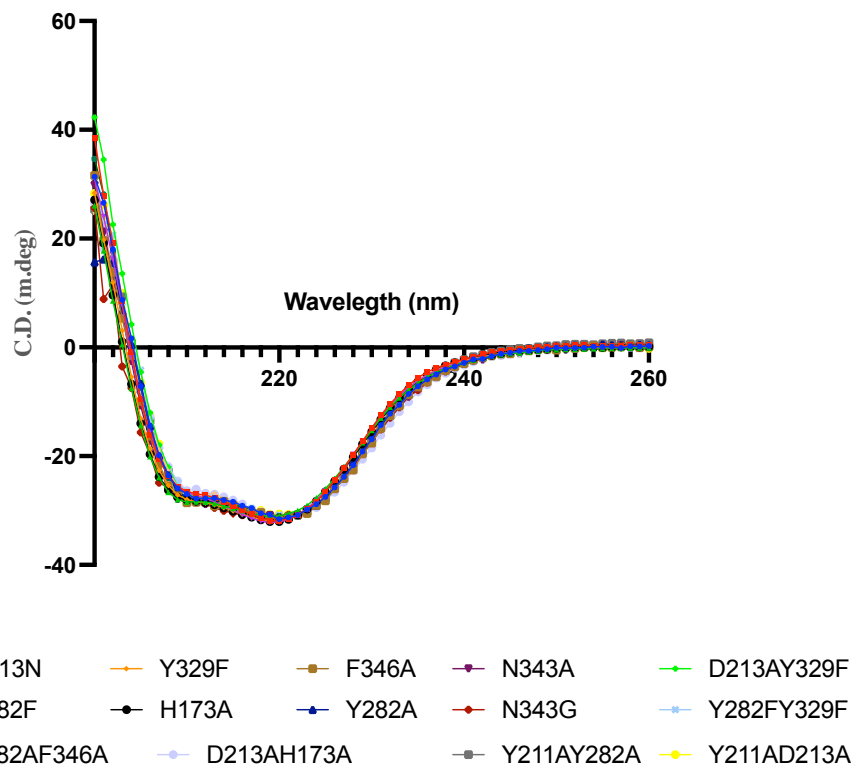
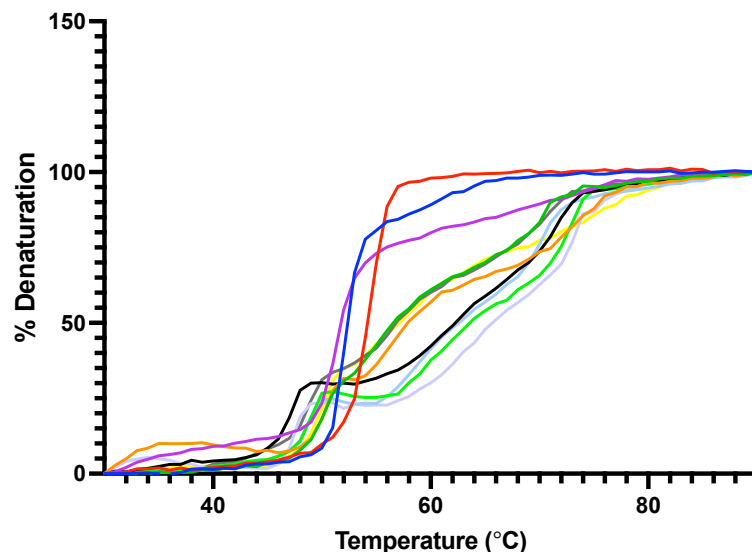


Figure. 2.3.2. Graphical representation of the data points collected by the C.D. spectrophotometer for the wild-type FmtA^{Δ27} compared to FmtA^{Δ27} variants. 200 μl of 10 μM protein samples were prepared in 50 mM sodium phosphate buffer (pH 7.2) and were subjected to CD spectroscopy for data acquisition. The data points are the average of three scans and were normalized for buffer signal

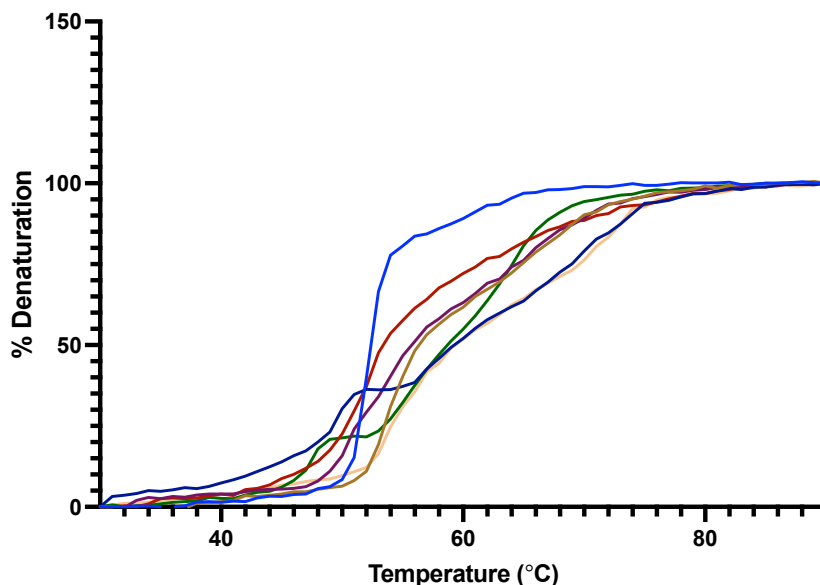
2.3.3 Analyzing how mutations affect the Thermal and Chemical stability of the FmtA^{Δ27} and its variants

The ability of a protein to maintain its structure in the presence of denaturants like heat or chemicals divulges its thermal or chemical stability. Heat-induced unfolding studies of wild-type FmtA^{Δ27} and its variants were performed to assess how the proposed substitutions, designed to probe the adaptations related to the protein's esterase activity and WTA binding, affect the thermal stability of the protein.. The value at 222 nm was monitored as it reflects the changes in the α-helix content of the protein.



— Wild Type — Y211F — Y282F — H173A — Y282FY329F — Y211AY282A
 — D213N — Y329F — D213AY329F — D213AH173A — Y211AD213A

Figure 2.3.3.1. Graph representing the data points collected at 222 nm by the CD spectrophotometer during thermal denaturation of the mutant proteins proposed to probe the adaptations in FmtA specific for its esterase activity, enabling the enzyme to hydrolyze ester bonds in wall teichoic acids.. 200 μ l of 10 μ M protein samples were prepared in 50 mM sodium phosphate (pH 7.2) for data acquisition



— Wild Type — F346A — N343A — L342H
 — Y282A — N343G — Y282AF346A

Figure 2.3.3.2. Graph representing the data points collected at 222 nm by the CD spectrophotometer during thermal denaturation of the mutant proteins proposed to probe the adaptations in FmtA specific for its WTA binding. 200 μ l of 10 μ M protein samples were prepared in 50 mM sodium phosphate (pH 7.2) for data acquisition

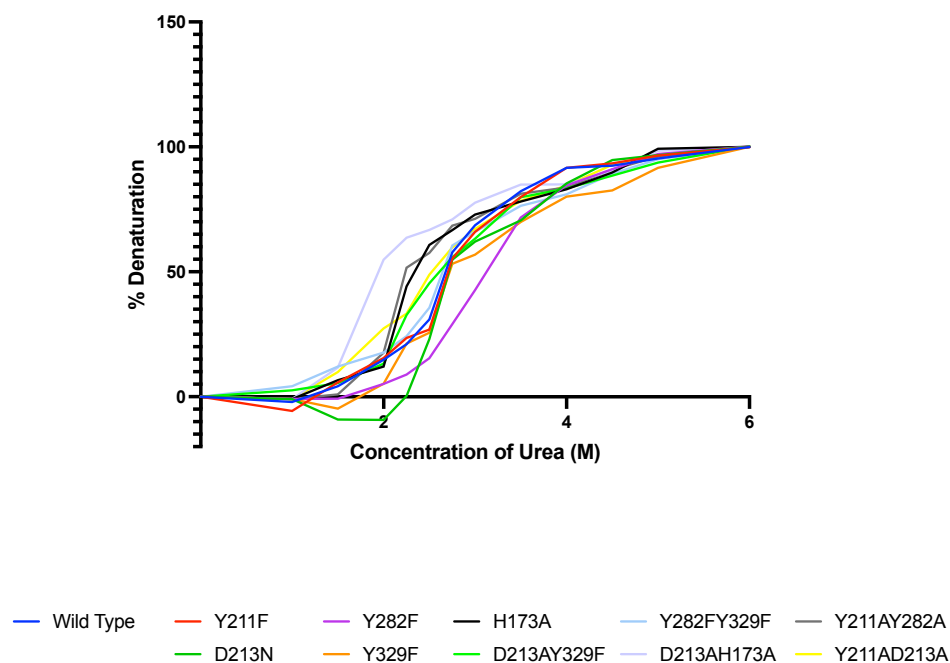


Figure 2.3.3.3. Graph representing the data points recorded by the CD spectrophotometer at 222 nm on 200 μ l of 10 μ M protein samples after 1-hour incubation at room temperature with an increasing concentration of urea (0-6 M) of the mutant proteins proposed to probe the adaptations in FmtA specific for its esterase activity.

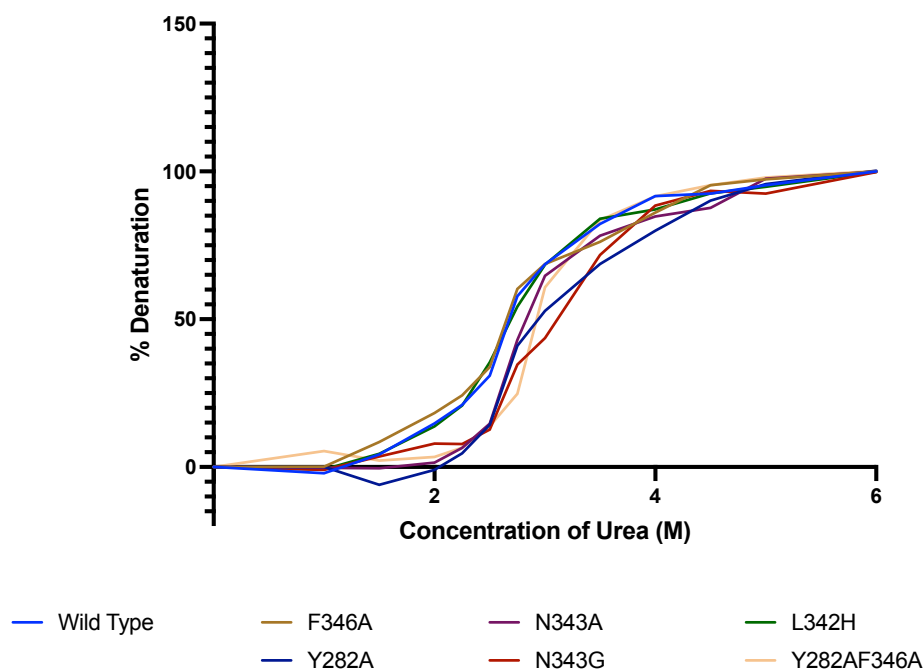


Figure 2.3.3.4. Graph representing the data points recorded by the CD spectrophotometer at 222 nm on 200 μ l of 10 μ M protein samples after 1-hour incubation at room temperature with an increasing concentration of urea (0-6 M) of the mutant proteins proposed to probe the adaptations in FmtA specific for its WTA binding.

Table 2.3.3.1: The table indicates the T_m and the chemical denaturation points of various FmtA variants with impacted esterase activity.

Protein	T_m (°C)	[Urea]_{50%} (M)
Wild Type	53	2.63
Y211F	56	2.63
D213N	57	2.63
Y282F	51	3.13
Y329F	58	2.63
H173A	62	2.3
D213AY329F	64	2.63
Y282FY329F	62	2.63
D213AH173A	66	2
Y211AY282A	57	2.13
Y211AD213A	58	2.4

Table 2.3.3.2: The table indicates the T_m and the chemical denaturation points of various FmtA variants with impacted WTA binding.

Protein	T_m (°C)	[Urea]_{50%} (M)
F346A	56	2.63
Y282A	59	2.9
N343A	55	2.8
N343G	53	3.13
L342H	58	2.63
Y282AF346A	59	2.9

Previous research by Qamar & Golemi-Kotra, (2012) established the thermal denaturation range of the wild-type protein to be within 50-52 °C. My analysis revealed that the wild-type protein exhibited 50% denaturation at 53°C, closely aligning with the established range. All other variants demonstrated increased thermal stability compared to the wild-type, except for the Y282F mutant, which showed a ~4% decrease in stability. The variants that exhibited enhanced thermal stability, namely Y211F, D213N, Y329F, H173A, F346A, Y282A, N343A, N343G, L342H, Y282AF346A, D213AY329F, Y282FY329F, D213AH173A, Y211AY282A, and Y211AD213A, displayed a ~6-20% increase in thermal stability where the most increase was recorded for the mutant D213AH173A (Table 2.3.3.1 and Table 2.3.3.2). Furthermore, except for the Y282F and Y211F variants, none of the other variants exhibited denaturation curves resembling that of the wild-type protein. Unlike the sharp transition observed in the wild-type, these variants displayed more gradual melting curves. Additionally, all variants except Y282F, Y211F, F346A, and Y282AF346A showed a distinct break around 25% denaturation before transitioning to a more gradual melting pattern (Fig. 2.3.3.1 and Fig 2.3.3.2).

Our group also established the chemical denaturation range for the wild-type protein to be between 2.5-2.75 M concentration of urea. In my experiments, the wild-type protein, along with the variants Y211F, D213N, Y329F, F346A, L343A, D213AY329F, and Y282FY329F, fell within this established range when their 50% denaturation points were compared to the range. However, the variants D213AH173A, Y211AY282A, and Y211AD213A exhibited a 9-24% decrease in stability, with the D213AH173A mutant demonstrating the most pronounced effect. Conversely, the variants Y282F, H173A, Y282A, N343A, N343G, and Y282AF346A showed increased stability, ranging from 6-16% (Table 2.3.3.1 and Table 2.3.3.2). In the case of the chemical denaturation curves, some discrepancies were also recorded when compared to the wild type. A steeper chemical denaturation curve was observed for the variants D213AH173A, H173A, D213N, Y282AY329F, Y211AY282A, and Y211AD213A compared to the wild-type protein. In contrast, the variants Y282A, F346A, N343A, N343G, Y282F, and Y282AF346A displayed a more gradual chemical denaturation curve relative to the wild type. The remaining variants exhibited chemical denaturation curves comparable to the wild type (Fig. 2.3.3.3 and Fig 2.3.3.4).

2.3.4 Analysis of the concentration-dependent binding between FmtA^{Δ27} and its variants with Bocillin

Our group focused on studying the binding characteristics of the FmtA^{Δ27} protein with Bocillin FL. Bocillin FL, a fluorescent derivative of penicillin, is widely used to investigate the interaction of β -lactam antibiotics with penicillin-binding proteins (PBPs), which are crucial for cell wall biosynthesis (G. Zhao et al., 1999). The binding of Bocillin FL to PBPs has been well-documented, revealing the specificity of these interactions (Qamar & Golemi-Kotra, 2012).

Komatsuzawa et al. (1997) suggested that FmtA^{Δ27}, although not a PBP, exhibits penicillin-recognizing properties, suggesting its potential role in cell wall metabolism and antibiotic

resistance. Studies by Qamar & Golemi-Kotra, (2012) have shown that FmtA^{Δ27} can bind to Bocillin FL, albeit with lesser kinetics and affinities compared to traditional PBPs. This differential binding indicates that while FmtA^{Δ27} shares some structural similarities with PBPs, it also possesses unique properties that contribute to its specific role in *S. aureus* physiology, particularly its D-amino esterase activity.

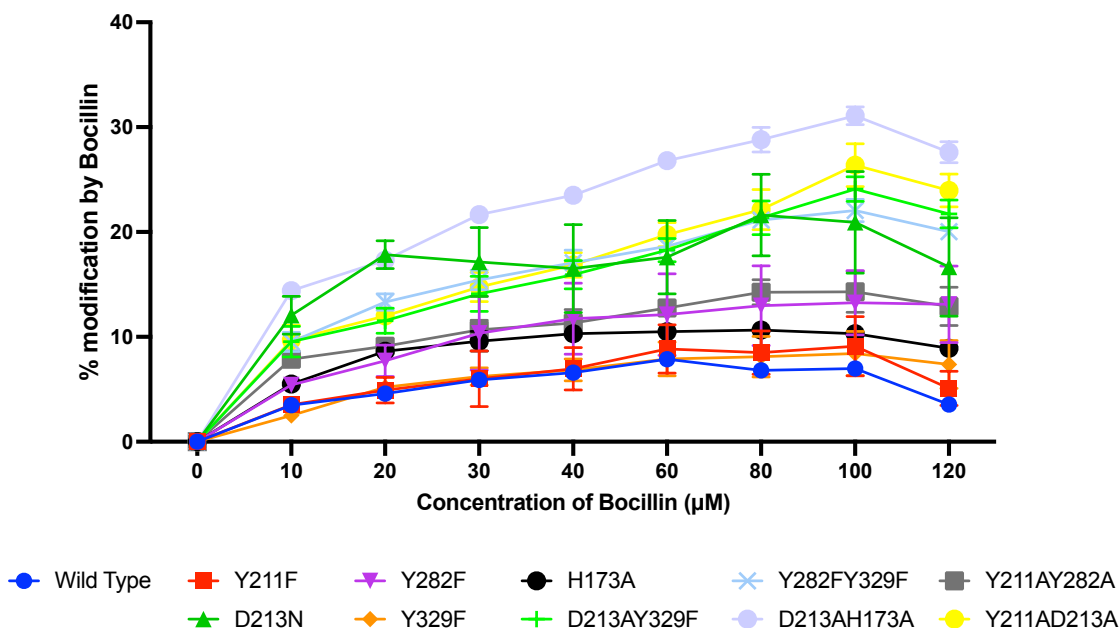


Figure 2.3.4.1. Binding curve representing the % modification by Bocillin on the FmtA^{Δ27} variants proposed to probe the adaptations in FmtA specific for its esterase activity. 10 μM of the protein samples were incubated with a gradient of Bocillin FL (0-120 μM) for an hour in the dark at 37 °C before running in a nPAGE/H⁺ gel. 4 μM BlaR incubated with 120 μM Bocillin was used as a control in these experiments. Error bars represent the standard deviation calculated from three independent trials. The values are relative to the control and the values were adjusted for the difference between the control and experimental concentration before presenting, taking the difference in concentration between the control and the proteins into account.

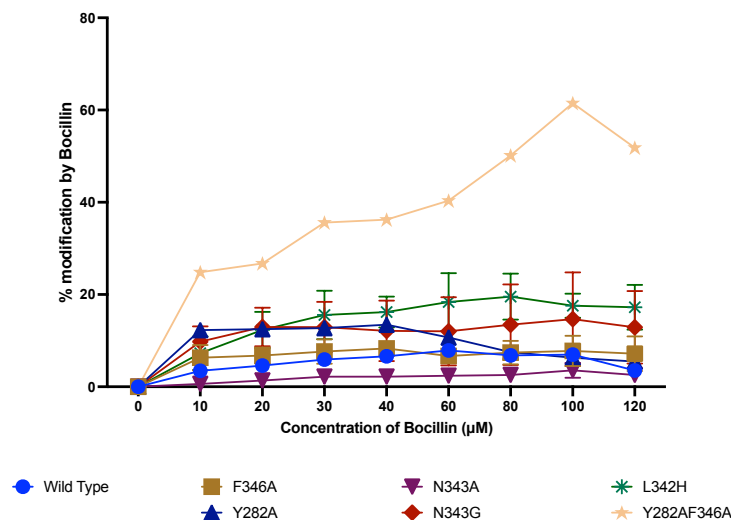


Figure 2.3.4.2. Binding curve representing the % modification by Bocillin on the FmtA^{Δ27} variants proposed to probe the adaptations in FmtA specific for its WTA binding. 10 μM of the protein samples were incubated with a gradient of Bocillin FL (0-120 μM) for an hour in the dark at 37 °C before running in a nPAGE/H⁺ gel. 4 μM BlaR incubated with 120 μM Bocillin was used as a control in these experiments. Error bars represent the standard deviation calculated from three independent trials. The values are relative to the control and the values were adjusted for the difference between the control and experimental concentration before presenting, taking the difference in concentration between the control and the proteins into account.

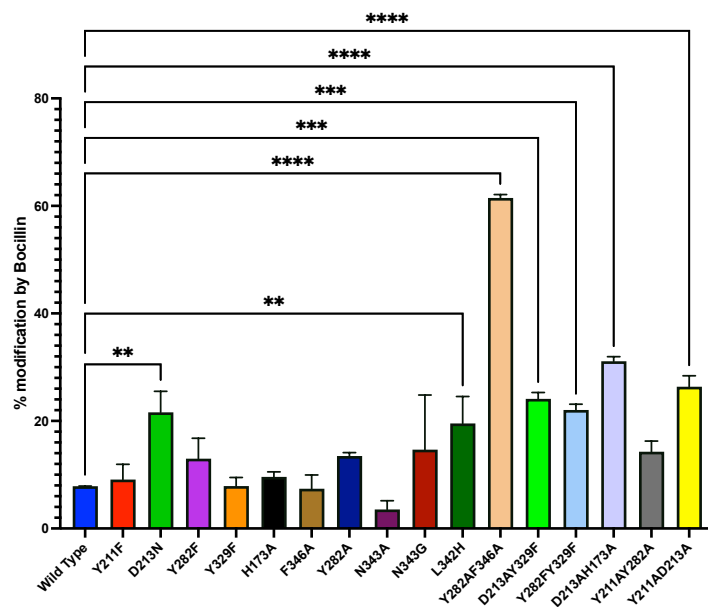


Figure 2.3.4.3. Statistical analysis of the % Bocillin modification for the FmtA^{Δ27} variants under their respective optimal conditions. Data represent the mean ± SD from three independent experiments. Statistical significance was evaluated using a parametric one-way ANOVA followed by Dunnett's multiple comparison test (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$). Variants without indicated pairwise comparisons were not statistically significant.

To draw a comparison, I used the BlaR protein as a reporter protein in my studies, known for undergoing complete modification with Bocillin FL (Golemi-Kotra et al., 2003). My results indicate that neither FmtA^{Δ27} nor the generated variants achieve complete modification by Bocillin FL, corroborating previous findings about their low binding affinity towards β-lactams such as Bocillin FL (Fan et al., 2007).

However, most variants exhibit altered binding to Bocillin FL compared to the wild-type, with the exception of Y211F, Y329F and F346A, which show binding curves comparable to the wild-type in this experiment. Among the variants designed to probe the enzymatic activity of FmtA^{Δ27}, D213A, Y282F, H173A, D213AY329F, D213AH173A, Y282AY329F, Y211AY282A, and Y211AD213A demonstrate increased binding of variable degrees (2.3-3.8 folds) to Bocillin FL compared to their wild-type counterparts (Fig. 2.3.4.1). Among the variants generated to probe the protein's modifications to accommodate WTA as a substrate binding, several variants—specifically Y282A, N343G, L342H, and Y282AF346A—exhibited varying degrees of enhanced affinity for FmtA^{Δ27} where the most prominent (7.6 folds) change was noticed in the Y282AF346A mutant. Notably, the N343A mutant uniquely displayed a reduced binding affinity (0.5 folds) relative to the wild type (Fig. 2.3.4.2).

A statistical analysis of Bocillin FL binding across the variants versus the wild type, each under their respective binding saturation points, reveals that only the variants D213N, L342H, Y282AF346A, D213AY329F, D213AH173A, Y211AD213A, and Y282AY329F exhibited statistically significant increases (Ranging from 3.8-7.6 folds) in binding compared to the wild-type (Fig. 2.3.4.3).

2.3.5 Analysis of the time-dependent interaction between FmtA^{Δ27} and its variants with Bocillin

As the binding studies with Bocillin FL demonstrated varying degrees of binding affinity of the variants, a time-course dependent interaction experiment was also performed to elucidate the time required for these variants to attain a maximum binding compared to the wild-type and determine and compare their rates of interaction.

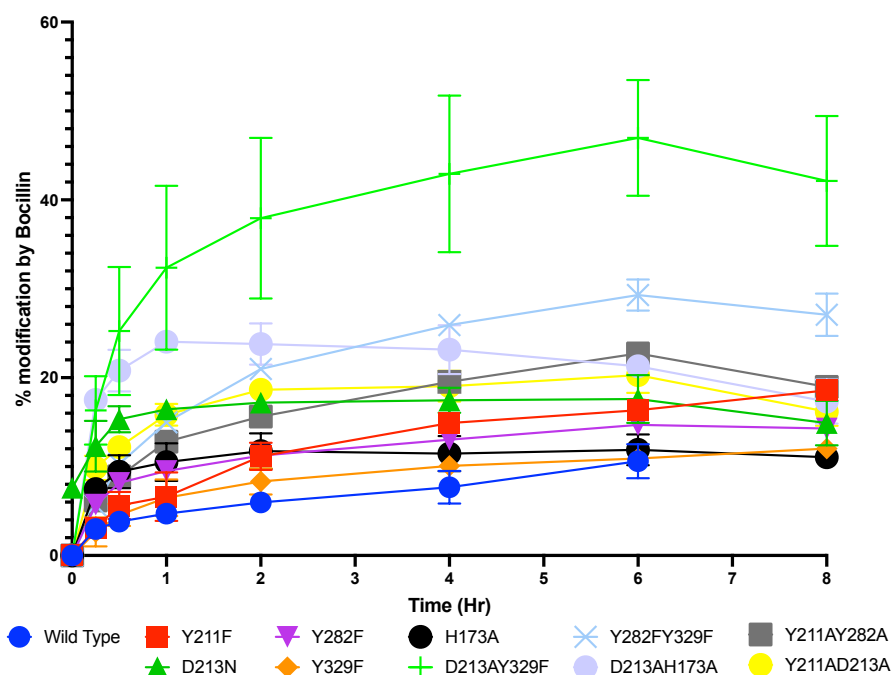


Figure 2.3.5.1. Analysis of the % modification by Bocillin on the FmtA^{Δ27} mutations proposed to probe the adaptations in FmtA specific for its esterase activity. 10 μM of the protein samples were incubated with 100 μM Bocillin FL for different periods (0-8 hours) in the dark at 37 °C before running in a nPAGE/H⁺ gel. 4 μM BlaR incubated with 120 μM Bocillin for an hour was used as a control in these experiments. Error bars represent the standard deviation calculated from three independent trials. The values are relative to the control, and the values were adjusted before presenting, taking the difference in concentration between the control and the proteins into account.

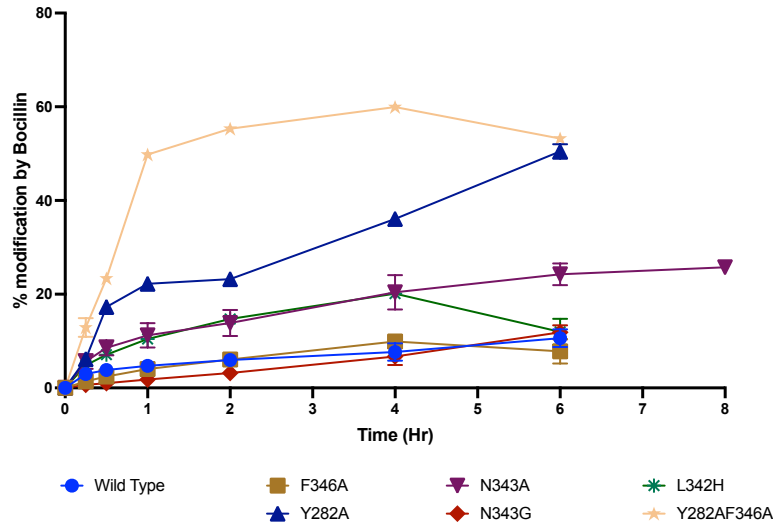


Figure 2.3.5.2. Analysis of the % modification by Bocillin on the FmtA^{Δ27} variants proposed to probe the adaptations in FmtA specific for its WTA binding. 10 μ M of the protein samples were incubated with 100 μ M Bocillin FL for different periods (0-8 hours) in the dark at 37 °C before running in a nPAGE/H⁺ gel. 4 μ M BlaR incubated with 120 μ M Bocillin for an hour was used as a control in these experiments. Error bars represent the standard deviation calculated from three independent trials. The values are relative to the control and the values were adjusted before presenting, taking the difference in concentration between the control and the proteins into account.

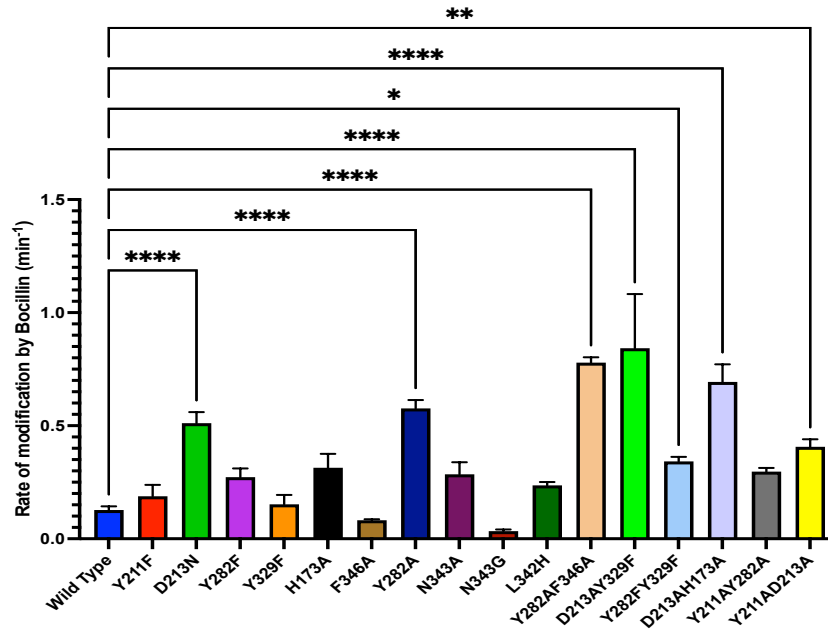


Figure 2.3.5.3. Statistical analysis of the Rate of complex formation between Bocillin and FmtA^{Δ27} variants. Rates were calculated by the modification percentage at each 30-minute interval by the corresponding time. Data represent the mean $\pm$ SD from three independent experiments. Statistical significance was evaluated using a parametric one-way ANOVA followed by Dunnett's multiple comparison test (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$). Variants without indicated pairwise comparisons were not statistically significant.

Variable times were observed for different variants in terms of achieving maximum binding between Bocillin FL and the proteins compared to the wild-type. Specifically, variants such as D213A, H173A, D213AH173A, and Y211AD213A reached binding saturation within 1-2 hours of incubation (Figure 2.3.5.1). Meanwhile, the variants L342H and Y282AF346A required approximately 3 and 4 hours, respectively, to achieve similar saturation, whereas the wild-type protein took about 6 hours (Figure 2.3.5.2). Notably, at 37°C, several variants, namely F346A, Y282A, N343G, L342H, and Y282AF346A, along with the wild-type protein, degraded after 6 hours of incubation at 37°C, and thus, data for these variants are only available up to 6 hours of incubation (Figure 2.3.5.2). Furthermore, the wild-type protein, along with variants Y211F, Y282A, Y329F, and N343A, did not reach saturation within the duration of the experiment. (Figure 2.3.5.1 and Figure 2.3.5.2).

A comparison of the Bocillin modification rates between the variants and the wild-type revealed a statistically significant increase in variants D213N, Y282A, Y282AF346A, D213AY329F, Y282FY329F, D213AH173A, and Y211AD213A (Figure 2.3.5.3), with rates showing an increase of 4- to 8-folds compared to the wild type.

2.3.6 Comparison of WTA binding by FmtA^{Δ27} and its variants

Structurally, FmtA^{Δ27} bears a close resemblance to PBPs (Dalal et al., 2019). However, its functional role diverges significantly from the typical functions associated with PBPs (which are predominantly involved in bacterial peptidoglycan synthesis) (Qamar & Golemi-Kotra, 2012). FmtA^{Δ27} exhibits D-amino esterase activity, specifically removing D-alanine from WTA (Rahman et al., 2016).

Here I investigated the variations in binding between different concentrations of WTA (isolated from RN4220 cells) with the mutant FmtA^{Δ27} proteins in comparison to the wild type (Fig. 2.3.6.1).

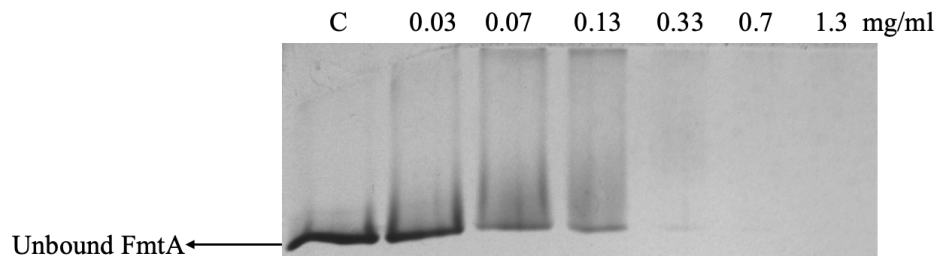
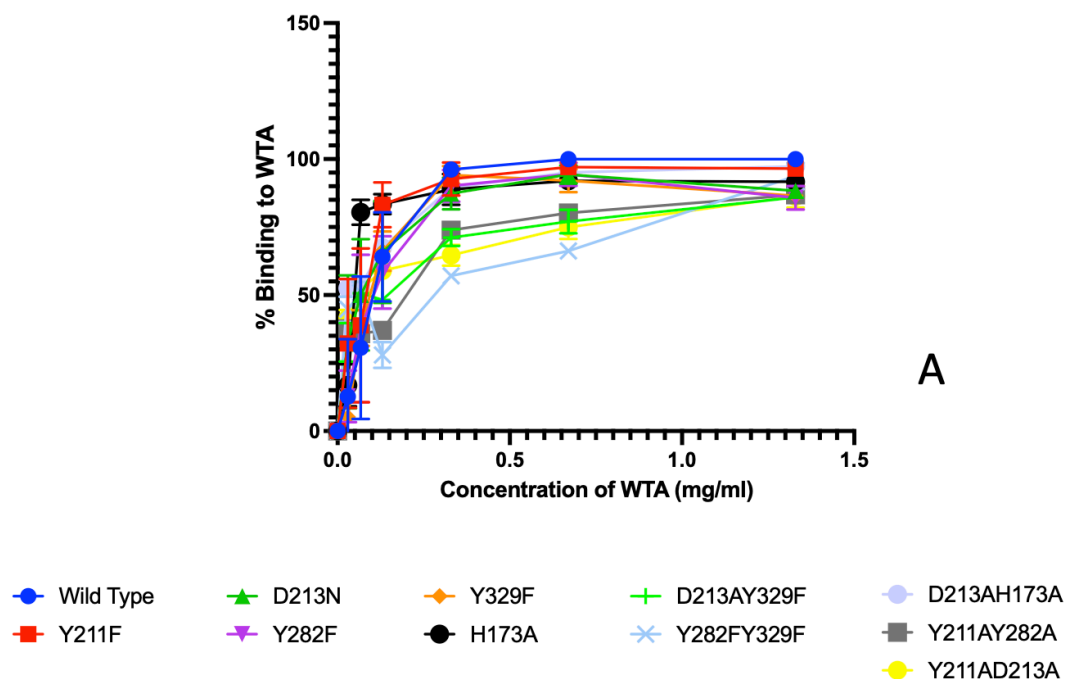


Figure 2.3.6.1. Exemplary 10% nPAGE/H⁺ gel run of the reaction mixtures consisting of 10 μ M FmtA^{Δ27} alone (C) and 10 μ M FmtA^{Δ27} with 0-1.3 mg/ml (0-67 μ M) WTA. The reactions were carried out in 50 mM Sodium phosphate buffer pH 7.2. 15 μ l reaction mixtures were incubated at 37 °C for 1 hour before electrophoresis. Here C is the control protein sample without WTA.



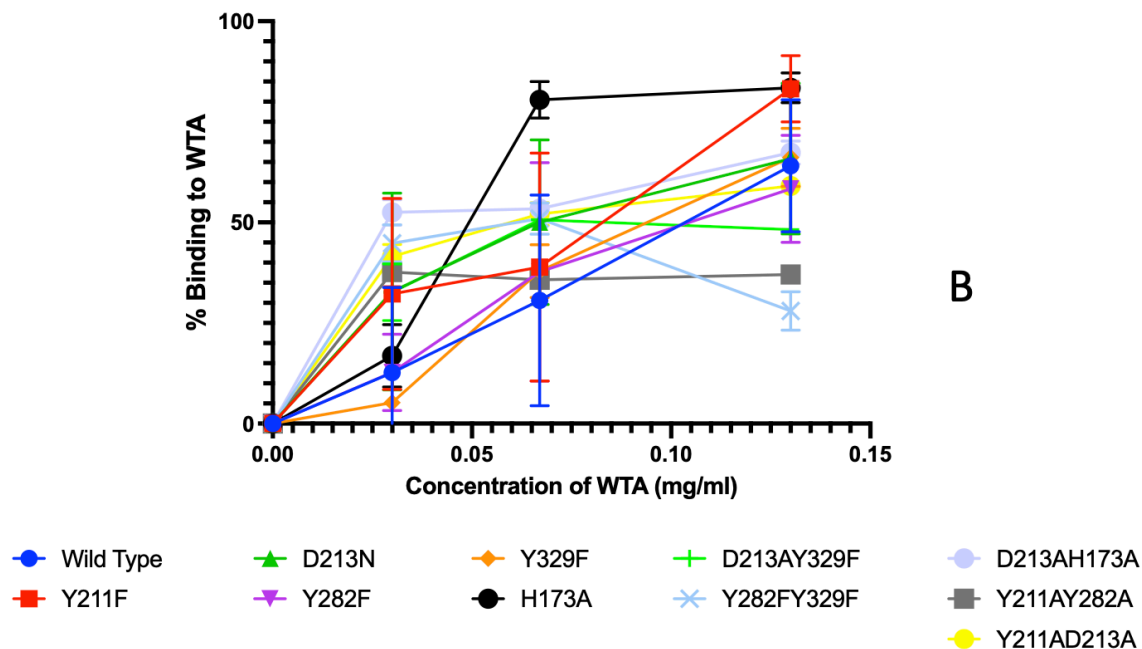
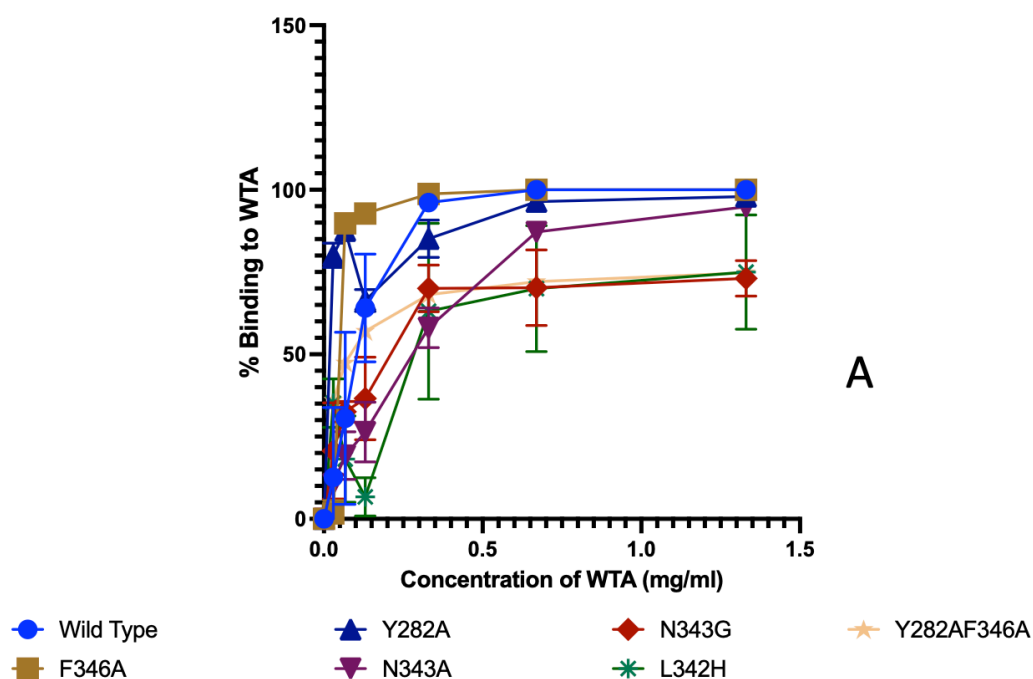


Figure 2.3.6.2. A. Binding curve representing the % binding between WTA and the FmtA^{Δ27} mutations proposed to probe the adaptations in FmtA specific for its esterase activity. **B.** An enlarged graph displaying data points up to 0.15 mg/ml (8 μM) for improved clarity and detail. 10 μM of the protein samples were incubated with (0-1.3 mg/ml WTA) (0-67 μM) for an hour at 37 °C before running in a nPAGE/H⁺ gel. 10 μM of the protein incubated without WTA for an hour was used as a control in these experiments. Error bars represent the standard deviation calculated from three independent trials.



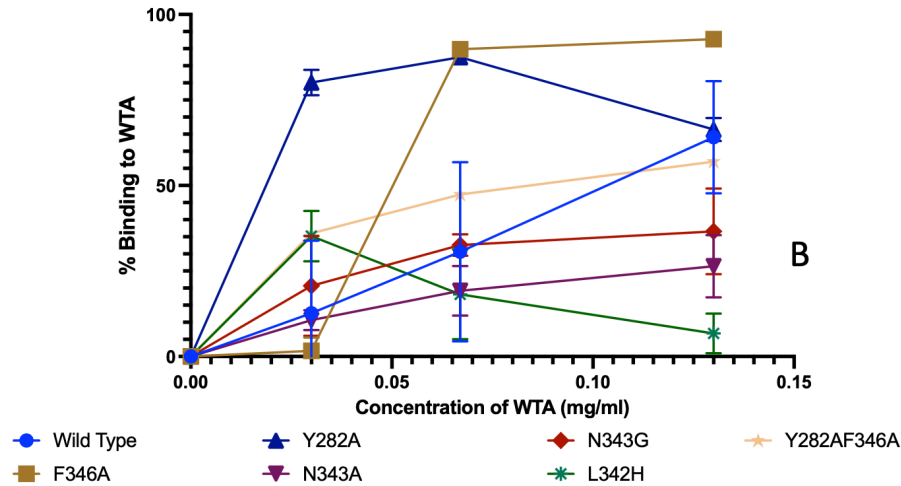


Figure 2.3.6.3. A. Binding curve representing the % binding between WTA and the FmtA Δ^{27} variants generated the mutations proposed to probe the adaptations in FmtA specific for its WTA binding. **B.** An enlarged graph displaying data points up to 0.15 mg/ml (8 μ M) for improved clarity and detail. 10 μ M of the protein samples were incubated with (0-1.3 mg/ml WTA) (0-67 μ M) for an hour at 37 °C before running in a nPAGE/H⁺ gel. 10 μ M of the protein incubated without WTA for an hour was used as a control in these experiments. Error bars represent the standard deviation calculated from three independent trials.

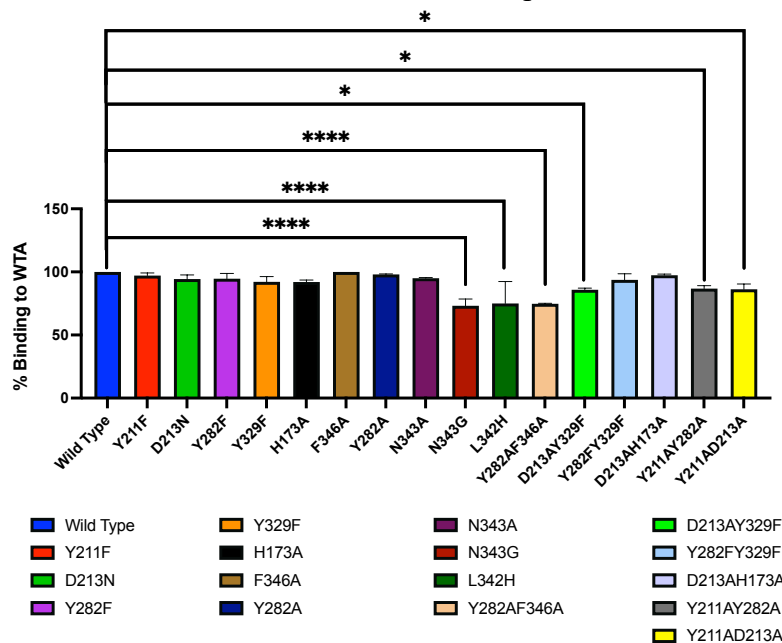


Figure 2.3.6.4. Statistical analysis of the % Binding of WTA with the FmtA Δ^{27} variants at their individual optimal conditions. Data represent the mean $\pm$ SD from three independent experiments. Statistical significance was evaluated using a parametric one-way ANOVA followed by Dunnett's multiple comparison test (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$). Variants without indicated pairwise comparisons were not statistically significant.

Among the mutations proposed to probe the adaptations that occurred in the FmtA conserved catalytic residues that led to the esterase activity towards WTA, none of the variants were able to reach a complete binding like the wild type, and the variants involved demonstrated a maximum of 10% reduction in WTA binding (Fig 2.3.6.2). In the variants designed to investigate adaptations in the FmtA active site for accommodating WTA as a substrate, a more pronounced decrease in WTA binding (approximately 30%) was observed, except in the F346A and Y282A variants. Specifically, the variants L342H, N343G, and Y282AF346A each showed around a 30% reduction in WTA binding (Fig 2.3.6.3).

When comparing all the variants based on their optimum WTA binding capabilities only the variants N343G, L342H, Y282AF346A, D213AY329F, Y211AY282A and Y211AD213A demonstrated a statistically significant reduction in WTA binding meanwhile the other variants seem to be comparable to the wild type in WTA binding (Fig 2.3.6.4).

2.3.7 Analysis of the esterase activity of FmtA^{Δ27} and its variants against WTA through fluorescence-based enzyme-coupled assay

This enzyme-coupled assay was employed to evaluate deviations in the esterase activity of FmtA variants compared to the wild-type protein.

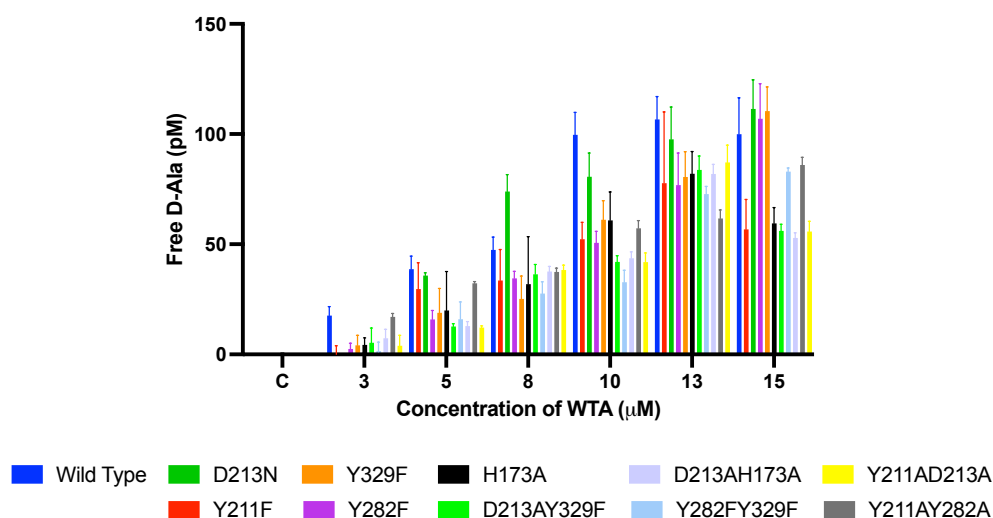


Figure 2.3.7.1. Analysis of the free D-Ala released from WTA in the presence of wild-type FmtA^{Δ27} and the mutations proposed to probe the adaptations in FmtA specific for its esterase activity. Increasing amounts of WTA (0-15 μ M/0-0.3 mg/ml) were incubated with 10 μ M of the proteins at 37 °C for 2 hours after which the reaction mixtures were again incubated in a 96-well plate with the reaction mixture consisting of DAO, HRP and Amplex red at RT in the dark for an hour. After which the resultant fluorescence was measured in the Synergy H4 Hybrid Reader at an excitation wavelength of 563 nm and emission wavelength of 584 nm. Experiments were done in triplets and the results were averaged. From the average data, the amount of Free D-Ala released was calculated from a D-Ala standard curve prepared beforehand.

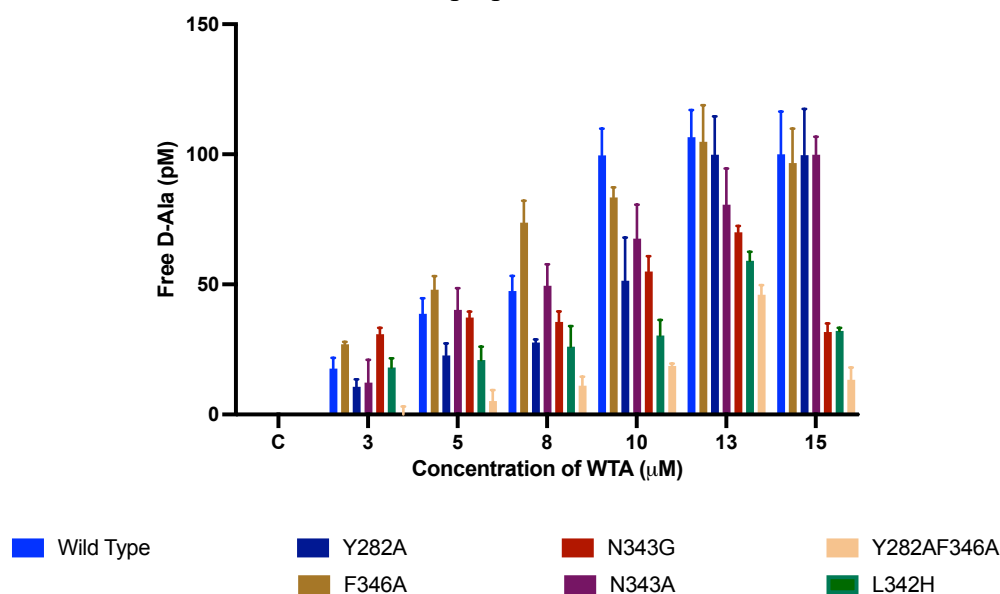


Figure 2.3.7.2. Analysis of the free D-Ala released from WTA in the presence of wild-type FmtA^{Δ27} and the mutations proposed to probe the adaptations in FmtA specific for its WTA binding. Increasing amounts of WTA (0-15 μ M/0-0.3 mg/ml) were incubated with 10 μ M of the proteins at 37 °C for 2 hours after which the reaction mixtures were again incubated in a 96-well

plate with the reaction mixture consisting of DAO, HRP and Amplex red at RT in the dark for an hour. After which the resultant fluorescence was measured in the Synergy H4 Hybrid Reader at an excitation wavelength of 563 nm and emission wavelength of 584 nm. Experiments were done in triplets and the results were averaged. From the average data, the amount of Free D-Ala released was calculated from a D-Ala standard curve prepared beforehand.

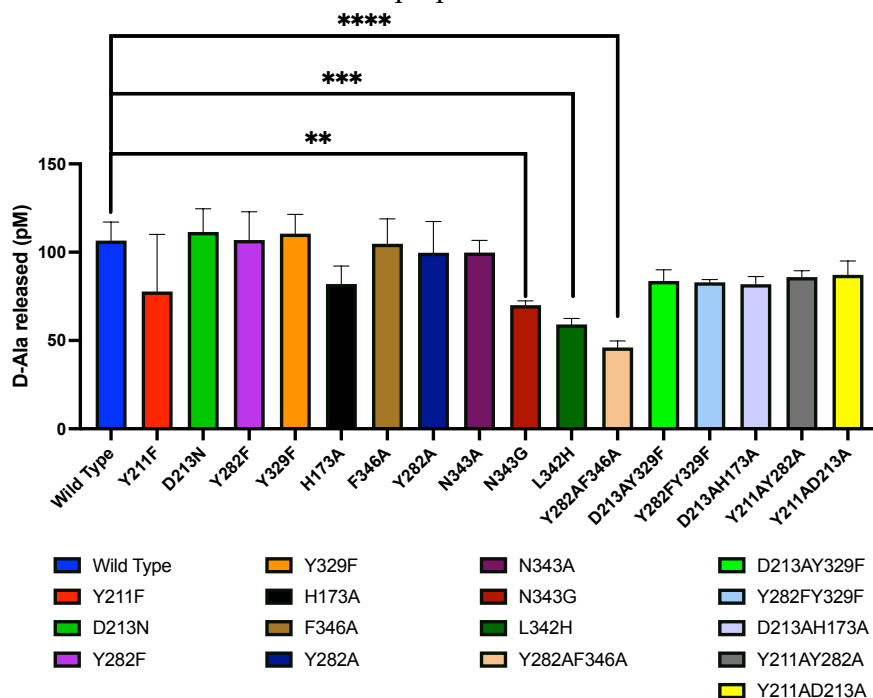


Figure 2.3.7.3. Statistical analysis of the free D-Ala released from WTA in the presence of the FmtA^{Δ27} variants at their individual optimal conditions. Data represent the mean $\pm$ SD from three independent experiments performed in triplicate. Statistical significance was evaluated using a parametric one-way ANOVA followed by Dunnett's multiple comparison test (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$). Variants without indicated pairwise comparisons were not statistically significant.

A variable release of D-Ala was observed among the listed variants, with only Y282A and F346A demonstrating levels comparable to the wild type. Notably, a subset of these variants, including Y211F, H173A, N343G, L342H, Y282AF346A, D213AY329F, D213AH173A, and Y211AD213A, reached a saturation point at 13 μ M (0.25 mg/ml) concentration of WTA, similar but to a lesser extent (According to the concentration of D-Ala released) than the wild type. In contrast, the remaining variants did not reach saturation within the experimental parameters (Fig. 2.3.7.1 and Fig. 2.3.7.2).

When comparing the maximum concentration of D-Ala released by each mutant within the experimental conditions, only the reduced WTA binding observed in the N343G, L342H, and Y282AF346A variants showed a statistically significant change compared to the wild type (Fig. 2.3.7.3).

2.3.8 Analysis of the esterase activity of FmtA^{Δ27} and its variants against WTA through ¹H-NMR

Rahman et al. (2016) outlined a method for assessing the enzymatic activity of FmtA^{Δ27} against WTA by tracking the chemical shift of the free D-Ala released from WTA in the presence of the protein over time. Following this approach, the impact of specific mutations on FmtA's D-amino esterase activity was examined. The release of D-Ala from WTA by FmtA^{Δ27} and its variants were monitored using ¹H-NMR. Rate constants were averaged from two independent trials to ensure the accuracy and reproducibility of the kinetic activity measurements.

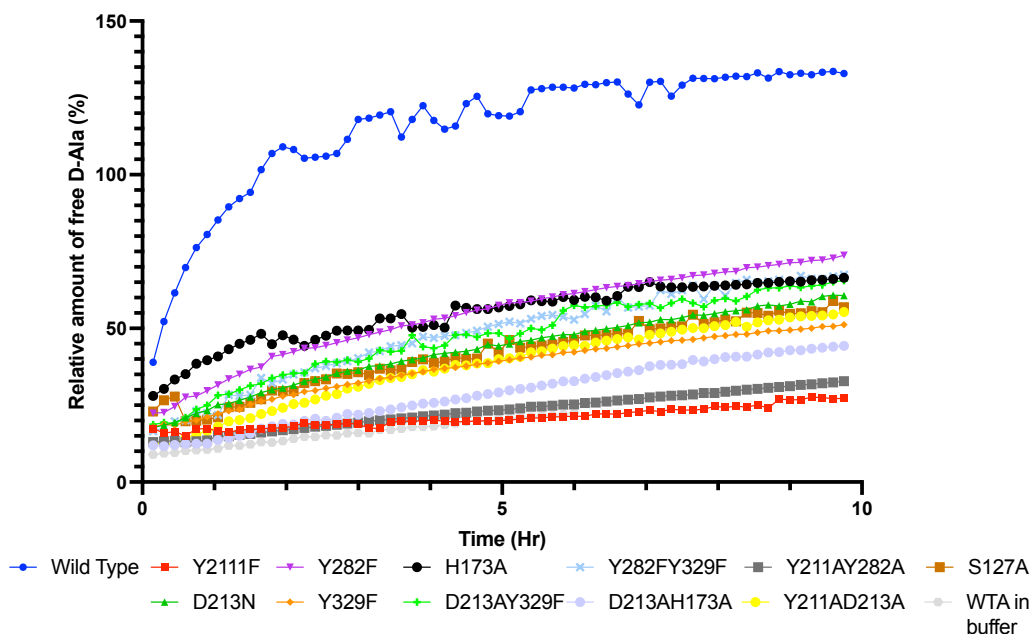


Figure 2.3.8.1. Graph representing the percent relative free D-Ala released from WTA by the FmtA^{Δ27} mutations proposed to probe the adaptations in FmtA specific for its esterase activity. The relative amount of free D-Ala was calculated in comparison to the initial bound D-Ala.

10 μ M of the wild-type FmtA protein and its variants were mixed with 130 μ M (2.5 mg/ml) WTA at 25 $^{\circ}$ C and the kinetics of the D-Ala released were monitored by NMR.

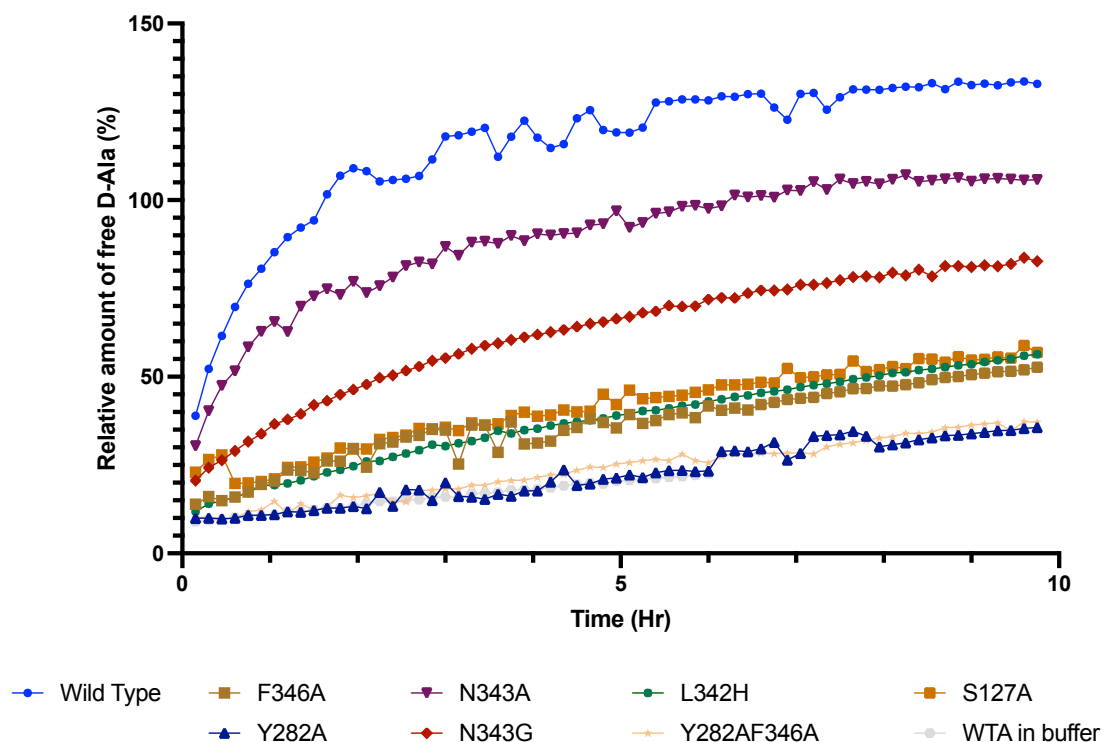


Figure 2.3.8.2. Graph representing the percent relative free D-Ala released from WTA by the FmtA ^{Δ 27} mutations proposed to probe the adaptations in FmtA specific for its WTA binding. The relative amount of free D-Ala was calculated in comparison to the initial bound D-Ala. 10 μ M of the wild-type FmtA protein and its variants were mixed with 130 μ M (2.5 mg/ml) WTA at 25 $^{\circ}$ C and the kinetics of the D-Ala released were monitored by NMR.

Table 2.3.8.1: The table shows the remaining activity of FmtA variants as measured by ^1H -NMR. This variants carry mutations which are proposed to be involved in the catalysis of FmtA.

Protein	Activity (%)	k (/h)
Wild Type	100	0.6±0.1
Y211F	38	0.23±0.03
D213N	20	0.12±0.03
Y282F	33	0.2±0.007
Y329F	23	0.14±0.05
H173A	38	0.23±0.03
D213AY329F	27	0.16±0.03
Y282FY329F	27	0.16±0.01
D213AH173A	10	0.06±0.04
Y211AY282A	7	0.04±0.01
Y211AD213A	23	0.14±0.02

Table 2.3.8.2: The table shows the remaining activity of FmtA variants as measured by $^1\text{H-NMR}$. This variants carry mutations which are proposed to be involved in binding of WTA.

Protein	Activity (%)	k (/h)
F346A	17	0.1±0.02
Y282A	7	0.04±0.02
N343A	67	0.4±0.07
N343G	39	0.23±0.007
L342H	16	0.1±0.01
Y282AF346A	7	0.04±0.02
S127A	15	0.09
WTA in Buffer	10	0.06

The kinetic analysis of FmtA $^{\Delta 27}$'s esterase activity on WTA via NMR revealed significant impacts on the rate constants for variants Y282A, Y282AF346A, Y211AY282A, and D213AH173A (Fig 2.3.8.1 and Fig. 2.3.8.2, Table 2.3.8.1 and Table 2.3.8.2). These variants exhibit only about 7-10% of the remaining activity compared to the wild type. Additionally, variants L342H, F346A, D213N, Y329F, Y282FY329F, and D213AY329F show a moderate decrease in activity, with a reduction of 70-80% compared to the wild type. The N343A mutant displayed an activity level closest to the wild type, retaining approximately 70% activity (a 30% decrease) compared to the wild type (Fig 2.3.8.1 and Fig. 2.3.8.2, Table 2.3.8.1 and Table 2.3.8.2).

2.6 Discussion

Based on the structural analysis of FmtA $^{\Delta 27}$ by Dalal et al. (2019), several hypotheses were proposed regarding the protein's ability to accommodate WTA as a substrate. These hypotheses

also explore how the active site of the protein is specifically adapted to exhibit catalytic activity against WTA, drawing parallels with other known PRPs to elucidate the mechanistic basis for this interaction. Specifically, PRPs with well-characterized functions and elucidated structures that share significant homology with FmtA^{Δ27} were closely examined. By juxtaposing their structural features with those of FmtA^{Δ27}, particularly the conserved amino acid residues within the active site, I sought to infer how these residues might influence the enzymatic function of FmtA^{Δ27}. The comparative analysis highlighted how specific amino acid residues, occupying critical positions within the protein's active site, could modulate FmtA's enzymatic activity, especially in terms of its adaptation to WTA as a substrate. In essence, these assumptions underscore the role of active site architecture in accommodating WTA and facilitating the catalytic D-amino esterase activity of FmtA.

The mutations performed were classified into two different categories. The first category consists of the residues subjected to targeted mutagenesis to elucidate the molecular adaptations that may have occurred to facilitate the enzyme's catalytic activity against WTA. Focusing on the first category of targeted residues, the rationale driving the proposed mutagenesis experiments is based on the structural predictions articulated in our lab's seminal work (Dalal et al., 2019). This study postulates that the tyrosine residue at position 211 serves a critical anchoring function, ensuring the precise spatial orientation of the residue Ser127, thereby enabling it to function as the active site nucleophile. Concurrently, Lys130 residue located in the active site is hypothesized to undertake the role of a general base, facilitating the catalytic activity of the enzyme. The targeted mutation of Tyr211 was proposed to assess its mechanistic role through site-directed mutagenesis.

Furthermore, the mutations proposed for tyrosine at position 329 and histidine at position 173 aim to investigate the hypothesized hydrogen-bonding present between these residues and the

Tyr211 residue. This network is believed to be crucial for donating electrons to Tyr211, compensating for its electron deficiency induced in the process of stabilizing the nucleophile Ser127. Finally, the mutagenesis of the aspartate at position 213 and tyrosine at position 282 is intended to elucidate their potential roles within this hydrogen-bonding network, specifically in stabilizing Lys130 during its function as a general base.

In the context of the double variants under investigation, strategic mutations were introduced in the Asp213 and Tyr329 residues concurrently, as well as in the Asp213 and His173 residues. The rationale behind this approach is predicated on the hypothesis that these mutations will provide deeper insights into the catalytic mechanism of FmtA as Asp213 is postulated to play a crucial role in stabilizing Lys130 during its function as a general base in catalysis, while Tyr329 and His173 are theorized to contribute to the stabilization of Tyr211. Simultaneously mutating two residues, the experimental design aims to disrupt both the pathways presumed necessary for catalysis, thereby offering a more comprehensive understanding of the indispensability of these residues in FmtA's enzymatic function.

A similar strategic rationale underlies the proposal to mutate Tyr282 and Tyr329 together. Tyr282 is implicated in the stabilization of Lys130, while the role of Tyr329 is documented before. Moreover, the simultaneous mutation of Tyr211 with Tyr282 and Tyr211 with Asp213 is intended to elucidate the interconnected roles of these residues in the catalytic mechanism of FmtA, potentially unveiling their cooperative interaction.

The second category comprises residues selected for targeted mutagenesis to unravel the molecular adaptations that may have evolved to accommodate WTA as a substrate. The rationale for mutating Tyr282 to alanine stems from insights presented in recent literature (Dalal et al., 2019), which propose that Tyr282 likely plays a critical role in WTA binding by contributing to

the solvent-exposed entrance of the active site. We assumed that altering this residue may disrupt the binding affinity between the protein and FmtA^{Δ27}. A similar hypothesis applies to the Phe346 residue, which is also targeted for mutation to assess its proposed role in configuring the active site entrance for optimal WTA binding. Finally, a double mutant was proposed to comprehensively investigate the combined effects of these residues on WTA binding.

Furthermore, the proposal to mutate the Asn343 residue to alanine is intended to test the hypothesis that this residue contributes to creating a spatial environment conducive to WTA binding. A further strategy involves mutating Asn343 to glycine, drawing inspiration from the structural analogs within the PRPs, where glycine occupies the second position of the KTG-box in DAP, facilitating a broader side chain substrate specificity (Remaut et al., 2001).

Lastly, the proposed mutation of Leu342 is suggested to explore its hypothesized role in forming a pocket adjacent to Tyr211, which is believed to accommodate the D-Ala moiety of teichoic acid. This mutation aims to examine whether Leu342 fills a structural space unique to FmtA, a feature absent in its structural homolog DD-carboxypeptidase (DDCP), which instead possesses a histidine at this position (Dalal et al., 2019). By replacing Leu342, we seek to validate its functional significance in the enzyme's interaction with its substrate.

Mutagenesis is an invaluable tool to investigate the roles of specific amino acid residues in the construction and functionality of an enzyme's active site. However, several critical considerations must be addressed after employing mutagenesis as an analytical approach. One of the paramount concerns is the preservation of the protein's secondary structure, as maintaining structural integrity is essential for the protein's proper function. It is imperative to verify that the introduction of the mutations does not alter the protein's overall conformation. My analysis revealed no significant differences in the secondary structures of the mutant proteins compared to their wild type

counterpart. This finding demonstrates that any functional deviations observed in the mutant proteins are attributable to the introduced mutations only rather than any unintended structural alterations.

Furthermore, the alterations in the thermal and chemical stability of the proteins were also thoroughly investigated. In terms of thermal stability, comparing the temperatures at which 50% denaturation occurs, the N343G mutant showed similar stability to the wild type, while Y211F and N343A variants exhibited a negligible change of ± 2 °C. Most variants demonstrated increased thermal stability, and except for the Y211A and Y282F variants, all others displayed a more gradual melting curve compared to the wild type's steep curve. This suggests that the introduced mutations confer greater thermal stability compared to the original amino acids at those positions, enhancing the overall stability of the protein relative to the wild type. Furthermore, the altered melting curves may indicate that the FmtA protein's active site core has been disrupted, potentially exposing it to temperature-induced denaturation as two separate domains, the all α -helical domain and the α/β domain each with distinct thermal stabilities. Moreover, these are likely indicative of hydrophobic regions being exposed due to the mutations, which in turn accelerates protein aggregation a common phenomenon reported by Wetzel, (1994). This aggregation likely results in increased heat resistance during the denaturation process and an altered melting curve.

In the chemical denaturation experiment, based on the concentration of urea at which 50% denaturation occurs, the variants Y211F, D213N, Y329F, F346A, L343A, D213AY329F, and Y282FY329F showed comparable stability to the wild-type, suggesting that these mutations did not affect the protein's chemical stability. In contrast, the variants D213AH173A, Y211AY282A, and Y211AD213A exhibited a 9-24% decrease in stability, suggesting that these mutations had a modest impact on the protein's chemical stability. These alterations may affect protein

conformation, increasing its susceptibility to denaturants while minimally exposing the active site to reduce the risk of aggregation. Conversely, the variants Y282F, H173A, Y282A, N343A, N343G, and Y282AF346A demonstrated a slight increase in chemical stability. Additionally, all variants displayed noticeable changes in their denaturation curves, supporting the idea that the FmtA protein's active site core may have been disrupted, potentially exposing the protein to chemical-induced denaturation as two distinct domains: the all- α helical domain and the α/β domain, each with its own chemical stability.

For decades, Bocillin FL has been a choice of reporter substrate to elucidate the penicillin-binding of PBPs (Gee et al., 2001; Shapiro et al., 2013; G. Zhao et al., 1999). Moreover, due to structural similarities with PBPs and demonstrating PRP structural characteristics, our lab also investigated the interaction between Bocillin FL and FmtA ^{Δ 27} (Fan et al., 2007; Qamar & Golemi-Kotra, 2012). In alignment with the findings of Fan et al, (2007) and Qamar & Golemi-Kotra, (2012), my report reaffirms that neither the wild-type FmtA ^{Δ 27} protein nor its respective variants exhibit an affinity for Bocillin FL comparable to that of an ideal PBP, BlaR (control). Among the variants, Y282AF346A displayed the highest binding affinity, yet even at its peak, it achieved only 60% of the control's binding capability. Amongst the other variants demonstrating significant increases in Bocillin FL binding, were limited to 20-30% of BlaR's binding, indicating that the mutations did not elevate the protein to the control's binding levels.

However, a discernible pattern emerges, particularly involving residues D213 and Y282. In my investigation, mutations in these residues, whether individually or in combination, were associated with enhanced Bocillin FL binding compared to the wild-type protein. For example, the D213 residue is frequently observed in the variants under investigation either as a single mutation or as part of a double mutation that exhibits an increased affinity for Bocillin FL. The D213

residue, which corresponds to a significant position in known PBPs where instead of aspartic acid (D), asparagine (N) residues play a crucial role in penicillin-binding, is particularly noteworthy. This suggests that the FmtA^{Δ27} protein, having evolved from a PBP, may regain some of its ancient functionality when this residue is mutated. Additionally, the Y282 residue, when mutated in conjunction with F346, showed the most notable increase in Bocillin FL affinity. Given that these residues are located at the entrance of the active site, their mutation might reduce the active site's entrance size, making it more conducive to Bocillin FL binding. Similarly, the mutant L342H, where the L342 residue is hypothesized to accommodate the methyl group of WTA, showed increased Bocillin FL affinity. This result supports the idea that mutating L342 to a histidine, akin to typical PBPs, might indeed enhance Bocillin FL binding, validating the predicted role of the L342 residue.

Along with binding studies, I also investigated a time-dependent interaction between Bocillin FL and the variants. Consistent with our lab's previous research, my results indicate that the wild-type FmtA^{Δ27} protein did not reach saturation within the 8-hour experimental period. Similarly, many of the variants also failed to achieve a binding saturation within this timeframe. This suggests that both the wild-type and these variants exhibit a slow acylation rate towards Bocillin FL and do not undergo a complete modification within the scope of the experiment. In an ideal infection model, this rate of acylation is considered slow. This is because *S.aureus* has a doubling time of 30 minutes whereas these variants along with the wild type only undergo a partial modification in 6 hours. This allows the protein to perform its enzymatic activity within each generational cycle of the bacteria uninterrupted even under antibiotic stress. However, some variants demonstrated early saturation with Bocillin FL, indicating an enhanced acylation rate compared to the wild type. Despite this, their modification in the presence of Bocillin FL was

limited to 60% of the control. While variants such as D213A, H173A, D213AH173A, and Y211AD213A showed acylation rates comparable to the control, and the variants L342H and Y282AF346A exhibited faster rates compared to the wild type (but slower than the control), none of these variants achieved modification levels like that of the control. This suggests that although these variants have increased affinity or faster acylation rates compared to the wild type, their overall effectiveness in binding penicillin derivatives remains suboptimal.

In this study, my primary focus was to probe the adaptations in the active site of the FmtA^{Δ27} protein that distinguishes it from other PBPs and contributes to its unique D-amino esterase activity. Building on the findings of Rahman et al. (2016), which demonstrated the enzymatic activity of FmtA^{Δ27} against WTA, and the structural elucidation by Dalal et al. (2019), this experiment aimed to determine whether mutations in amino acid residues proposed to be involved in either the enzymatic activity or binding of WTA would indeed affect their interaction with WTA. Among the engineered variants, N343G, L342H, and Y282AF346A supported our hypothesis, showing a significant reduction in WTA binding compared to the wild type. The N343 residue, located in the β13 strand region of FmtA^{Δ27}, is believed to have a role in determining the side chain of the substrate that the protein can accommodate. In a known PBP; DAP, this position is occupied by glycine, opening the space for proteins binding to substrates with larger side chains. A similar substitution in FmtA^{Δ27} led to a notable decrease in WTA binding suggesting that this residue is important in WTA binding. The L342 residue is proposed to shape the active site to accommodate the methyl group of WTA. In this study, the statistically significant decrease in WTA binding observed in the L342H mutant supports the validity of this hypothesis. Similarly, mutations in the Y282 and F346 residues, which form the entrance to the active site, may have partially obstructed WTA entry, leading to a significant reduction in binding.

Additionally, the variants D213AY329F, Y211AY282A, and Y211AYD213A intended primarily to disrupt the protein's enzymatic activity also exhibited a statistically significant reduction in WTA binding, albeit around 10%, which is less pronounced than the 30% decrease observed in the earlier-mentioned variants. This reduction can likely be attributed to the disruption of the hydrogen bonding network within the active site caused by these mutations. These disruptions may induce subtle changes in the active site that affect enzymatic activity and partially hinder substrate binding.

In addition to analyzing the difference in binding between the variants and WTA, I also investigated their differences in enzymatic activity through an enzyme-coupled assay. This enzyme-coupled assay provides an effective tool for quantifying the D-Ala released by the mutant proteins, allowing us to assess the impact of specific mutations on enzymatic activity in comparison to the wild type. The observed reduction in activity, except in variants Y282A and F346A, suggests that the mutations being investigated are indeed critical for substrate binding and catalysis. Furthermore, variants such as D213N, Y282A, Y329F, N343A, Y282AY329F, and Y211AY282A did not reach saturation within the experimental capacity, indicating a slower hydrolysis rate, underscoring the significance of these residues within the active site. Notably, only the N343G, L342H, and Y282AF346A variants showed a statistically significant decrease in D-Ala release, which aligns with their reduced WTA binding observed in previous experiments, further validating their role in enzyme activity.

In comparison to the FBEC assay, ¹H-NMR is a more effective and precise experimental tool for evaluating enzymatic activity and detecting differences in the interactions between FmtA^{Δ27} variants and WTA relative to the wild type. Following the experimental framework established by Rahman et al. (2016) and later utilized by Dalal et al. (2019), the chemical shift

peaks of free D-Ala resulting from the interaction between the protein and WTA and recorded over 10 hours provide a valuable means to determine and compare their kinetic rates. Two negative controls were also included for reference: the inactive mutant S127A, which retains 15% of protein activity, and WTA in buffer, showing 10% activity. These controls help establish a baseline for characterizing the variants based on changes in their enzymatic activity.

All the variants under investigation exhibited varying degrees of rate kinetics, with N343A (67%) being the closest to wild-type enzyme activity. Compared to the negative control, the variants Y282A, F346A, L342H, D213AH173A, Y282AF346A, and Y211AY282A, which demonstrated enzymatic activities ranging from 7% to 20%, showed the most significant reduction in enzymatic activity, rendering these variants functionally similar to the inactive S127A mutant. Most of these variants also displayed significantly reduced WTA binding in the WTA binding experiment and were confirmed by the FBEC assay to release significantly less D-Ala compared to the wild type, which supports the observed reduction in activity.

A notable addition to this list is the Y211AY282A mutant. The Y211 residue is within hydrogen bonding distance and is assumed to stabilize the S127 residue (the active site nucleophile), while Y282 is near the K130 residue (the general base). When both Y211 and Y282 are mutated together, the functions of these key residues (S127 and K130) are likely disrupted, leading to the observed significant reduction in enzymatic activity.

In summary, the Bocillin FL experiments provide compelling evidence that FmtA has evolved from PBPs to specifically bind WTA, as mutations at key positions mirroring those found in PBPs highly sensitive to β -lactams enhanced Bocillin FL binding in the mutant proteins compared to the wild type. However, no significant improvements were observed in their acylation rates against Bocillin FL, indicating that while these mutations increase Bocillin FL uptake in the

variants, they do not make the protein functionally equivalent to a PBP. Additionally, these residues were also hypothesized to play a crucial role in WTA binding and enzymatic activity, with some variants showing activity levels comparable to an inactive mutant, highlighting the importance of these residues in WTA binding and hydrolysis. The variants Y282A, F346A, L342H, D213AH173A, Y282AF346A, and Y211AY282A, which fit this category, are further investigated in the next chapter to explore how these mutations affect the physiological activity of *S. aureus* in an *in vivo* setting.

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Chapter Three

Probing the activity of FmtA^{Δ27} through *in vivo* experimentation

3.1 Introduction

In *Staphylococcus aureus*, the bacterial cell wall plays a crucial role in maintaining structural integrity, mediating environmental interactions, and resisting external stresses, with WTAs as key components (Nikolic & Mudgil, 2023). Like all Gram-positive bacteria, the *S. aureus* cell wall is primarily composed of peptidoglycan (PG), which forms a mesh-like structure around the bacterial membrane. PG acts as the bacterium's primary defense against osmotic pressure, mechanical stress, and antimicrobial agents, such as lysozyme, lysostaphin, and detergents like Triton X-100 (Shimada et al., 2010; V. K. Singh et al., 2010).

The synthesis and modification of PG are highly regulated, involving several essential genes (e.g., the *mur* group, *pbp* group, *ftsW*, *rodA*, *oatA*), many of which are critical for bacterial growth, survival, and virulence (Egan et al., 2020). Among these, *oatA* encodes an enzyme that modifies peptidoglycan, enhancing *S. aureus*' ability to evade host defenses (Sychantha et al., 2017). Additionally, *S. aureus* WTAs are modulated by the incorporation of D-alanine esters (Neuhaus & Baddiley, 2003). This modification reduces the overall negative charge of the cell wall, influencing how *S. aureus* interacts with its environment and responds to external stressors (R. Sobral & Tomasz, 2019). Furthermore, biofilm formation is a key survival strategy for *S. aureus*, allowing the bacterium to thrive under adverse conditions. These biofilms provide a protective barrier against host immune defenses and significantly increase resistance to antimicrobial treatments (Guo et al., 2022).

3.1.1 Interaction of Triton X-100 with *S. aureus* Cell Wall and their Mechanisms of Resistance

Triton X-100 is a nonionic detergent widely used in microbiology for lysing cells by disrupting cell membranes (Cornett & Shockman, 1978). In *S. aureus*, Triton X-100 disrupts the cell wall and membrane integrity by solubilizing lipids and destabilizing the cell envelope (Cordwell et al., 2002). While *S. aureus* is sensitive to Triton X-100 at higher concentrations, certain strains resist its lytic effects, often through modifications to the cell wall structure, including changes in the charge of WTAs via D-alanylation (Raychaudhury & Chatterjee, 1985). By modifying the charge through D-alanylation, *S. aureus* can reduce the negative charge of WTAs, thereby decreasing the cell surface's affinity for cationic molecules and other stress-inducing agents like Triton X-100 (Rawlinson et al., 2011). This is because Triton X-100 is more effective in disrupting negatively charged membranes, the lower surface negativity decreases the detergent's ability to bind to and solubilize the membrane, thus conferring resistance (Cordwell et al., 2002). D-alanylation has also been shown to enhance the stability of the cell wall by promoting tighter cross-linking within the PG layer (Cordwell et al., 2002). This additional structural integrity helps the cell resist the lytic effects of detergents like Triton X-100, which would otherwise weaken the cell wall, leading to cell lysis (Song et al., 2021).

Triton X-100 is also known to activate endogenous autolysins (Cornett & Shockman, 1978). D-alanylation of WTAs has been shown to inhibit the activity of autolysins by altering the charge environment around the PG layer, thus reducing the likelihood of autolysis upon exposure to Triton X-100 (Neuhaus & Baddiley, 2003). Here, D-alanylation acts as a protective mechanism against internal (autolytic) stresses. Strains of *S. aureus* that are deficient in the *dlt* operon, and thus lack D-alanylation, exhibit increased sensitivity to Triton X-100 (Koprivnjak et al., 2006). This heightened susceptibility is attributed to the unmodified WTAs, which retain their full

negative charge, making the cell wall more vulnerable to lysis by Triton X-100 (Vergara-Irigaray et al., 2008). The absence of D-alanylation also increases the accessibility of autolysins to the PG layer, leading to higher rates of autolysis in response to detergent exposure (Grishin et al., 2020).

3.1.2 Interaction of Lysozyme with *S. aureus* Cell Wall and their Mechanisms of Resistance

Lysozyme is an antimicrobial enzyme that forms part of the innate immune response in humans where its primary role is to degrade the PG in bacterial cell walls, thereby causing cell lysis (Ragland & Criss, 2017). Lysozyme targets the β -(1,4) glycosidic bonds between N-acetylmuramic acid and N-acetylglucosamine in the peptidoglycan, which is critical for the structural integrity of bacterial cells (Brott & Clarke, 2019). Being a cationic protein, lysozyme carries a net positive charge. By reducing the negative charge on the bacterial cell surface through D-alanylation, *S. aureus* decreases electrostatic attraction between lysozyme and the bacterial cell wall (Saar-Dover et al., 2012) thus reducing its enzymatic activity and decreasing the likelihood of cell wall degradation. WTAs are covalently linked to the peptidoglycan and, by their proximity, can shield the glycan strands that lysozyme targets (Caudill et al., 2020). When D-alanylated, WTAs form a more protective barrier around the PG, making it physically harder for lysozyme to access and cleave the critical β -(1,4) glycosidic bonds (Brown et al., 2013). This shielding effect is particularly important in resisting the host's immune response, allowing *S. aureus* to evade lysozyme-mediated killing.

S. aureus strains deficient in the *dlt* operon display increased sensitivity to lysozyme, as the heightened negative charge promotes stronger electrostatic interactions between lysozyme and the cell surface, enhancing lysozyme's ability to bind and degrade the PG (Herbert et al., 2007). The absence of D-alanylation also leads to a weaker, more vulnerable cell wall structure (Bertsche et al., 2011). This demonstrates that the presence of D-alanine esters on WTAs is crucial for

reducing lysozyme susceptibility. In addition to altering the WTA charge, *S. aureus* can modify its PG to reduce lysozyme activity. For example, the O-acetylation of the peptidoglycan, catalyzed by the *oatA* gene, prevents lysozyme from cleaving its glycan strands (Sythantha et al., 2017).

3.1.3 Interaction of Lysostaphin with *S. aureus* Cell Wall and their Mechanisms of Resistance

Lysostaphin is a bacteriolytic enzyme that specifically targets *S. aureus* by cleaving the pentaglycine cross-bridges in the peptidoglycan layer of the bacterial cell wall (Bastos et al., 2010). This cleavage leads to rapid cell lysis and death, making lysostaphin a potent antimicrobial agent against *S. aureus*, including methicillin-resistant strains (MRSA) (Bastos et al., 2010). While lysostaphin is highly effective, *S. aureus* has developed resistance mechanisms, through modifications to WTAs, such as D-alanylation. (Climo et al., 2001)

Lysostaphin resistance in *S. aureus* is closely linked to the charge and structural configuration of the cell wall, both of which are modulated by WTAs and their D-alanylation (Brown et al., 2012). Lysostaphin, like many antimicrobial peptides, is positively charged (Bastos et al., 2010) and by reducing the negative charge of the cell wall through D-alanylation, *S. aureus* creates a repulsive electrostatic environment that hinders the binding of lysostaphin to the cell wall. This decreases the likelihood of lysostaphin coming into close proximity with its target, the pentaglycine cross-bridges, thereby impairing its ability to cleave the peptidoglycan effectively (Climo et al., 2001). Moreover, by modifying WTAs through D-alanylation, *S. aureus* can indirectly affect the accessibility of the pentaglycine cross-links that lysostaphin targets. The presence of D-alanine residues in WTAs can create a more compact and stabilized cell wall structure, making it more difficult for lysostaphin to access and degrade the pentaglycine bridges (R. Sobral & Tomasz, 2019; Wysocka et al., 2022). This increased structural integrity further enhances lysostaphin resistance (Wysocka et al., 2022). Furthermore, D-alanylation of WTAs

modulating autolysin activity is important in lysostaphin resistance. A more stable cell wall is less susceptible to degradation by autolysins and external agents like lysostaphin (Grishin et al., 2020).

S. aureus strains that are deficient in the *dlt* operon, thus exhibit increased sensitivity to lysostaphin (Koprivnjak et al., 2006). The lack of D-alanine modification enhances lysostaphin's ability to bind and cleave the pentaglycine cross-links in the peptidoglycan (Peschel et al., 2000). This increased sensitivity highlights the protective role of D-alanylation in modulating cell wall charge and resistance (Peschel et al., 2000). Restoration of the *dlt* operon in D-alanylation-deficient variants rescues lysostaphin resistance, confirming that D-alanylation is a key factor in mediating resistance (Kiriukhin & Neuhaus, 2001).

3.1.4 The Regulation of O-Acetylation and their protective role in *S. aureus* Physiology

The OatA enzyme catalyzes the transfer of an acetyl group from acetyl coenzyme A (acetyl-CoA) to the C6 hydroxyl group of N-acetylmuramic acid (MurNAc), a critical component of the PG backbone (Jones et al., 2021). This modification takes place after the PG has been incorporated into the cell wall and cross-linked during the final stages of cell wall biosynthesis (Jones et al., 2021). The OatA-mediated O-acetylation is known to increase the resistance of *S. aureus* peptidoglycan to enzymatic degradation (Baranwal et al., 2017). For example, the O-acetylation of the MurNAc residue in *S. aureus* sterically hinders lysozyme access to this bond, preventing the enzyme from effectively degrading the PG (Pushkaran et al., 2015). O-acetylation also increases the overall stability of the PG layer by reducing the activity of endogenous autolysins (Brott & Clarke, 2019).

Once inside the host, *S. aureus* must contend with the immune system's efforts to clear the infection. O-acetylation reduces the effectiveness of lysozyme and other PG-degrading enzymes, enabling *S. aureus* to persist in the host and avoid destruction by immune cells (Ragland & Criss,

2017). The *oatA* gene also contributes directly to the virulence of *S. aureus* (Jones et al., 2021). Strains lacking functional *oatA* or deficient in O-acetylation are more susceptible to lysozyme and are generally less virulent in animal infection models (Jones et al., 2021). This suggests that the ability to modify peptidoglycan through O-acetylation is a key modulator of *S. aureus* pathogenicity.

The expression of the *oatA* gene is tightly regulated and can be influenced by environmental factors and host interactions (Jones et al., 2021). Two-component systems (TCS) are common regulatory mechanisms in bacteria that allow for rapid adaptation to changing environmental conditions (Rapun-Araiz et al., 2020). These systems typically consist of a sensor kinase and a response regulator (Rapun-Araiz et al., 2020). In *S. aureus*, certain TCSs regulate the expression of cell wall-related genes, including *oatA*. For example, the WalkR two-component system, which responds to cell wall stress, modulates *oatA* expression in response to antibiotics or immune challenges (J. Zhu et al., 2021). In addition to TCS, global virulence regulators, such as the *agr* (accessory gene regulator) and *sarA* (staphylococcal accessory regulator A) systems, can also influence the expression of *oatA* (Patel & Rawat, 2023). These regulators control a wide array of virulence genes and may fine-tune the expression of *oatA* depending on the stage of infection or the specific host environment (Herbert et al., 2007).

3.1.5 FmtA's Role in Regulating Cell Division in *S. aureus*

In *S. aureus*, cell division is a highly orchestrated process that depends on the precise synthesis and remodelling of the cell wall (Qamar & Golemi-Kotra, 2012). Mutant strains lacking the *fmtA* gene (Δ *fmtA*) display abnormal cell morphology and defects in maintaining regular cell division (Qamar & Golemi-Kotra, 2012). Additionally, FmtA influences the D-alanylation WTAs, which modulate the overall charge of the cell wall (Rahman et al., 2016). This affects the

localization and function of key enzymes involved in cell division, such as PBPs and autolysins (Rahman et al., 2016). By regulating the electrostatic environment, D-alanylation ensures these enzymes' correct spatial organization and activity, promoting proper cell division (Qamar & Golemi-Kotra, 2012). Inhibiting FmtA and disrupting D-alanylation could compromise *S. aureus* cell wall integrity, making the bacterium more susceptible to existing antibiotics.

3.1.6 The role of *fmtA* in Biofilm formation by *S. aureus*

FmtA also plays a significant role in *S. aureus* biofilm formation by modulating key aspects of the cell wall that are critical for bacterial adhesion and biofilm maturation (Qamar & Golemi-Kotra, 2012). Specifically, FmtA regulates the D-alanylation of WTAs, a process that alters the overall charge of the bacterial cell wall (Rahman et al., 2016). This electrostatic modulation is essential for initial adhesion in biofilm formation, where *S. aureus* attaches to surfaces (Guo et al., 2022). WTAs, which are anchored to the peptidoglycan layer of the cell wall, interact with surface proteins, extracellular DNA, and other biofilm components to promote cell-to-cell adhesion and structural integrity (Schilcher & Horswill, 2020).

Mutant strains of *S. aureus* lacking *fmtA* ($\Delta fmtA$) exhibit deficiencies in biofilm formation, as the disruption of WTA D-alanylation affects the ability of the cells to adhere to surfaces and form stable intercellular connections necessary for biofilm maturation (Boles et al., 2010; Tu Quoc et al., 2007). Furthermore, the negative charge imparted by WTAs can influence the binding of cationic molecules, including antibiotics, making the biofilm environment more susceptible to antimicrobial agents (Schilcher & Horswill, 2020).

3.1.7 Objectives of Chapter 3

The influence of WTA charge and D-alanylation on resistance to agents like Triton X-100, lysozyme, and lysostaphin underscores the importance of these pathways in the survival of *S.*

aureus, particularly MRSA. Targeting D-alanylation through mutating FmtA could enhance the effectiveness of both the host immune response and existing antibiotics, offering a promising strategy for combating infections. A deeper understanding of FmtA's role in cell division and its regulation of D-alanylation broadens our knowledge of *S. aureus* physiology and highlights potential targets for antimicrobial therapies. Additionally, disrupting WTA modifications presents an attractive avenue for drug development, potentially weakening *S. aureus* and making it more susceptible to various antimicrobial treatments.

Moreover, D-alanylation is essential for regulating cell wall dynamics and turnover (Koprivnjak et al., 2006). The altered charge properties of D-alanylated WTAs can interfere with the activity of autolysins (Weidenmaier & Peschel, 2008). Furthermore, (Qamar & Golemi-Kotra, 2012) highlighted the dual role of FmtA in both biofilm formation and autolysis, underscoring its importance in the regulation of these critical processes. Dysregulation of autolysin activity due to inhibition of WTA can lead to aberrant cell division, thickening of the cell wall, and altered bacterial morphology, thereby compromising bacterial health and virulence (Jensen et al., 2019). Here, my investigation focuses on comprehending the impact of *fmtA*, its deletion mutant, and its mutant complements on biofilm formation, cell wall integrity, peptidoglycan structure, and cell division dynamics using an *in vivo* approach. My study aims to correlate these biochemical activities with the physiological functions of *S. aureus* RN4220.

3.2 Materials and Methods

3.2.1 Materials and Chemicals

The growth media, primers, Bouin's solution, Trypsin, Triton-X-100, and Lysozyme were procured from Sigma-Aldrich (Canada). The *fmtA* Y211AY282A gene, cloned into the pUC18 vector, was purchased from GeneScript (USA). Safranin was supplied by Westlab

(Canada), while Lysostaphin was acquired from Cedarlane (Canada). A phosphate assay kit was sourced from BioAssay Systems (Canada), and an electron microscopy resin kit was purchased from Electron Microscopy Sciences (EMS) (USA).

3.2.2 Construction of *fntA* mutant variants

The entire gene sequence of *fntA* was previously cloned into a pMK4 vector (5575 bp) by a former lab member (Rahman et al., 2016). Using this construct as the template, specified variants were generated (as outlined in Table 3.2.2) using the QuikChangeTM Site-Directed Mutagenesis method (by Stratagene).

The process was initiated by introducing one of the target mutations to generate the double variants. Subsequently, the mutated construct served as a template for introducing the second mutation. Following PCR amplification, the resulting products underwent digestion with Dpn1, a restriction endonuclease utilized to remove the original methylated template DNA.

Following digestion, *E. coli* DH5 α cells were transformed with the digested products and were selected based on Ampicillin resistance. The correct construction by mutagenesis was validated through DNA sequencing conducted at the Center for Applied Genomics at the Hospital for Sick Kids in Toronto, Canada.

After sequencing confirmation, the validated correct constructs were introduced into RN4220 *fntA* electro-competent cells and selected based on Chloramphenicol resistance for subsequent experimentation.

Table 3.2.2. The table showing the list of variants prepared from the pMK4::*fmtA* construct where the mutation site is italicized

Construct	Designed primers
Y211A	Dir-5' GCACATGGCTAACGATGGGAA 3' Rev-5' ATTCCCATCGTTAGCCATGTG 3'
Y282A	Dir-5' ATTTTGGACCAAGCCTATGGTGCAGG 3' Rev-5' CCTGCACCATAGGCTTGGTCCAAAAT 3'
L342A	Dir-5' CCAACATTGAATAGACATAACGGGGG 3' Rev-5' CCCCCGTTATGTCTA <i>TT</i> CAATGTTGG 3'
F346A	Dir- 5' TTAACGGGGGAGCCTTTGGACAAG 3' Rev- 5' CTTGTCCAAAGGCTCCCCCGTTAA 3'
D213A	Dir-5' GCACATGTATAACGCTGGGAATTATTTAGTACT 3' Rev-5' GCAAGTACTAAATAATTCCCAGCGTTATACATG 3'
H173A	Dir-5' CCCATTGAAAGATTTAATGTTGGCTCAAAGTGG 3' Rev-5' CCACTTTGAGCCAACATTAAATCTTTCAATGGG 3'

3.2.3 Cloning and transformation of the *fmtA* Y211AY282A gene into a pMk4 vector

The synthesized construct of *fmtA* Y211AY282A (1644 bp) was obtained from Genescript Biotech, which was already cloned into a pUC18 vector. This construct included Sall and EcoRI digestion sites compatible with those present in the pMK4 vector. Initially, both the pUC18 *fmtA* Y211AY282A and pMK4 plasmids were subjected to a double digestion with Sall and EcoRI enzymes, followed by an agarose gel electrophoresis to confirm a successful digestion. DNA fragments of the correct size (1644 bp for *fmtA* Y211AY282A including the promoter region and 5575 bp for pMK4) were then extracted from the gel and ligated overnight. Subsequently, the ligated product was transformed into DH5 α cells, and the success of the transformation was confirmed through ampicillin selection. The presence of the desired insert and the specific mutations (Y211AY282A) was confirmed by sequencing. Upon confirmation of

the correct construct, the pMK4 plasmid was further transformed into RN4220 *AfmtA* cells for subsequent experimentations (Fig. 3.2.3).

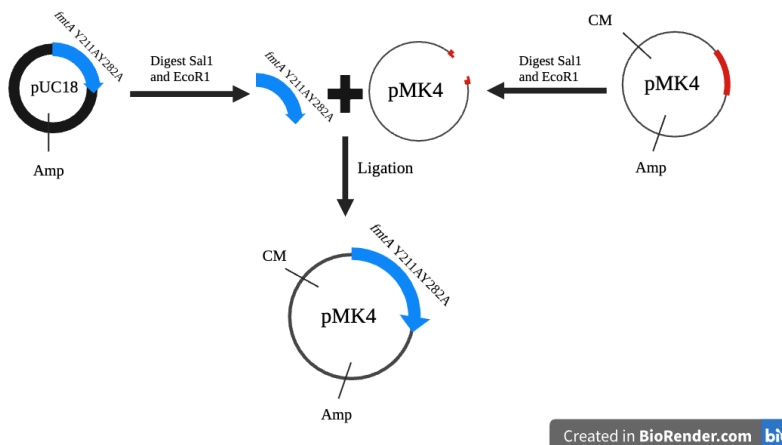


Figure 3.2.3 Illustration demonstrating the cloning process of the *fmtA* Y211AY282A construct into a pMK4 vector.

3.2.4 Biofilm formation in RN4220 cells in the presence of exogenous FmtA^{Δ27} and its variants

The impact of exogenous FmtA^{Δ27} and its variants on the biofilm formation by the RN4220 strain was evaluated following the protocol by Qamar & Golemi-Kotra, (2012) established under static conditions. The RN4220 strain cells were cultured in TSB media (without any supplements) until reaching an O.D.₆₀₀ of 1 from an overnight culture. The bacterial culture was diluted 1:50 (to a final volume of 25 ml) in TSB media supplemented with 0.2% glucose, where the final O.D.₆₀₀ = 0.02. Aliquots of 200 µl were dispensed into sterile, flat-bottom tissue culture plates (Corning Incorporated). FmtA^{Δ27} was subsequently introduced in a gradient of increasing concentrations (0–2 µM). The plates were incubated at 37°C for 24 hours before further experimental procedures.

After incubation, the solutions from the wells were carefully aspirated using a pipette. The wells were then washed four times with sterile 1x Phosphate Buffered Saline (PBS), which

contains 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄, (Purchased from the University science store) by gently aspirating the washing solution with a pipette. The adherent biofilms in the wells were fixed with Bouin's fixative (Purchased from Sigma) for 4 hours and stained with 0.1% safranin for 10 minutes. Afterward, the wells were rinsed in running tap water, and photographs were taken using a phone camera. The remaining biofilms were dissolved in 30% glacial acetic acid for 15 minutes, and the relative biofilm formation was quantified by measuring absorbance at 600 nm wavelength using a Hybrid H₄ plate reader ensuring the recorded O.D.₆₀₀ ranges between a reliable reading range (0-2 O.D.₆₀₀) (Fig. 3.2.4). Each assay was conducted in triplicates.

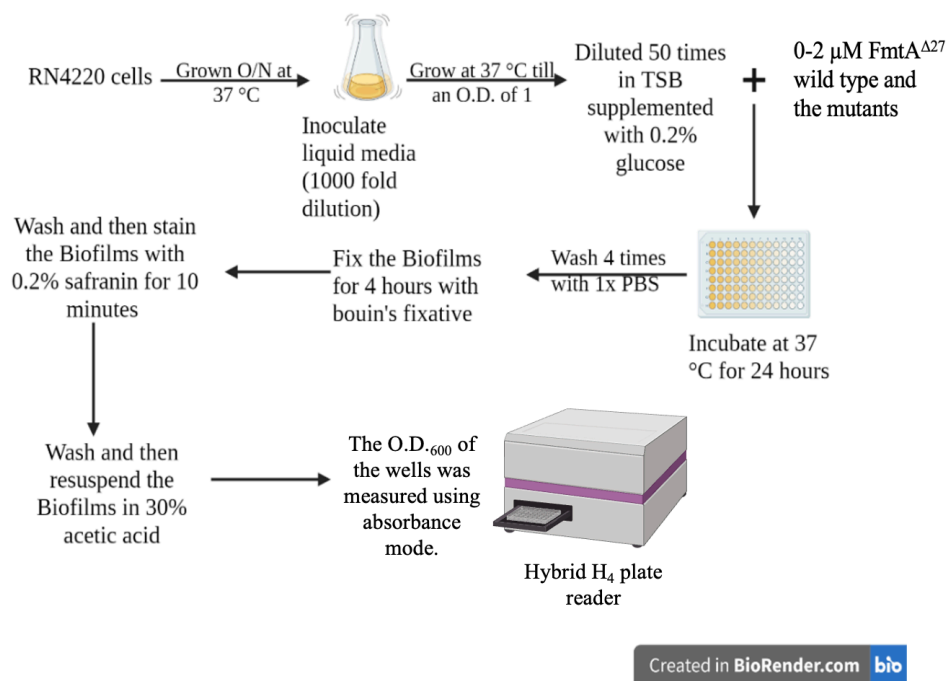
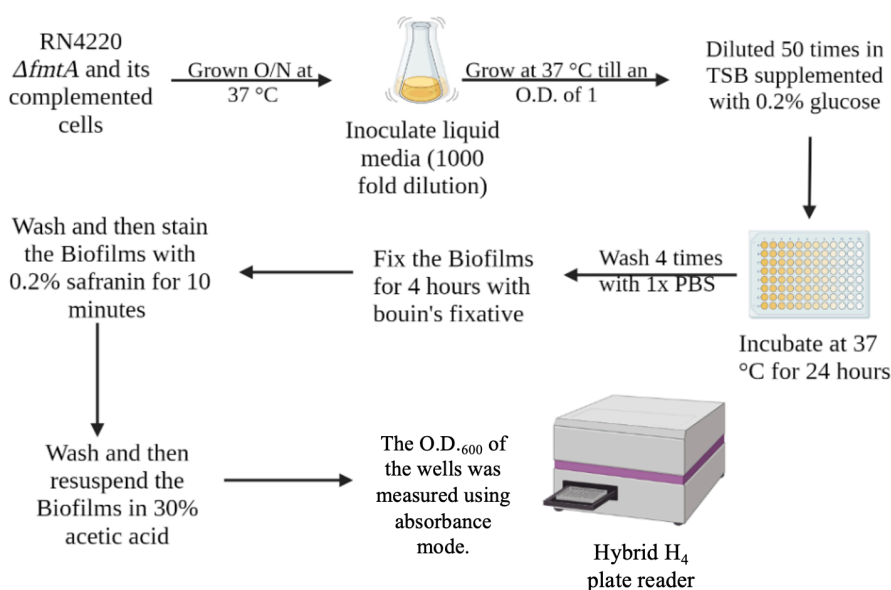


Figure 3.2.4. Illustration representing the experimental parameters followed during the biofilm formation assay performed with RN4220 cells of O.D.₆₀₀ = 0.02 supplemented with 0.2% glucose, incubated with different concentrations of (0-2 μM) exogenous FmtA^{Δ27} and its selected variants for 24-hours at 37 °C in a 95-well flat bottom plate for biofilms to form.

3.2.5 Effects in biofilm formation in RN4220 *ΔfmtA* and its complemented strains

Similar to the biofilm formation assay described earlier, this experiment followed identical steps with two key distinctions. Firstly, instead of using only the RN4220 strain, the RN4220 *ΔfmtA* strain and mutant *fmtA* genes incorporated into RN4220 *ΔfmtA* cells via the pMK4 plasmid were employed in the assay. Secondly, except for the *ΔfmtA* strain, the other variants were grown in 10 µg/ml Chloramphenicol. Notably, the exogenous introduction of FmtA^{Δ27} and its variants were omitted for this set of experiments, allowing for the observation of the effects solely attributable to endogenous *fmtA* expression through mutagenesis (Figure 3.2.5).



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Figure 3.2.5. Illustration depicting the experimental parameters of the biofilm formation assay conducted with RN4220, RN4220 *ΔfmtA* and the variants where cells were grown for 24 hours at 37°C.

3.2.6 WTA isolation from RN4220 *ΔfmtA* and its complemented strains

WTA from the RN4220 *ΔfmtA* and its complemented strains were isolated to be able to characterize them based on their D-Ala content with respect to their Phosphate (Pi) content. The

WTA isolation process followed the previously described protocol (page 87), with a modification for the variants containing the pMK4 vector. Specifically, these variants were grown in a media supplemented with 10 µg/mL chloramphenicol (Figure 3.2.6). The rest of the isolation protocol remains the same.

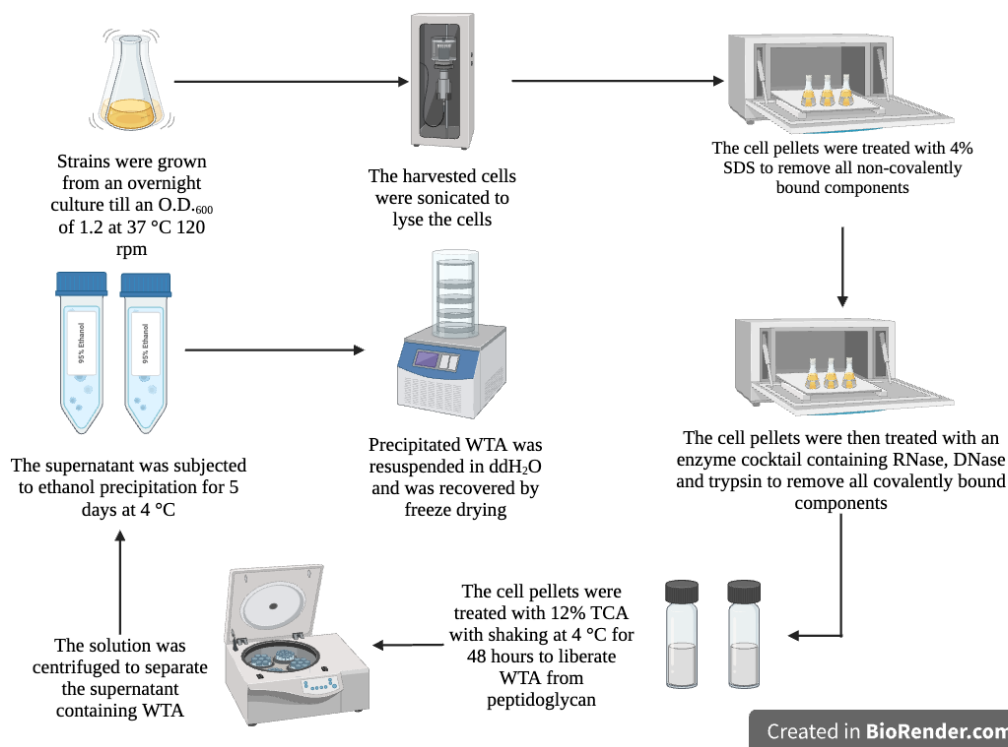


Figure 3.2.6. Illustration depicting the experimental parameters of the process of WTA isolation

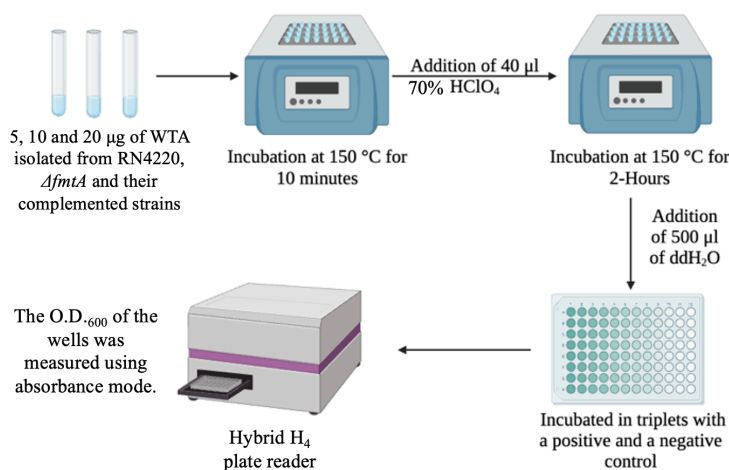
3.2.6.1 Inorganic phosphate analysis on mutant WTAs

The determination of inorganic phosphate in my study was conducted using the Phosphate Assay Kit (Bioassay Systems), which employs a colorimetric method based on the malachite green reagent. This assay quantifies inorganic phosphate resulting in a vivid green color. The intensity of the green colour, measured spectrophotometrically at an absorbance of 620 nm, is directly proportional to the concentration of inorganic phosphate present in the sample.

The underlying principle of this assay involves the initial reaction of inorganic phosphate with ammonium molybdate to form a phosphomolybdate complex. This complex then reacts with malachite green, producing a stable green-coloured complex. The method is highly sensitive and specific for inorganic phosphate, enabling the detection of phosphate concentrations (Feng et al., 2011).

In this assay, 5, 10 and 20 µg samples (as solutions) of the respective WTAs, were subjected to the following treatment, where the samples were prepared in ddH₂O and were incubated in glass tubes at 150°C for 10 minutes in a dry bath. Provided initially the samples were quantified by comparing the isolated WTAs to a 5 mg/ml WTA standard sample (this comparative quantification was determined and calculated using ¹H-NMR). Following this, 40 µl of 70% Perchloric acid was added to the tubes, and the samples were further incubated for 2 hours at 150°C. After the incubation, 500 µl of autoclaved ddH₂O was introduced into the experimental tubes. Subsequently, 50 µl samples were prepared in triplicates, alongside positive controls (30 µM inorganic phosphate (Pi)) and negative controls (ddH₂O), each also in triplicates, in a 96-well flat-bottom plate. To each replicate, 100 µl of the detection reagent was added, and the plate was further incubated at room temperature for 30 minutes before measuring the O.D.₆₂₀ in a Synergy H4 Hybrid plate reader (Fig. 3.2.6.1). Finally, the inorganic phosphate concentrations for the experimental WTAs were calculated according to the equation provided in the kit which as follows:

$$Pi = \frac{(O.D._{Experimental} - O.D._{Blank})}{(O.D._{Positive\ Control} - O.D._{Blank})} \times 30 \mu M$$



Created in BioRender.com

Figure 3.2.6.1. Illustration depicting the experimental setup for quantifying inorganic phosphate using the malachite green method.

3.2.6.2 Preparation of D-Ala/DSS standard curve from NMR spectra

To quantify the amount of D-Ala in a WTA sample following hydrolysis, a D-Ala standard curve was established. D-Ala samples ranging from 200 to 1000 μM were prepared in H_2O , 10% D_2O , and 50 $\mu\text{g}/\mu\text{l}$ Sodium trimethylsilylpropanesulfonate (DSS) (prepared in D_2O), the final volume being 300 μl . These samples were then incubated with 0.1 M NaOH overnight (16 hours). DSS is widely used as an internal standard in NMR spectroscopy for chemical shift referencing, where the chemical shifts of the sample are measured relative to the DSS signal, which is set at 0 ppm (Westwood et al., 2019). This standardization enables consistent and comparable results across various NMR experiments, and as DSS is chemically inert, it does not interact with or alter the sample's NMR signals (Westwood et al., 2019).

Subsequently, the samples were subjected to 700 MHz ^1H -NMR analysis at 25 $^\circ\text{C}$; the resulting data were analyzed with Topspin 4.3.0 software (Bruker Ltd.), and the D-Ala peaks were integrated against DSS to generate a D-Ala/DSS ratio. Finally, the D-Ala/DSS ratio was

plotted against the D-Ala concentrations to generate a standard curve (Fig. 3.2.6.1 and Table 3.2.6.2).

Table 3.2.6.2: Table showing the D-Ala/DSS vs D-Ala concentration data

D-Ala concentration	D-Ala/DSS ratio
200 μ M	0.2865
400 μ M	0.5279
600 μ M	0.8492
800 μ M	1.1773
1000 μ M	1.43

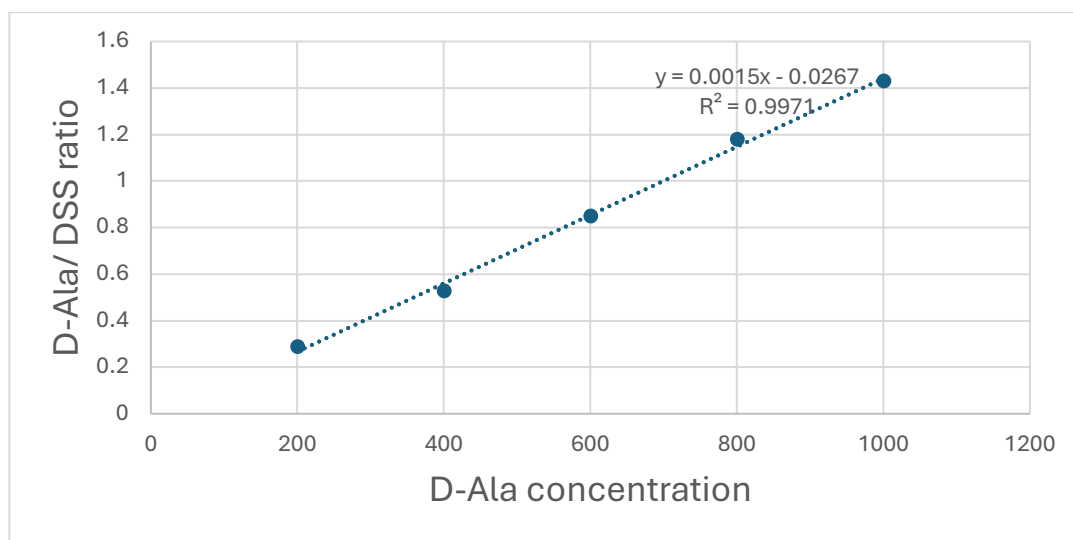


Figure 3.2.6.2. Graph representing the D-Ala/DSS vs D-Ala concentration standard curve

3.2.6.3 Quantification of D-Ala in the isolated WTAs

To quantify the amount of D-Ala present in the WTA isolated from different *S. aureus* RN4220 strains that carry a particular *fntA* variant, WTAs were subjected to hydrolysis overnight (16 hours) in the presence of 0.1 M NaOH. A protocol involving the incubation of 200 μ g and 300

μg of the WTA samples (from a stock solution) with 0.1 M NaOH, 10% D₂O, H₂O, and 50 μg/μl DSS overnight was followed, resulting in a final volume of 300 μl (Fig. 3.2.6.3).

Subsequently, the entire sample was subjected to ¹H-NMR analysis, where the ¹H NMR spectra were collected on a Bruker AV III 700 NMR spectrometer equipped with a 5 mm, liquid nitrogen-cooled H/C/N Prodigy probe. The 1D spectra were acquired using the standard “zgesgp” excitation sculpting pulse program, which used 32,768 data points, a spectral width of 16 ppm, with a 7-second relaxation delay and 64 scans. The resulting data were analyzed with Topspin 4.3.0 software (Bruker Ltd.). The DSS peak was integrated with the free D-Ala peak from the recorded NMR chemical shift and then used in the standard curve equation of D-Ala (explained before) to calculate the concentration of D-Ala present in the original stock.

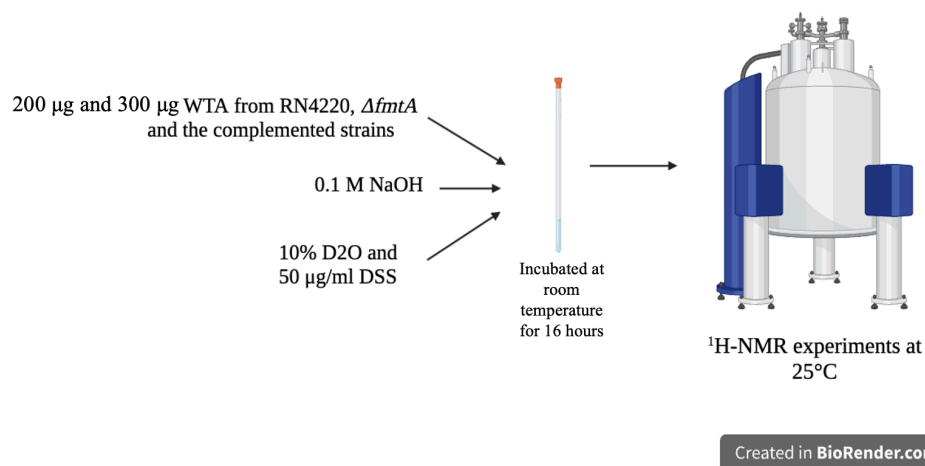


Figure 3.2.6.3. Illustration showcasing the experimental parameters involved in determining D-Ala concentrations within the WTA samples.

3.2.7 Investigation of cell wall integrity in RN4220 *ΔfmtA* and its complemented strains with Triton X-100

To investigate the role of *fmtA* in the overall cell wall integrity, a lysis assay with Triton X-100 was conducted. RN4220 *ΔfmtA* strain, along with RN4220 *ΔfmtA* strain complemented

with the *fmtA* gene and its variants, were subjected to Triton-X-100 treatment and compared to the wild-type strain.

For this assay, cells were cultured in TSB media (supplemented with 10 µg/ml chloramphenicol for the variants containing the pMK4 vector) until reaching an O.D.₆₀₀ of 0.8. 50 mg of the cells were harvested by centrifuging ~50 ml of the cell culture at 5000 g (6825 rpm) for 3 minutes at room temperature. The cells were then washed three times with 10 ml of 50 mM Tris-HCl buffer (pH 7.5), with each wash followed by recovery via centrifugation (5000 g, 3 minutes, RT). Then the pellets were resuspended in 50 ml of 50 mM Tris-HCl buffer (pH 7.5) to achieve a concentration of 1 mg/ml. Subsequently, 1 mg/ml of the cells were treated with 0.05% Triton X-100 prepared in 50 mM Tris-HCl (pH 7.5) (final volume being 200 µL) and incubated at 37°C, 180 rpm for 8 hours in a sterile, flat-bottom 96-well plate (Corning incorporated). O.D.₆₀₀ measurements were recorded in the Synergy H4 Hybrid Reader at 30-minute intervals for the first 4 hours of incubation and hourly thereafter (Fig. 3.2.7). All experiments were performed in triplicates, and a control experiment was conducted with wild-type RN4220 cells without Triton X-100.

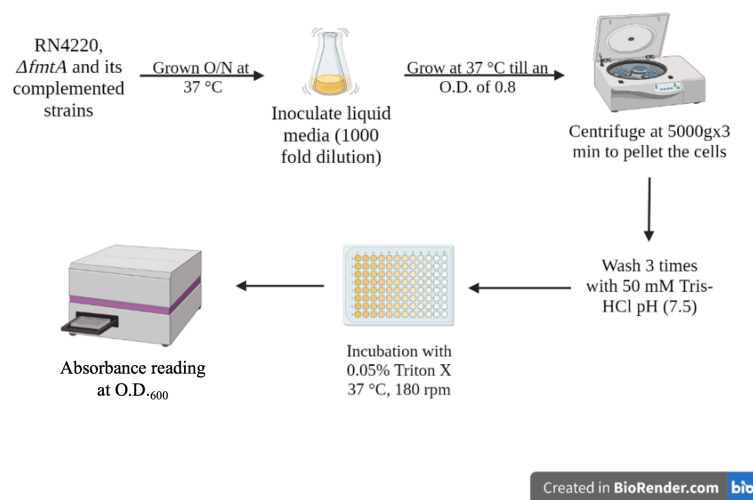
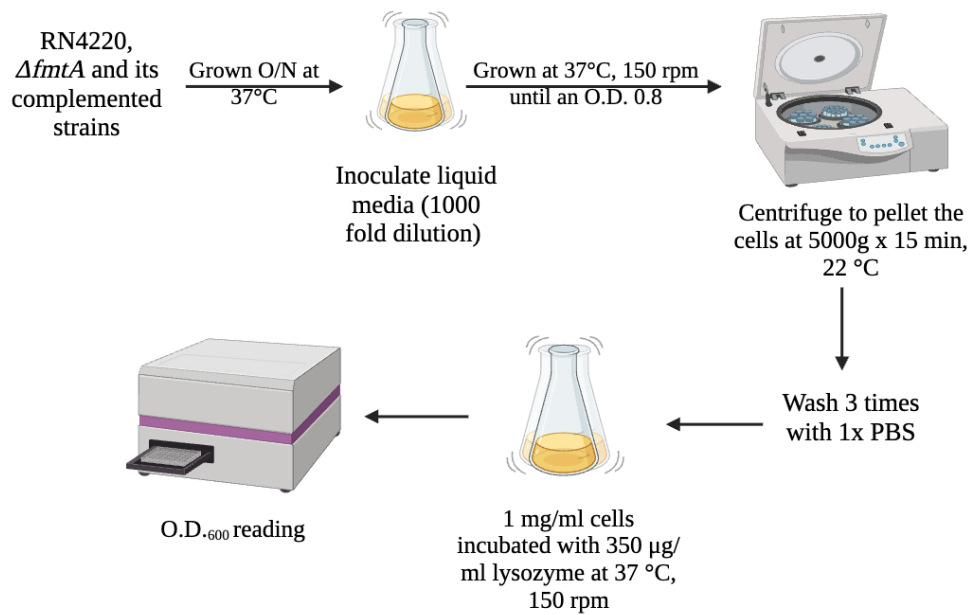


Figure 3.2.7. Illustration representing the experimental parameters analyzing the effects of Triton X-100 on the RN4220, *ΔfmtA* and its complemented strains

3.2.8 Investigation of peptidoglycan integrity in RN4220 Δ *fmtA* and its complemented strains in the presence of lysozyme

To investigate the impact of lysozyme stress on the peptidoglycan integrity of mutant RN4220 strains with varying levels of D-Ala in their WTAs, an assay was conducted where the mutations were achieved through *fmtA* deletion and complementation with wild-type and mutant *fmtA* genes.

The experimental protocol involves culturing the strains in TSB media (supplemented with 10 µg/ml chloramphenicol for the variants containing the pMK4 vector) until an O.D.₆₀₀ of 0.8 is reached from overnight cultures. 50 mg of the cells were harvested by centrifuging ~50 ml of the cell culture at 5000 g (6825 rpm) for 15 minutes at room temperature. The cells were washed thrice with 10 ml 1X PBS buffer to eliminate residual growth media. Thereafter, 1 mg/ml of the samples were treated with 350 µg/ml lysozyme prepared in 1X PBS and were incubated at 37°C, 150 rpm, where the final volume was 50 ml. The changes in O.D.₆₀₀ were monitored hourly for 8 hours to assess the impact of lysozyme exposure on the cells (Fig.3.2.8). This approach allows us to assess how alterations in WTA composition, mediated by FmtA activity influence RN4220's susceptibility to lysozyme-induced cell wall degradation over time.



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Figure 3.2.8. Illustration representing the experimental parameters analyzing the effects of lysozyme on the RN4220, $\Delta fmtA$ and its complemented strains

3.2.9 Investigation of peptidoglycan integrity in RN4220 $\Delta fmtA$ and its complemented strains in the presence of lysostaphin

The objective of this experiment mirrors that of the lysozyme investigation, aiming to explore how mutations in *fmtA* or its complete removal influence the susceptibility of RN4220 peptidoglycan to lysostaphin-induced degradation. Following a similar experimental protocol described previously, fresh cultures of the strains were grown in a TSB media (supplemented with 10 μg/ml chloramphenicol for the variants containing the pMK4 vector) to an O.D.₆₀₀ of 0.8 from overnight cultures. Thereafter, 100 mg of cells were harvested by centrifuging ~100 ml of the cell culture at 5000 g (6825 rpm) for 15 minutes at room temperature. The harvested cells were then thoroughly washed thrice with 10 ml of the 50 mM Tris-HCl buffer (pH 7.5) and recovered by centrifugation.

Finally, 2mg/ml of samples were exposed to 0.1 μ g/ml lysostaphin prepared in 50 mM Tris-HCl buffer (pH 7.5) (final volume being 50 ml), and changes in O.D.₆₀₀ were monitored every 40 minutes up to 200 minutes. Additionally, specific dilutions of each strain at each time point were plated on TSB agar plates for colony counts, allowing us to correlate observed changes in O.D.₆₀₀ with the viability of the cells over time (Fig. 3.2.9).

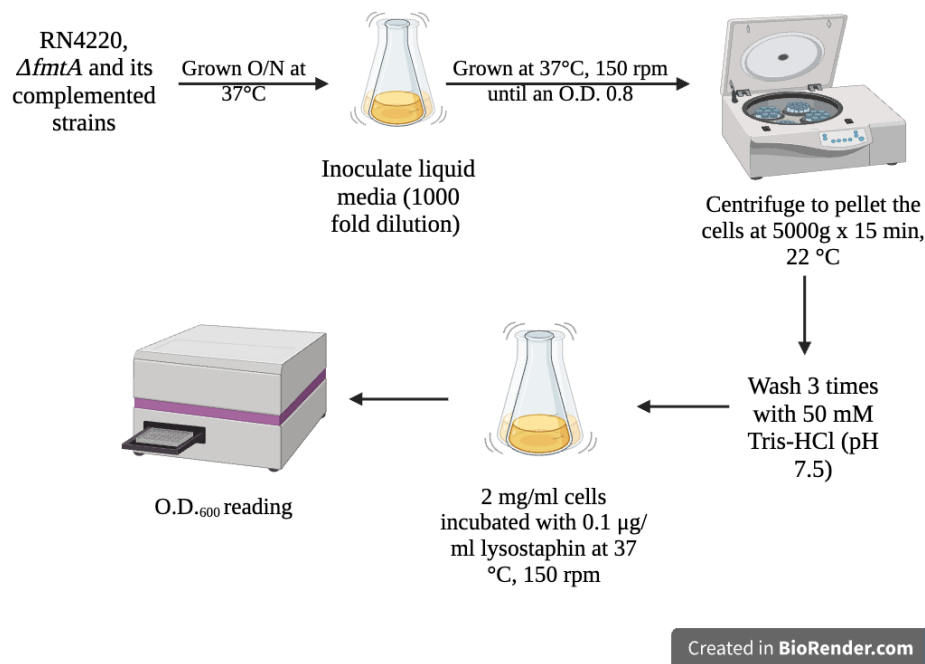


Figure 3.2.9. Illustrative representation of the experimental parameters analyzing the effects of lysostaphin on the RN4220, Δ *fmtA* and its complemented strains

3.2.10 Transmission electron microscopy

Research conducted by Qamar et al. (2012) shed light on the localization of the *fmtA* gene within the division septum of *S. aureus* using fluorescence microscopy, underscoring its role in the cell division process. Building upon this foundation, my study focuses on the role of *fmtA* deletion and subsequent complementation with wild-type and mutant *fmtA* genes. Through this investigation, we aim to elucidate the role of *fmtA* in regulating the cell division of *S. aureus* RN4220 cells.

My experimental procedures involve a series of steps to prepare samples for transmission electron microscopy (TEM) analysis. Initially, bacterial cells are cultured at 37°C from an overnight seed culture, where for the pMK4::*fntA* variants, the media is supplemented with 10 µg/ml chloramphenicol, until reaching an O.D.₆₀₀ of 0.6.

Subsequently, cells from 5 ml of the culture are harvested by centrifugation at 6000 rpm for 1 minute at room temperature (RT). They undergo four washes in 0.1 M sodium cacodylate buffer (pH 7.4). Then Primary fixation is performed by incubating the cells in 3.5% glutaraldehyde prepared in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 hour with gentle stirring at RT. Following this, cells are subjected to secondary fixation with 1% osmium tetroxide prepared in 0.1 M sodium cacodylate buffer (pH 7.4) for an additional hour.

After fixation, cells are recovered by centrifugation and resuspended overnight in 0.1 M sodium cacodylate buffer (pH 7.4) at RT. The following day, cells undergo ethanol gradient treatment (30-90%) for 10 minutes each, with recovery by centrifugation after each step. Subsequently, they are treated with 100% ethanol for an additional hour. Samples are then recovered by centrifugation and incubated at RT overnight with Spurr's resin (prepared according to the protocol provided in the kit) mixed with 100% ethanol in a 1:1 ratio.

On the final day of sample preparation, the resin-ethanol mixture is removed without disturbing the cell pellets, and the pellets are transferred to special plastic casts. Freshly prepared pure resin is then added to the casts, and they are incubated at 70°C in an oven for 48 hours. Finally, the hardened resins containing the cell pellets are removed from their casts and thinly sliced to an appropriate size with a microtome for TEM analysis. Before analysis, the grids are stained with Uranylless and lead acetate solution to enhance contrast (Fig. 3.2.10).

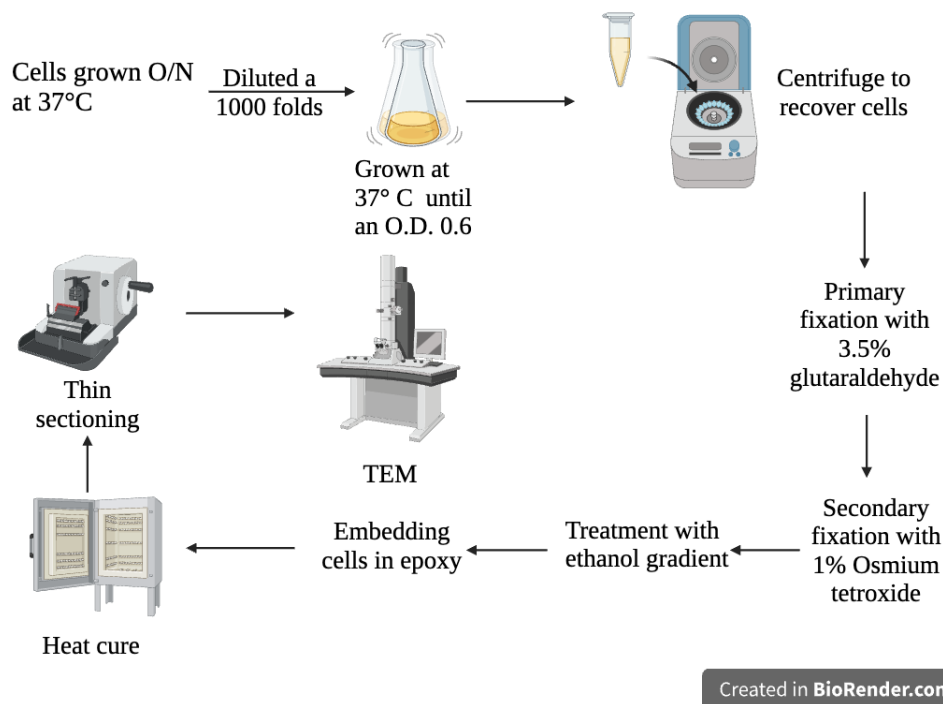


Figure 3.2.10. Illustration depicting the parameters of the Transmission electron microscopy experiment on the RN4220, $\Delta fmtA$ and its complemented strains.

3.3 Results

3.3.1 Selection of FmtA variants for *in vivo* experiments based on enzymatic activity

From the pool of variants generated for *in vitro* experiments, a few variants were chosen based on their diminutive enzymatic activity (between 7-20 %) against WTA compared to the wild type (Table 3.3.1) assessed by $^1\text{H-NMR}$. For a couple of experiments, a mutant namely N343A demonstrating 67% of the wild-type activity was used as a partial loss-of-function control. WTA in buffer and an enzymatically inactive mutant (FmtA $^{\Delta 27}$ S127A) showed 10% and 15% activity, respectively (Fig. 3.3.1)

Table 3.3.1: Table shows the remaining activity of different FmtA variants

Mutant	Activity (%)	k (h⁻¹)
Wild Type	100	0.6±0.1
L342H	17	0.1±0.01
Y282A	7	0.04±0.02
F346A	17	0.1±0.02
D213AH173A	10	0.06±0.04
Y282AF346A	7	0.04±0.02
Y211AY282A	7	0.04±0.01
N343A	67	0.4±0.07

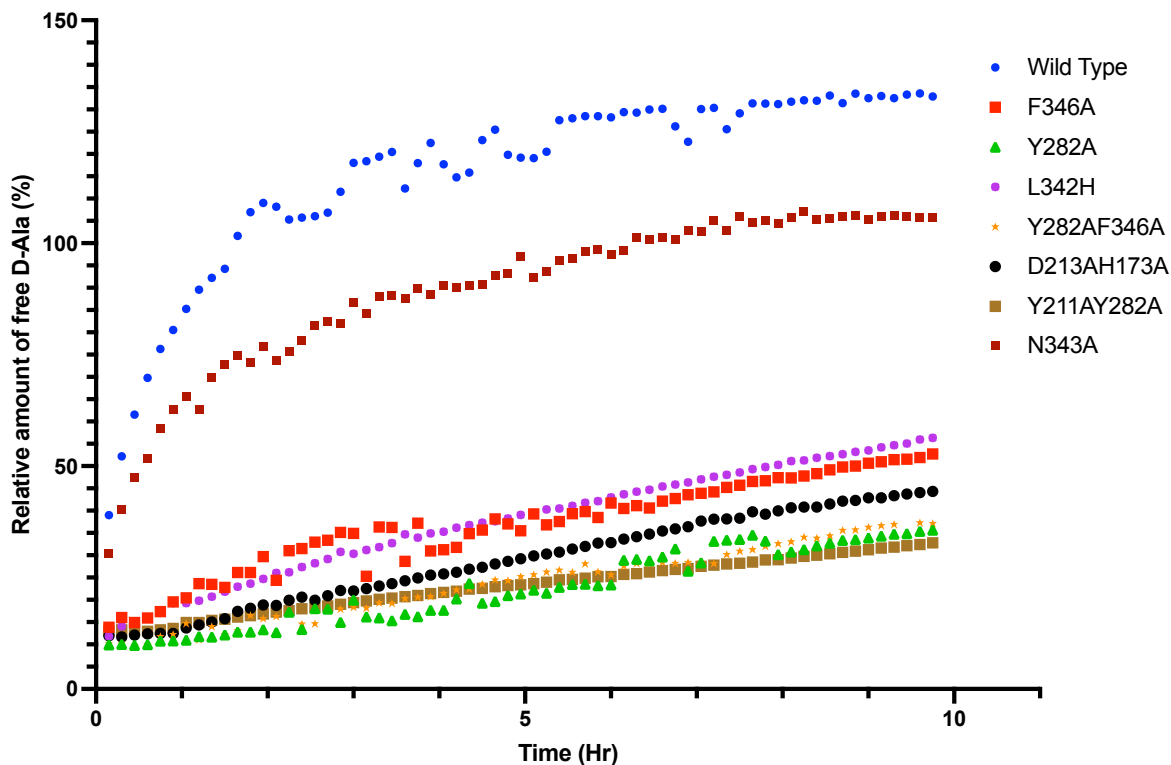


Figure 3.3.1. Illustration depicting the relative amount of free D-Ala released by the wild-type FmtA^{Δ27} enzyme compared to the variants selected for *in vivo* experimentation. It visually represents the differences in D-Ala release among these enzyme variants.

3.3.2 Effects of exogenous FmtA^{Δ27} and its variants on *S. aureus* RN4220 biofilm formation

Previous studies, as highlighted by Hou et al., 2022 and Zhu et al., 2018, suggested the significance of WTA in the biofilm formation process of *S. aureus*. Additionally, (Qamar & Golemi-Kotra, 2012) demonstrated a concentration-dependent increase in biofilm formation of the *S. aureus* RN4220 strain when exposed to exogenous FmtA^{Δ27} at concentrations of $\leq 0.2 \mu\text{M}$. Since FmtA regulates the D-Ala content in *S. aureus* WTA, the present investigation aims to evaluate the impact of exogenously administered wild-type and mutant FmtA^{Δ27} proteins on biofilm-forming cell cultures to elucidate the effects of the mutation on the protein.

For this study, the biofilm formation assay originally developed by Qamar & Golemi-Kotra, (2012) was adopted and described in the methods section (Fig. 3.2.4).

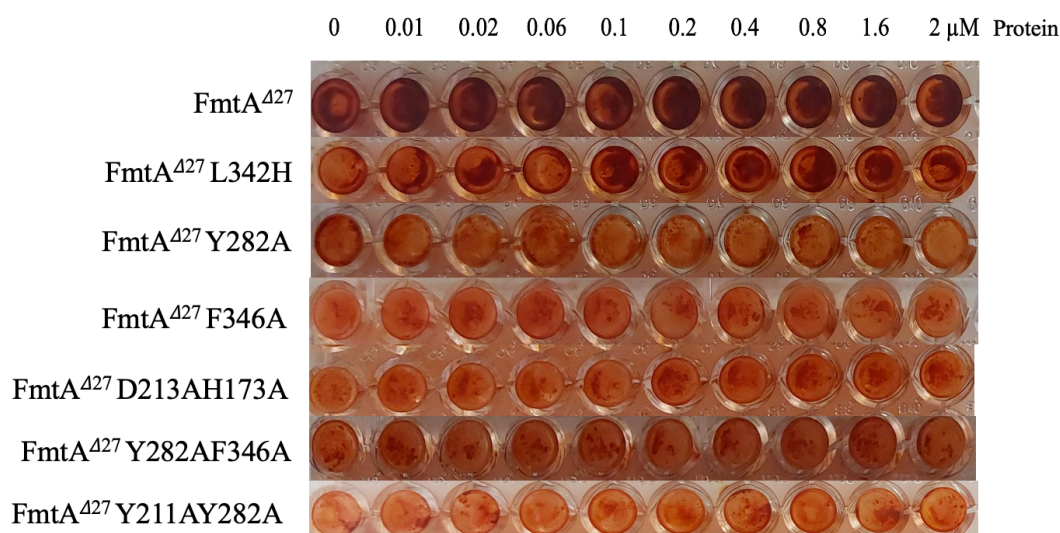


Figure 3.3.2.1. Biofilm formation by *S. aureus* RN4220 after 24 hrs of growth in polystyrene flat bottom culture plates in the presence of various concentrations of FmtA^{Δ27} and the selected variants.

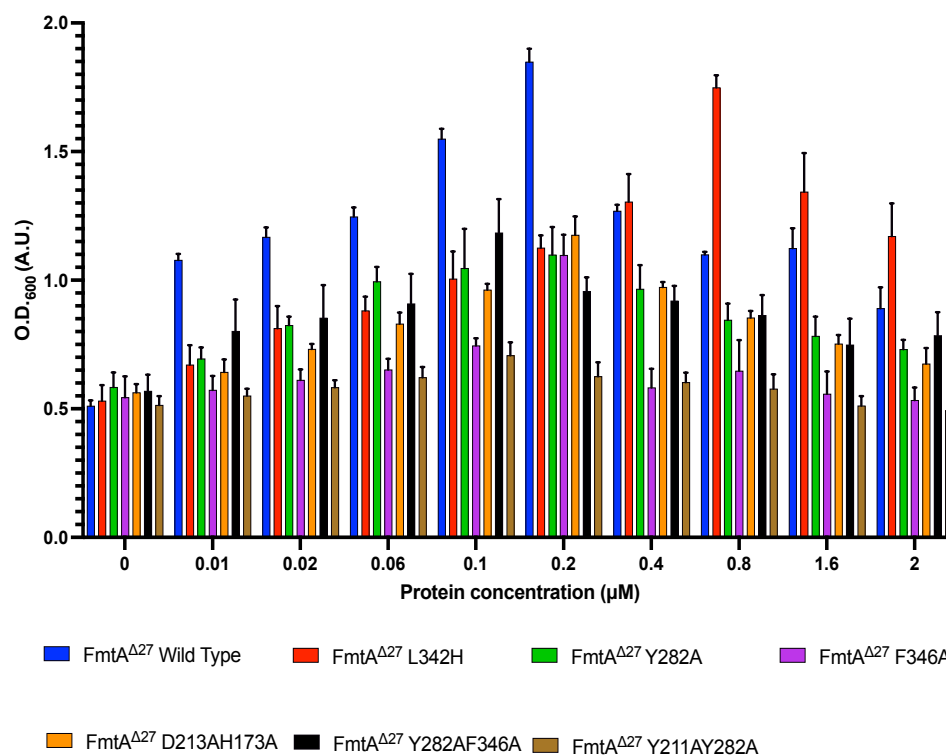


Figure 3.3.2.2. The analysis of biofilm formation by RN4220 strain in the presence of FmtA^{Δ27} and its variants. RN4220 cells were grown to an O.D.₆₀₀=1 and were diluted 50-folds in a TSB

medium supplemented with 0.2% glucose and were incubated in a 96-well plate with an increasing concentration of FmtA and its variants (0-2 μM) for 24-hours at 37 °C (the final volume being 200 μl). The biofilms formed were then subjected to wash four times with 1xPBS and were then subjected to fixation with Bouin's fixative for 4-hours. The wells were washed in running tap water were stained with 0.2% safranin for 10 minutes. The excess stain was removed under running tap water and the biofilms stained with safranin were dissolved in 30% acetic acid for 15 minutes. Finally, absorbance at O.D.₆₀₀ was measured for the plate in the Synergy H4 Hybrid Reader. Experiments were performed three times in triplets and the results were averaged. Error bars represent the standard deviation calculated from three independent trials performed in triplicates.

Consistent with the findings of (Qamar & Golemi-Kotra, 2012) RN4220 cells exhibited increased biofilm formation upon exposure to exogenous wild-type FmtA ^{$\Delta 27$} ($[\text{FmtA}^{\Delta 27}] \leq 0.2 \mu\text{M}$) (Fig. 3.3.2.1 and Fig. 3.3.2.2). This served as the baseline for comparison with the variants. The variants demonstrated a concentration-dependent enhancement in biofilm formation in RN4220 cells, albeit to a much lesser extent than the wild type and the L342H mutant. Except for the variants Y282AF346A, Y211AY282A (at 0.1 μM protein concentration) and L342H (at 0.8 μM protein concentration), the rest of the variants reached optimal biofilm levels at a protein concentration of 0.2 μM , mirroring the performance of the wild type in their respective trials (Fig. 3.3.2.2).

Upon further comparison of biofilm formation between the selected variants and the wild type under optimal conditions for each specific mutant (i.e., 0.8 μM protein concentration for the L342H mutant, 0.1 μM for the Y282AF346A and Y211AY282A variants, and 0.2 μM for the others), all variants exhibited a statistically significant reduction in biofilm formation, ranging from approximately 35% to 72% compared to the wild type, except for the L342H mutant (Fig. 3.3.2.3). These findings indicate that, when applied externally, none of the variants, except for the L342H mutant (which required a four-fold higher concentration compared to the wild type), can enhance biofilm formation in the RN4220 strain to the same extent as the wild type. This is

particularly evident with the Y211AY282A mutant, which exhibited biofilm formation levels comparable to the negative control, where cells were incubated without the protein, even under optimal conditions.

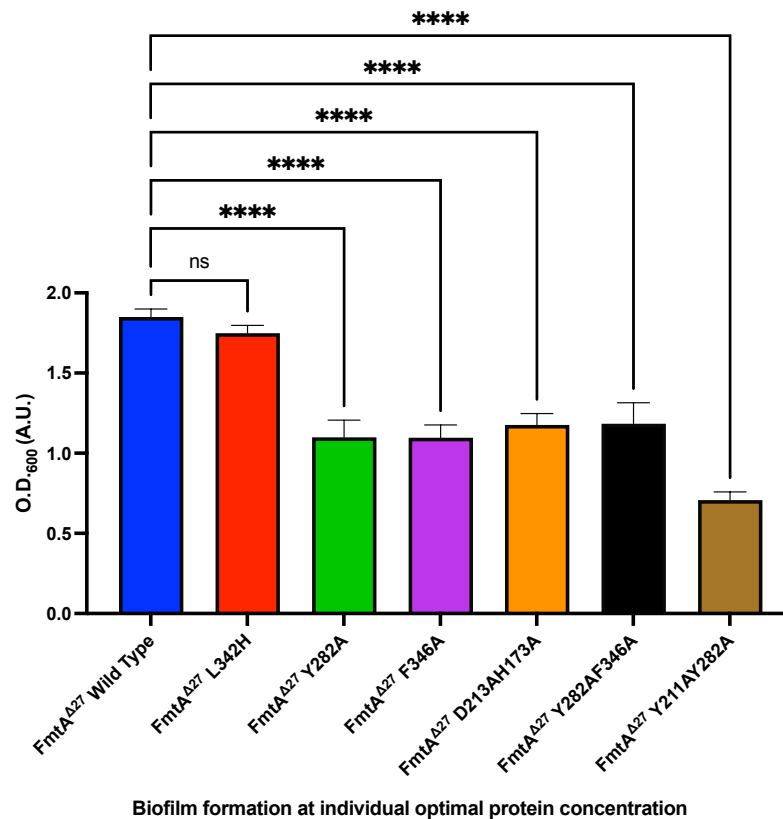


Figure 3.3.2.3. The analysis of biofilm formation by RN4220 strain in the presence of FmtA^{Δ27} and its variants at individual optimal conditions, i.e., in the presence of 0.8 μM L342H, 0.1 μM Y282AF346A and Y211AY282A and 0.2 μM of the rest of the proteins. Data represent the mean ± SD from three independent experiments performed in triplicate. Statistical significance was evaluated using a parametric one-way ANOVA followed by Dunnett’s multiple comparison test (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001; ****p ≤ 0.0001, and “ns” is not significant).

3.3.3 Effects of the RN4220, *ΔfmtA* and its complemented strains on *S. aureus* RN4220 biofilm formation

Similar to my investigation into the variations in biofilm formation of RN4220 cells in the presence of exogenous FmtA^{Δ27} and its variants, I also explored the effects of the *fmtA* gene on the

biofilm-forming capabilities of the RN4220 strain. This involved examining the impact of *fntA* deletion, complementation with the wild-type gene, or variants.

To assess the potential involvement of *fntA* in the biofilm formation process, a similar assay as previously described (Fig. 3.2.5) was conducted but without exogenously administering any protein. This assay involved the RN4220 $\Delta fntA$ mutant, its complemented wild-type strain, and the variants namely L342H, Y282A, F346A, D213AH173A, Y282AF346A, and Y211AY282A (Fig. 3.3.3.1). The *fntA* genes were introduced to the RN4220 $\Delta fntA$ cells via cloning in a pMK4 vector. Furthermore, to determine if the pMK4 vector influences the biofilm formation process, a blank vector was introduced into the RN4220 and RN4220 $\Delta fntA$ strains and these transformed strains, labelled as RN4220 pMK4 and RN4220 $\Delta fntA$ pMK4 respectively, were subjected to a similar biofilm formation assay.

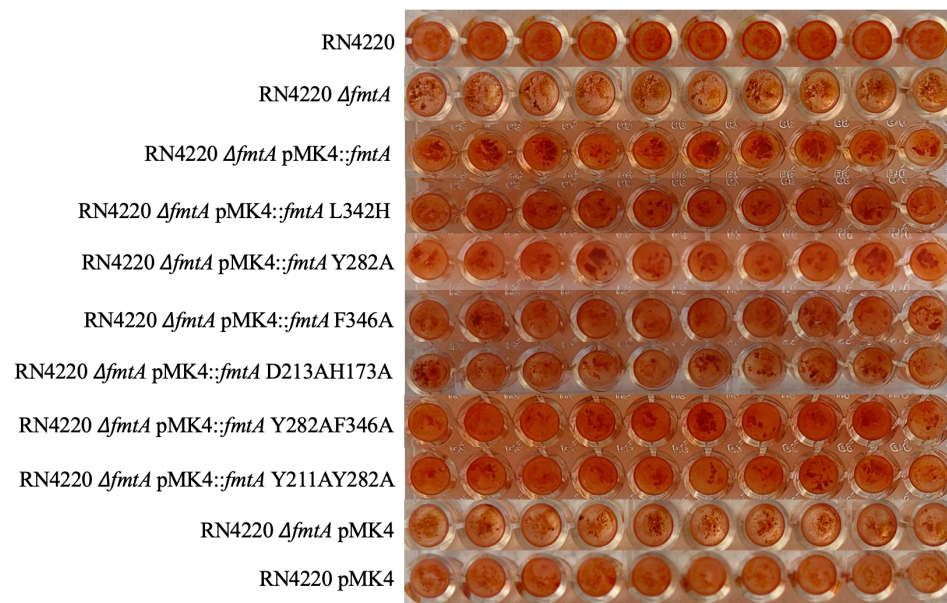


Figure 3.3.3.1. The formation of biofilms by *S. aureus* RN4220, its $\Delta fntA$ mutant, and the corresponding complemented strains, including the wild type and the selected variants.

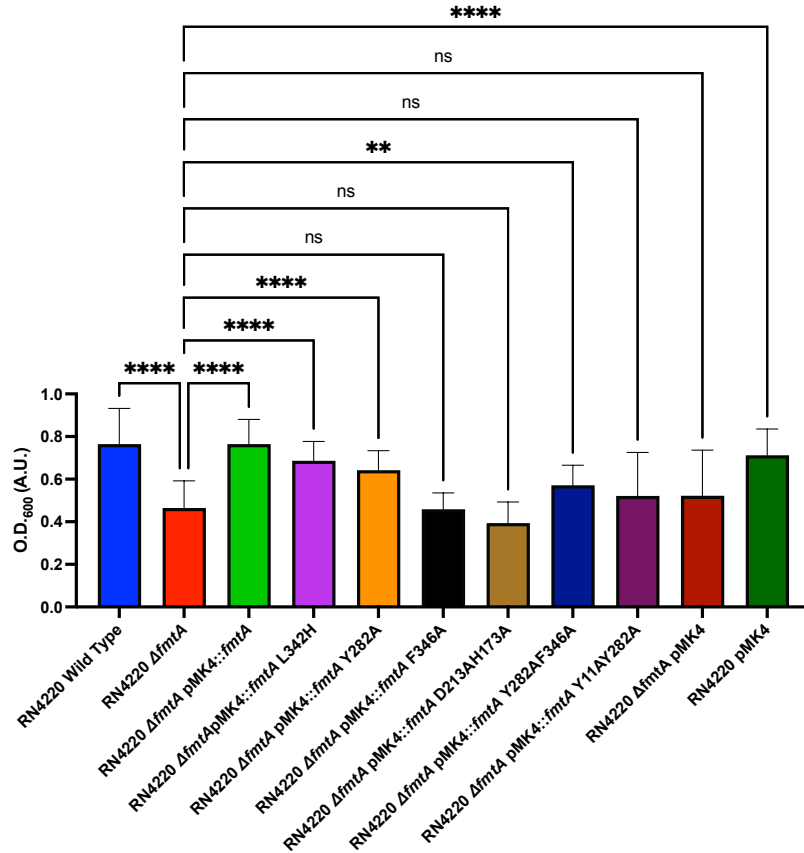


Figure 3.3.3.2. The analysis of biofilm formation by RN4220 Δ fmtA and its *fmtA* complemented strains of O.D.₆₀₀ = 0.02 supplemented with 0.2% glucose which was incubated for 24 hours at 37 °C in a 95-well flat bottom plate for biofilms to form. The biofilms were then washed 4 times with 1x PBS and fixed for 4 hours with Bouin's fixative. After this, the biofilms were stained with 0.2 % safranin for 10 minutes and then dissolved in 30% acetic acid for 15 minutes. Finally, O.D.₆₀₀ was measured in the Synergy H₄ Hybrid Reader. Data represent the mean $\pm$ SD from three independent experiments, where each experiment is an average of 30 wells. Statistical significance was evaluated using a parametric one-way ANOVA followed by Dunnett's multiple comparison test (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$, and "ns" is not significant).

Table 3.3.3. Table showing the relative biofilm formation by the RN4220 $\Delta fmtA$ mutant and its complemented strains to the wild-type RN4220 cells.

Strain	Relative biofilm formation (%)
RN4220 wild type	100
RN4220 $\Delta fmtA$	66
RN4220 $\Delta fmtA$ pMK4:: <i>fmtA</i> complemented	100
RN4220 $\Delta fmtA$ pMK4:: <i>fmtA</i> L342H	90
RN4220 $\Delta fmtA$ pMK4:: <i>fmtA</i> Y282A	84
RN4220 $\Delta fmtA$ pMK4:: <i>fmtA</i> F346A	60
RN4220 $\Delta fmtA$ pMK4:: <i>fmtA</i> D213AH173A	52
RN4220 $\Delta fmtA$ pMK4:: <i>fmtA</i> Y282AF346A	75
RN4220 $\Delta fmtA$ pMK4:: <i>fmtA</i> Y11AY282A	69
RN4220 $\Delta fmtA$ pMK4	68
RN4220 pMK4	93

Through a comparative analysis of biofilm formation, a statistically significant difference was observed between the RN4220 $\Delta fmtA$ strain and the wild-type RN4220 strain. The RN4220 $\Delta fmtA$ cells exhibited approximately a 34% decrease in biofilm formation compared to the wild-type cells (Fig. 3.3.3.2 and Table 3.3.3). However, when the $\Delta fmtA$ cells were complemented with the wild-type *fmtA* gene, the biofilm-forming capability of the strain mirrored that of the wild-type. The complemented strain regaining its biofilm-formation capabilities suggests that the wild-type gene successfully restored the biofilm-forming phenotype.

Additionally, compared to the wild-type cells, the biofilm formation exhibited by cells complemented with *fmtA* variants displayed significant variations. Notably, the variants Y282A

and L342H exhibited biofilm formation of approximately 80-90% when compared to the wild type. On the other hand, variants F346A and D213AH173A demonstrated the lowest activity, ranging between 50-60% of the wild type's biofilm formation while the Y282AF346A mutant displayed 75% activity. Statistical significance data suggests that the complementation with the mutant genes F346A, D213AH173A and Y211AY282A does not improve the strain's capability of biofilm formation greater than the deleted strain, suggesting that these mutations render the strain's capability similar to the deleted strain (Fig. 3.3.3.2 and Table 3.3.3).

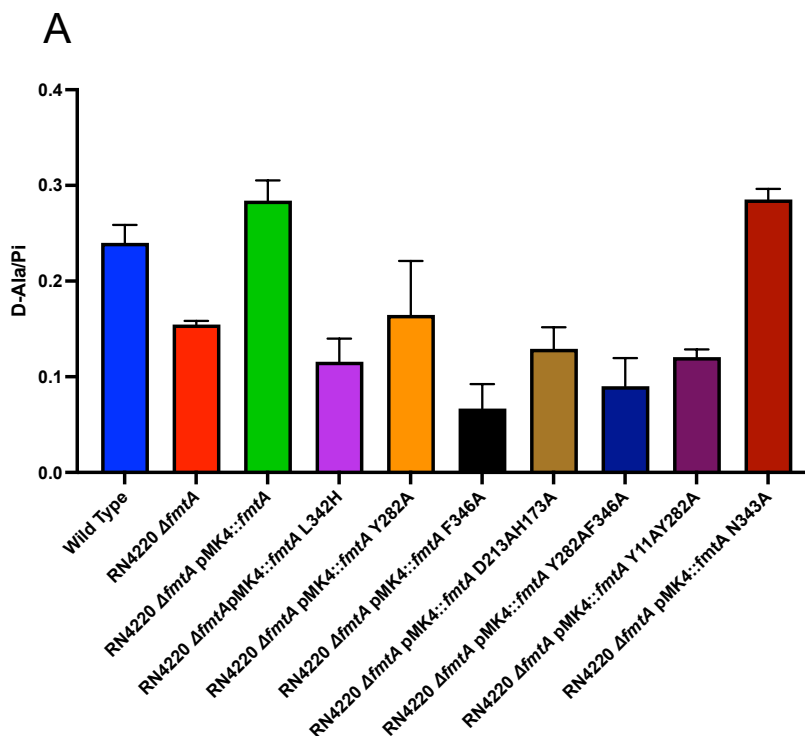
Meanwhile, the biofilm formation data for the L342H, Y282A, and Y282AF346A variants indicate a partial recovery of biofilm formation capabilities compared to the *fntA* deletion strain. However, this recovery does not restore biofilm formation to wild-type levels. It is also worth noting that there were no statistically significant differences in biofilm formation between the RN4220 $\Delta fntA$ pMK4 and RN4220 $\Delta fntA$ strains, nor between the RN4220 and RN4220 pMK4 strains highlighting that the pMK4 vector does not interfere with the biofilm formation process (Fig. 3.3.3.2 and Table 3.3.3).

3.3.4 Characterizing the WTAs isolated from *S. aureus* RN4220, $\Delta fntA$ and its complemented strains

Here my objective was to characterize the WTAs isolated from variants (this includes the *fntA* strain, the *fntA* strain complemented with the wild-type gene, and the mutant strains L342H, Y282A, F346A, D213AH173A, Y282AF346A, and Y211AY282A). These variants were selected based on their diminished enzymatic activity (7-17% remaining activity compared to the wild type), as determined by kinetic experiments conducted via $^1\text{H-NMR}$. As a partial control, the mutant N343A (demonstrating 67% remaining activity by $^1\text{H-NMR}$ compared to the wild type) was also subjected to a similar treatment. The characterization of WTA focused on the D-Ala

content relative to their phosphate concentration [Pi]. Since the mutations introduced in FmtA do not affect the [Pi] of the strain, and the [Pi] is solely dependent on the amount of WTA, the [Pi] serves as an accurate indicator of the WTA levels present (Fig. 3.3.4).

In my analysis, the $\Delta fmtA$ strain exhibited a D-Ala/Pi ratio approximately 1.6-fold lower compared to the wild type. Meanwhile, the mutants L342H, Y282A, F346A, D213AH173A, Y282AF346A, and Y211AY282A exhibit a D-Ala/Pi ratio ranging from 1.6 to 3.4 folds lower than the ratio observed in the wild-type strain. Among these, the lowest ratio was observed in the F346A (3.4 folds less compared to the wild type) mutant strain, and the highest ratio was observed for the Y282A (1.6 folds less compared to the wild type) mutant strain. Additionally, complementing the $\Delta fmtA$ strain with the wild-type *fmtA* gene and the N343A mutant demonstrates a D-Ala/Pi ratio which is 1.2 folds higher than the wild-type strain. Notably, except the N343A mutant (which retained 67% of wild-type activity), all other variants showed no more than 20% remaining activity as measured by $^1\text{H-NMR}$ *in vitro* (Fig. 3.3.4).



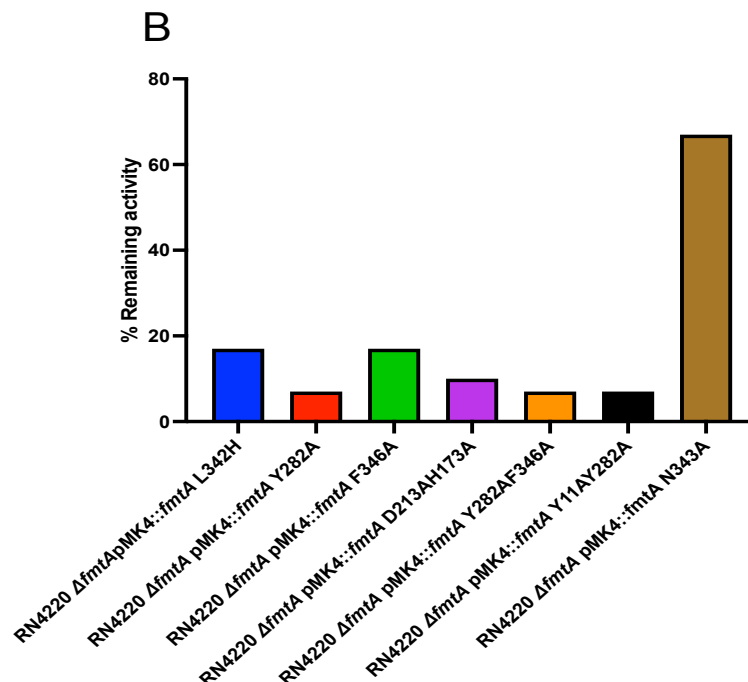


Figure 3.3.4. A. Analysis of the D-Ala/Pi ratio of the WTAs isolated from the RN4220, Δ *fmtA* and its *fmtA*-complemented strains. Pi for the WTAs of the individual strains were determined following the protocol provided in the QuantiChrome Phosphate Assay Kit and the amount of D-Ala for the same strains was determined by ^1H -NMR. Error bars represent the standard deviation calculated from two independent trials. **B.** % Remaining activity determined in the *fmtA* mutant strains compared to the wild-type strain determined by ^1H -NMR.

3.3.5 Effects of Triton X-100 on the *S. aureus* RN4220, Δ *fmtA* and its complemented strains

My study aims to investigate the role of *fmtA* in maintaining overall cell wall integrity when subjected to chemical stress. Triton X-100 is a commonly known detergent widely used in biological experiments for disrupting cell walls and exposing intercellular components (Koley and Bard, 2010).

My experimental findings provide insights that are consistent with previous research (Komatsuzawa et al., 1999), where the Δ *fmtA* strain of *S. aureus* was more susceptible to Triton X-100. Notably, the data in this study show when the Δ *fmtA* strain is complemented with the wild-type *fmtA*, the Triton X-100 susceptibility values revert to wild-type levels. During the initial 0-2

hours of incubation, a transient rise in O.D.₆₀₀ is observed across all treated cells, followed by a subsequent steady decline.

The most significant impact was observed for the variants L342H, F346A, and Y211AY282A, which resembled the $\Delta fmtA$ strain in terms of lysis extent. Within 8 hours of exposure, these variants demonstrated 46% more lysis compared to the wild-type. The rest of the variants namely Y282A, D213AH173A, and Y282AF346A showed a smaller improvement in resistance, demonstrating 34% more lysis compared to the wild-type (Fig. 3.3.5).

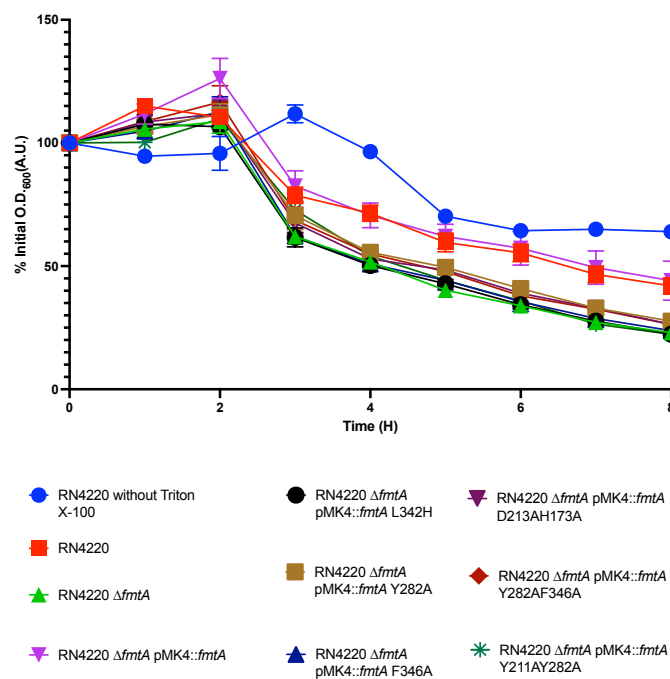


Figure 3.3.5. Analysis of the impact of Triton X-100 on RN4220 $\Delta fmtA$ and its *fntA* complemented strains. The cells were grown from an overnight culture till O.D.₆₀₀ = 0.8 and were pelleted and washed in 50 mM Tris-HCL (pH 7.5). Then the cells were incubated with 0.05% Triton X-100 and were incubated at 37°C, 180 rpm. O.D.₆₀₀ was measured in a Synergy H4 Hybrid Reader at different time points in the incubation process. A control experiment was performed with the wild-type cells without Triton X-100 and each data point is an average of triplets. % initial O.D.₆₀₀ was calculated by considering their individual 0-Hour O.D.₆₀₀ data as 100%. Error bars represent the standard deviation calculated from three independent trials performed in triplicate.

3.3.6 Effects of lysozyme on the *S. aureus* RN4220, $\Delta fmtA$ and its complemented strains

In this study, the strains were exposed to lysozyme for 8 hours where the experimental findings indicate minimal impact on wild-type RN4220 cells, with approximately 3% lysis observed during this period compared to untreated wild-type cells. In contrast, deletion of the *fmtA* gene makes the strain considerably more susceptible to lysozyme, with approximately 55% lysis observed over the same duration.

Interestingly, complementing the RN4220 $\Delta fmtA$ strain with the *fmtA* wild-type gene confers lysozyme resistance nearly comparable to wild-type levels, with around 13% lysis observed during the 8-hour exposure period. Remarkably, variants F346A and Y211AY282A displayed heightened resistance to lysozyme during the initial hour of incubation, distinguishing these two mutations from the rest. However, after 2 hours of exposure for F346A and 3 hours for Y211AY282A, these strains begin to mimic the susceptibility pattern observed in the $\Delta fmtA$ strain, similar to the other mutant strains (Fig. 3.3.6).

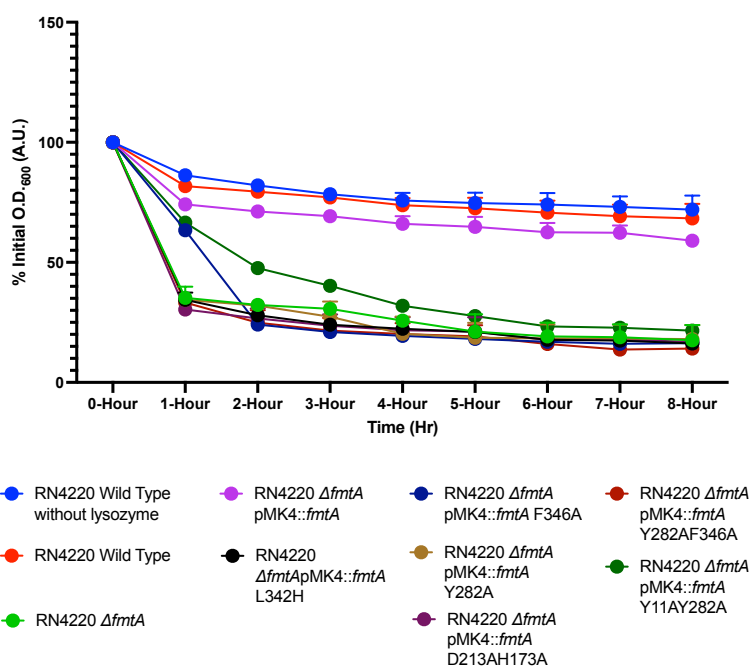


Figure 3.3.6. Graphical representation of the % initial O.D.₆₀₀ values illustrating the extent of lysis observed in RN4220, $\Delta fmtA$, and complemented strains upon exposure to 350 μ g/ml

lysozyme for 8 hours. Error bars represent the standard deviation calculated from three independent trials performed in triplicate.

3.3.7 Effects of lysostaphin on the *S. aureus* RN4220, *ΔfmtA* and its complemented strains

The aim of this study is similar to that of my lysis assay with lysozyme. Here, similar to the lysozyme-treated strains, my initial analysis centred on examining the % initial O.D.₆₀₀ data for the lysostaphin-treated strains. However, I observed no discernible trends in the data (Fig. 3.3.7.1). Consequently, the attention was redirected to investigating the decrease in colony-forming units/ml (CFU/ml) over time in response to lysostaphin stress (Fig. 3.3.7.2).

According to the graph representing the CFU/ml data (Fig. 3.3.7.2), after 200 mins of lysostaphin exposure, variants Y282A and D213AH173A exhibited the highest susceptibility, with complete lysis occurring within 120 minutes. Among the strains that were able to endure 200 minutes of lysostaphin stress, the highest susceptibility was observed in strain Y282AF346A (approximately 20-fold compared to the wild-type), followed by strain F346A (approximately 5-fold compared to the wild-type), followed by the L342H strain (approximately 2-fold compared to the wild-type). After 200 minutes of exposure, the N343A strain exhibited behavior similar to the F346A strain. However, up until 160 minutes of lysostaphin exposure, it closely resembles the wild-type strain. The remaining strains showed a trend similar to the wild-type strain, although a slightly elevated resistance against lysostaphin was observed for the complemented strain.

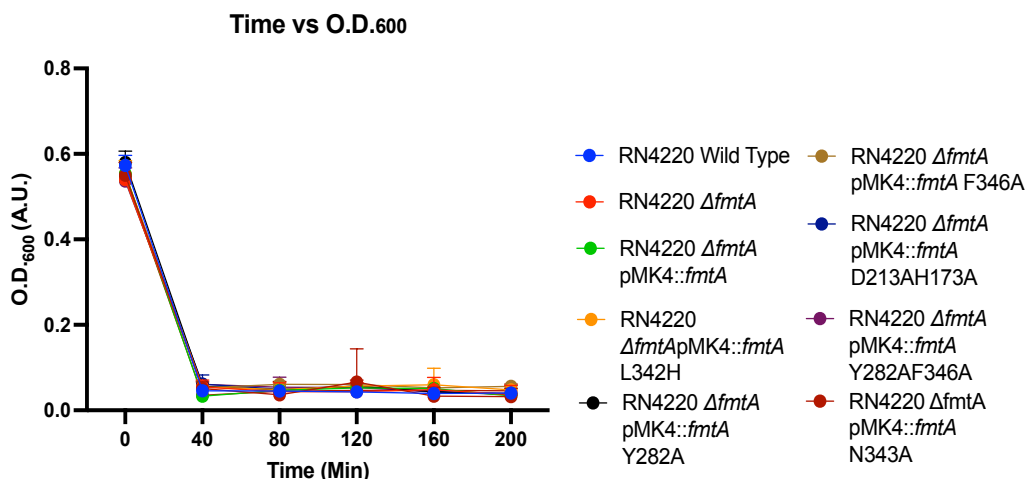


Figure 3.3.7.1. Graphical representation of the % initial O.D.₆₀₀ illustrating the extent of lysis observed in RN4220, *ΔfmtA*, and its complemented strains upon exposure to 0.1 μg/ml lysostaphin over 200 minutes. Error bars represent the standard deviation calculated from three independent trials performed in triplicate.

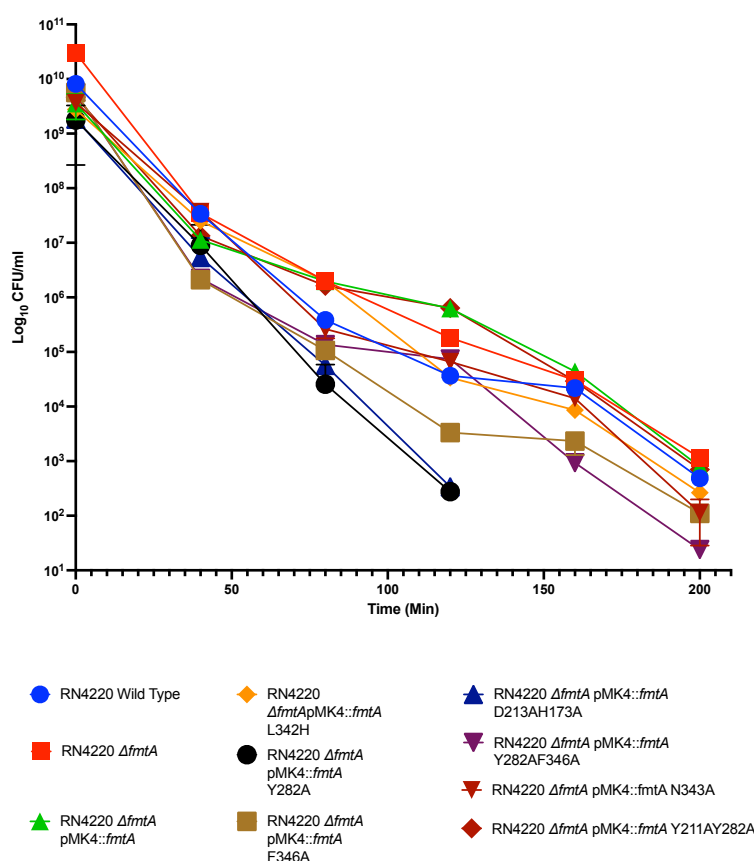


Figure 3.3.7.2. Graph illustrating the log decrease in CFU/ml over time, demonstrating the observed effects on RN4220, *ΔfmtA*, and its complemented strains following exposure to 0.1 μg/ml lysostaphin for 200 minutes. Error bars represent the standard deviation calculated from three independent trials performed in triplicate.

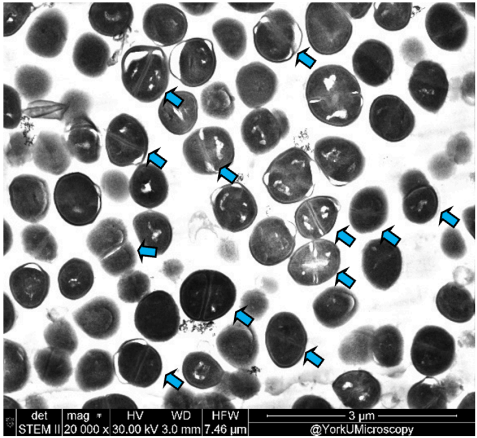
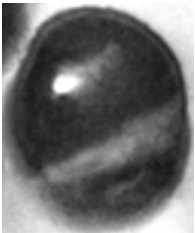
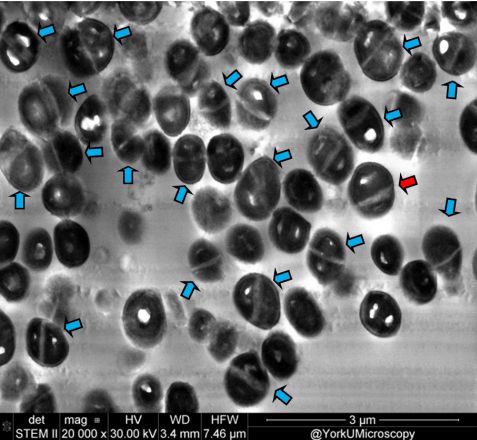
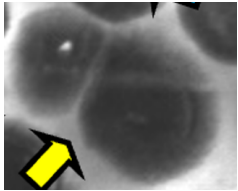
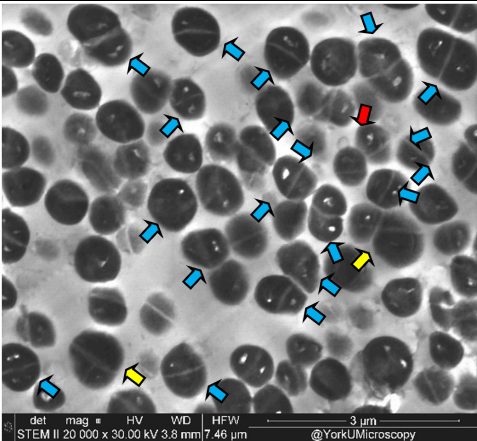
3.3.8 Effects of *fntA* deletion and its complementation with mutant genes in *S. aureus* RN4220 cell division

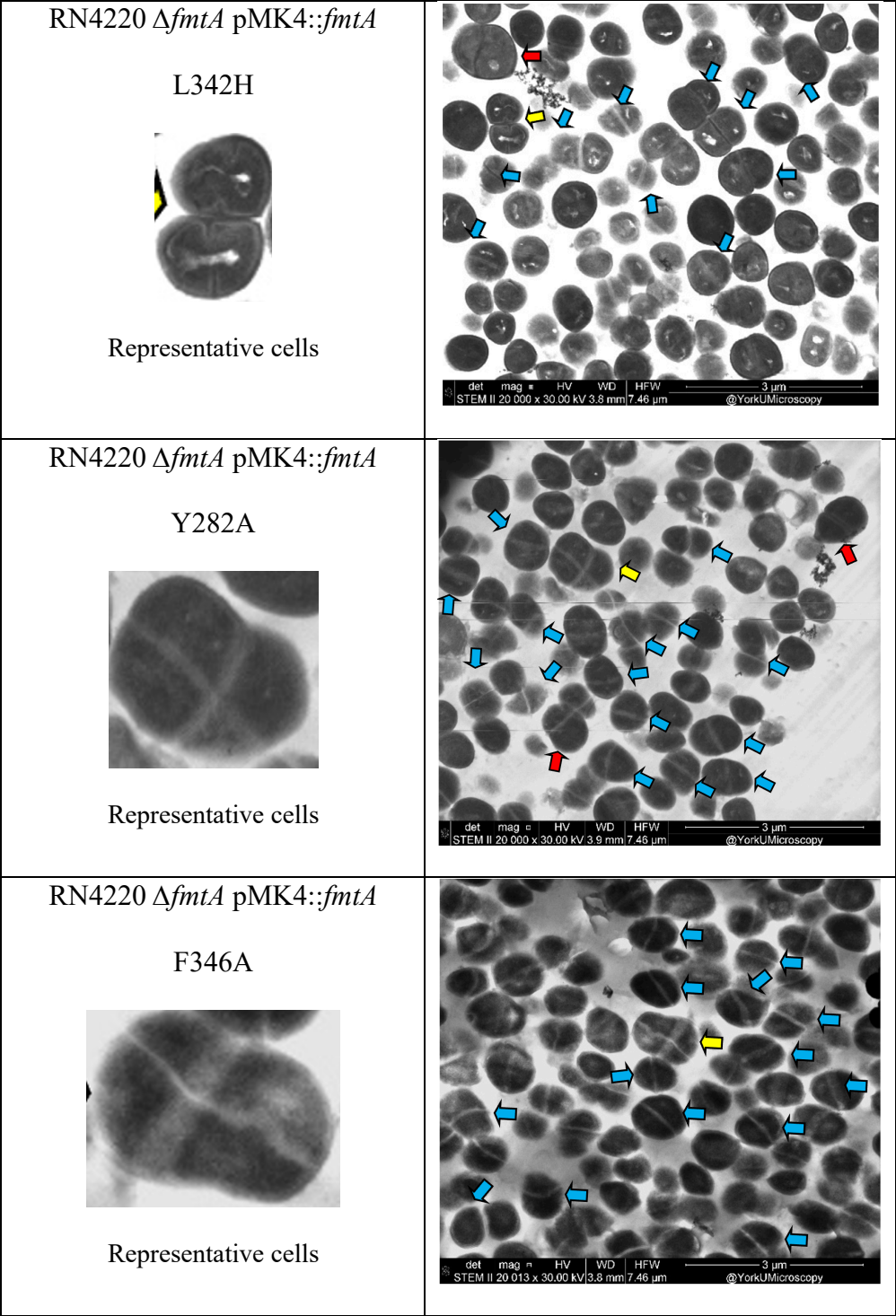
In this study, we investigated the impact of *fntA* gene deletion and its complementation with either wild-type or mutant genes on cell division patterns, particularly focusing on two distinct categories: asymmetrically dividing cells and those forming multiple division septa (Table 3.3.8).

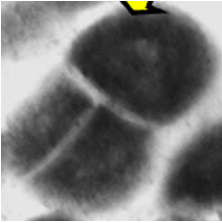
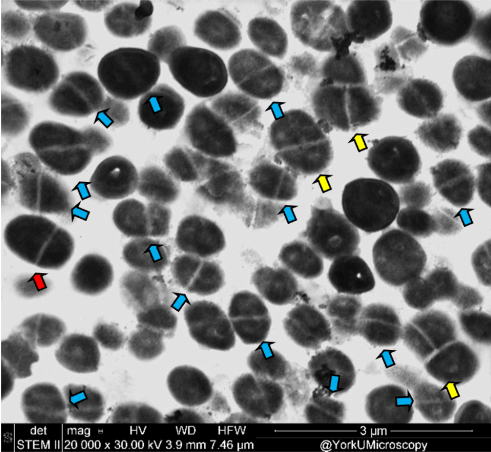
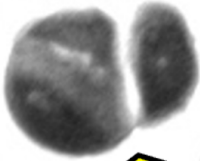
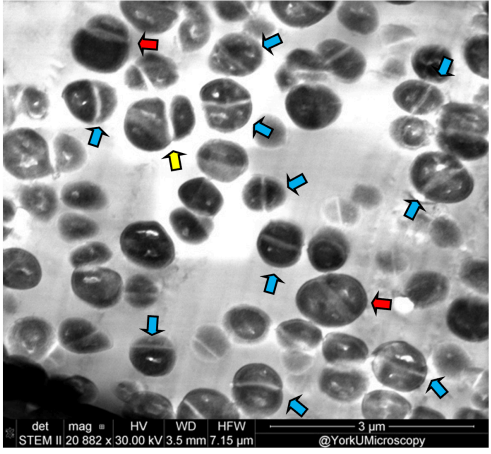
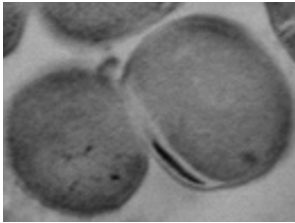
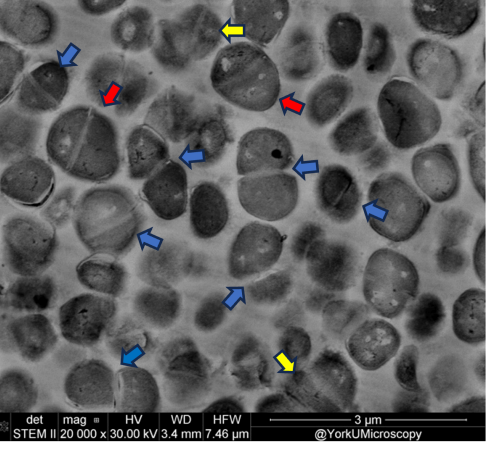
Regarding the asymmetric cell divisions, I observed a diminutive increase (0.5-2%) in the frequency of asymmetrically dividing cells amongst the variants $\Delta fntA$, Y282AF346A, and Y211AY282A compared to the wild type. Conversely, a decrease (1-3%) in the frequency of asymmetrically dividing cells was recorded in the complemented strain and variants L342H, Y282A, F346A, and D213AH173A. However, these differences were not statistically significant (Fig. 3.3.8.1).

Focusing on the formation of multiple-division septa, the $\Delta fntA$ mutant exhibited the lowest frequency (~ 5%) compared to the wild type (where no multiple-division septa-forming cells were recorded). In contrast, other variants displayed a higher frequency (7-13%) of multiple division septa formation relative to the wild type. These changes were statistically significant compared to the wild type (Fig. 3.3.8.2).

Table 3.3.8. The table showcases examples (one each) of TEM images captured for each of the RN4220 *ΔfmtA* variants, as well as for the wild type and the complemented strains. Dividing cells are indicated by blue arrows, while asymmetrically dividing cells are marked with red arrows. Dividing cells with multiple division septa are highlighted with yellow arrows.

Mutant	Image
RN4220 wild type  A single representative cell	
RN4220 <i>ΔfmtA</i>  A single representative cell	
RN4220 <i>ΔfmtA</i> pMK4::<i>fmtA</i> complemented  Representative cells	



RN4220 $\Delta fmtA$ pMK4::<i>fmtA</i> D213AH173A  Representative cells	 det mag = HV WD HFW 3 μm STEM II 20 000 x 30.00 kV 3.9 mm 7.46 μm @YorkUMicroscopy
RN4220 $\Delta fmtA$ pMK4::<i>fmtA</i> Y282AF346A  Representative cells	 det mag = HV WD HFW 3 μm STEM II 20 882 x 30.00 kV 3.5 mm 7.15 μm @YorkUMicroscopy
RN4220 $\Delta fmtA$ pMK4::<i>fmtA</i> Y11AY282A  Representative cells	 det mag = HV WD HFW 3 μm STEM II 20 000 x 30.00 kV 3.4 mm 7.46 μm @YorkUMicroscopy

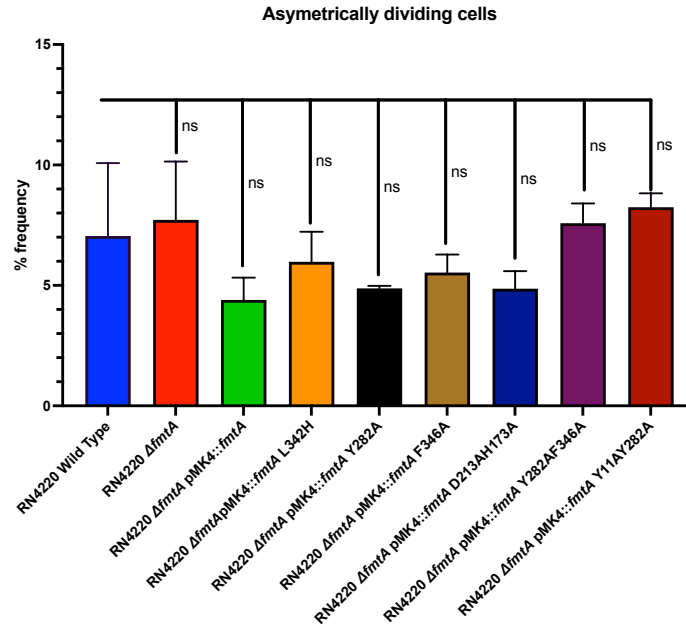


Figure 3.3.8.1. Graphical representation of the % frequency of the asymmetrically dividing cells observed in RN4220, Δ fmtA, and its complemented strains analyzed by TEM. Data represent the mean $\pm$ SE from two independent experiments, each with a sample size of 100. Statistical significance was evaluated using a parametric one-way ANOVA followed by Dunnett's multiple comparison test (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$, and “ns” is not significant).

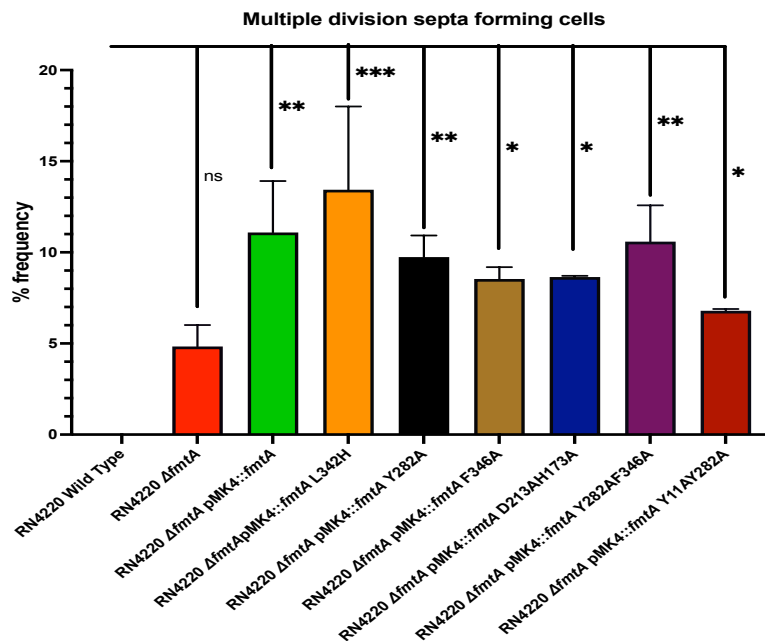


Figure 3.3.8.2. Graphical representation of the % frequency of the multiple division septa forming cells observed in RN4220, Δ fmtA, and its complemented strains analyzed by TEM. Data represent the mean $\pm$ SE from two independent experiments, each with a sample size of 100.

Statistical significance was evaluated using a parametric one-way ANOVA followed by Dunnett's multiple comparison test (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$, and "ns" is not significant).

3.4. Discussion

In this chapter, my examination involves an *in vivo* approach to investigate the effects of the specific active site residues of FmtA, focusing on those critical for its catalytic activity and ability to bind WTA. The targeted mutations were selected based on their behaviour, resembling that of a catalytically inactive protein as observed in enzymatic activity assays via $^1\text{H-NMR}$. By systematically replacing the wild-type *fmtA* gene with these mutant variants, I explored how these alterations impact various physiological processes in *S. aureus*, including biofilm formation, cell wall integrity, resistance to lysozyme and lysostaphin, and cell division.

Biofilms are communities of microorganisms, consisting of similar species, that adhere to surfaces and form on various biotic or abiotic substrates such as medical equipment, infection sites, or implants (Davey & O'toole, 2000). These biofilms are encased in a matrix-like film, known as extracellular polymeric substance (EPS), which is self-produced by the bacteria. The cells within biofilms, termed planktonic cells, exhibit distinct gene expression patterns compared to those responsible for biofilm formation (Shemesh et al., 2007; Toyofuku et al., 2016). The *fmtA* gene plays a crucial role in this process (Qamar & Golemi-Kotra, 2012). FmtA, has been shown to influence biofilm formation through its enzymatic activity against WTA by affecting cell wall integrity in the process (Qamar & Golemi-Kotra, 2012; Y. Zhao et al., 2012). The hypothesis is based on the fact that the mutations in the *fmtA* gene which renders the protein almost functionally inactive *in vitro* can affect the biofilm formation process. Understanding the role of FmtA in biofilm formation may provide new insights into combating biofilm-associated infections and developing strategies to target these resilient bacterial communities.

Based on the study reported by (Qamar & Golemi-Kotra, 2012) the addition of up to 0.2 μM of exogenous FmtA ^{$\Delta 27$} to RN4220 cells enhances biofilm formation compared to controls lacking exogenous FmtA. Beyond this concentration, a reduction in biofilm production was observed. My study with wild-type FmtA ^{$\Delta 27$} aligns with these findings, confirming similar biofilm-enhancing effects. Furthermore, I investigated the potential effects of active site mutations on this process by analyzing the corresponding mutant proteins. The results suggest that the L342H mutation did not significantly affect biofilm formation, whereas mutations in Y282A, F346A, D213A H173A, and Y282A F346A led to a ~45% reduction in biofilm formation, suggesting partial inactivation of the process. Notably, the Y211A Y282A mutant completely diminished the enzyme's ability to enhance biofilm formation, rendering it similar to the control levels (without exogenous FmtA ^{$\Delta 27$}) indicating a total loss of biofilm enhancement activity. These results suggest that specific residues within the FmtA active site are critical for its role in biofilm formation, and their alteration can severely impair the enzyme's function in assisting in biofilm formation.

As my research demonstrated the different degrees of impact by the wild-type FmtA ^{$\Delta 27$} and its selected variants on the biofilm formation process of *S. aureus* when applied exogenously, I also incorporated the investigation of the role of the *fmtA* gene in its cellular level. In a past study, Boles et al., 2010 demonstrated an impaired biofilm formation in *S. aureus* strain SH1000 when the function of *fmtA* was disrupted by transposon insertion. In a parallel study, I observed a reduction in biofilm formation by the RN4220 Δ *fmtA* strain. However, the biofilm levels were partially or fully restored when the wild type *fmtA* gene or its mutant variants were reintroduced into the Δ *fmtA* strain using the pMK4 vector, underscoring the gene's critical role in the process of biofilm formation.

The *ΔfmtA* strain complemented with the wild-type gene demonstrates a biofilm formation level comparable to the wild-type, suggesting a full recovery. In contrast, the F346A, D213AH173A, and Y211AY282A variants exhibited no significant difference in biofilm formation compared to the *ΔfmtA* strain, indicating that these mutations render the strain as inactive as the deletion strain itself. The L342H, Y282A, and Y282AF346A variants exhibited partial inactivity (20-30% reduction) in biofilm formation compared to the wild type. Although the L342H variant exhibits an enzymatic inactivity as confirmed by ¹H-NMR, the incorporation of this mutated gene into the *ΔfmtA* strain results in biofilm formation levels comparable to the wild type. This suggests that while this mutation leads to an enzymatic inactivation *in vitro*, its impact may be less pronounced *in vivo*, allowing the strain to retain its biofilm formation capabilities. Notably, the pMK4 vector itself did not influence biofilm formation, as biofilm levels remained consistent between the *ΔfmtA* strain and the *ΔfmtA* pMK4 strain, as well as between the RN4220 (wild type) and RN4220 pMK4 strain. This consistency indicates that the observed changes in biofilm formation were solely attributable to the specific mutations.

I further investigated the selected mutant genes by characterizing their D-Ala/Pi ratio, given the critical role of the *fmtA* gene in regulating the amount of D-Ala in *S. aureus* strains. The D-Ala levels in *S. aureus* serve as a crucial parameter as it influence various physiological activities, including virulence, surface attachment, biofilm formation, cell division, and autolysis (Nikolopoulos et al., 2023; Rahman et al., 2016). The D-Ala/Pi ratio obtained from the variants serves as a key metric for assessing the impact of specific mutations on the D-Ala modifications of WTAs within these strains.

My results indicate that the D-Ala/Pi ratio between the *ΔfmtA* strain and the Y282A variant was similar, with both showing approximately a 38% reduction compared to the wild type. While

the L342H, D213AH173A, and Y211AY282A variants exhibited comparable ratios, each demonstrating about a 45% decrease relative to the wild type. In contrast, the mutant strains N343A and the $\Delta fmtA$ strain complemented with the wild-type gene demonstrated D-Ala/Pi ratios comparable to those of the wild-type. Notably, the Y282AF346A and F346A variants exhibited the lowest D-Ala/Pi ratios, showing reductions of approximately 63% and 75%, respectively, compared to the wild-type strain.

It is proposed by Rahman et al. (2016) that the *fmtA* gene regulates D-Ala levels in teichoic acids by removing D-Ala from LTA, making it available for WTA under specific conditions. Additionally, they propose that *fmtA* facilitates D-Ala removal from WTA to assist the cell in resetting its surface charge, likely in response to environmental changes. My data suggest that the complete deletion of the *fmtA* gene or complementation with the L342H, Y282A, F346A, D213AH173A, Y282AF346A, and Y211AY282A mutants likely impairs the strain's ability to varying degrees to remove D-Ala from LTA for incorporation into WTA. As a result, these mutants exhibit a 38–75% lower D-Ala/Pi ratio in WTA compared to the wild type. In contrast, the D-Ala/Pi ratio in WTA remains comparable to those of the wild type when the $\Delta fmtA$ strain is complemented with either the wild-type gene or the N343A mutant where the N343A mutant demonstrated a partial inactivation *in vitro*, and in these experiments serves as a hypomorphic control. This suggests that these variants retain *fmtA* function similar to that of the wild-type gene.

Earlier studies conducted by Komatsuzawa et al., (1997, 1999) have demonstrated that strains with a deleted *fmtA* gene exhibit increased susceptibility to Triton X-100, a commonly used detergent, compared to their wild-type counterparts. This finding highlights the significance of the *fmtA* gene in maintaining cellular integrity under stress conditions, which was also confirmed by Qamar & Golemi-Kotra, (2012). The *fmtA* gene plays a crucial role in the proper functioning of

the *S. aureus* cell wall of *S. aureus*. It is essential for preserving cell wall integrity, responding to environmental stress, and regulating cell division Qamar & Golemi-Kotra, (2012). Research has shown that *fmtA*-deleted variants display defects in cell wall composition and are more susceptible to cell wall-targeting antibiotics (R. Sobral & Tomasz, 2019). Furthermore, (Bibek et al., 2019) stated that *fmtA* expression is upregulated in response to cell wall stressors. *fmtA* gene is often co-regulated with other key cell wall stimulon components, such as autolysins and penicillin-binding proteins, which are essential for remodelling and repairing the cell wall during growth and division (Lee et al., 2015).

Similar to the previous research, our results confirm that the Δ *fmtA* strain is twice as susceptible to Triton X-100 compared to the wild-type strain. When the wild-type *fmtA* gene was reintroduced into the Δ *fmtA* strain, the Triton X-100 resistance of the strain reverted to wild-type levels, indicating that the change in susceptibility was due to the deletion of the gene. However, complementing the Δ *fmtA* strain with the variants under investigation did not alter susceptibility levels, which remained consistent with the deleted strain. This suggests that the FmtA protein must demonstrate peak performance *in vivo* to maintain cellular integrity against Triton X-100, highlighting the critical role of these specific residues. The findings underscore the importance of the *fmtA* gene in preserving the overall integrity of the cell wall in *S. aureus*.

Similarly, while investigating the impact of the variants on the overall cell wall integrity of *S. aureus*, I extended my research to examine the effects of peptidoglycan-targeting enzymes such as lysozyme and lysostaphin. Lysozyme is known for its ability to target the β -1,4-glycosidic bonds between N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) in bacterial peptidoglycan, and it plays a crucial role in disrupting the structural integrity of bacterial cell walls (Nawaz et al., 2022; Ragland & Criss, 2017). In *S. aureus*, where peptidoglycan integrity is

essential for cell viability, lysozyme poses a significant threat (Wecke et al., 1982). WTAs are important components that contribute to the overall integrity of *S. aureus* peptidoglycan (M. Wang et al., 2022), and the enzymatic activity of FmtA, which hydrolyzes D-Ala from WTA, is critical in modulating the cell wall composition (Rahman et al., 2016). The incorporation of D-alanine residues into WTAs is facilitated by the *dlt* operon, which neutralizes the negative charge of WTAs (Wu et al., 2021). This modification significantly impacts the interaction between bacterial cells and cationic antimicrobial agents. For instance, positively charged lysozyme (Li et al., 2019) is unable to bind to D-alanylated WTA, thereby reducing its effectiveness (Wu et al., 2021).

My results show an approximately two-fold increase in lysozyme susceptibility for the *ΔfmtA* strain and other mutant strains, except for the strain complemented with the wild-type gene, where lysozyme resistance is found to be similar to the wild type. Earlier in this thesis, I demonstrated that the D-Ala/Pi ratio in the variants were lower than those in the wild type, except for the strain complemented with the wild-type gene and the N343A mutant. This suggests that the variants carrying fewer D-Ala residues result in a more negatively charged cell surface. Since lysozyme is positively charged, an increase in negative charge likely enhances electrostatic interactions, making the mutants more susceptible to lysozyme. This likely explains the observed increase in lysozyme susceptibility in the *fmtA* variants compared to the wild-type strain.

Lysostaphin, much like lysozyme, targets the bacterial peptidoglycan of *S. aureus*, but it operates through a distinct mechanism. While lysozyme disrupts the β -1,4-glycosidic bonds between N-acetylglucosamine and N-acetylmuramic acid in the peptidoglycan, lysostaphin specifically targets and breaks the pentaglycine bridge that crosslinks peptidoglycan strands (Wang et al., 2022). Positively charged lysostaphin is a zinc metalloenzyme with potent bacteriolytic

activity, uniquely designed to hydrolyze the glycine-rich cross-bridges in the peptidoglycan structure of *S. aureus*, which leads to cell lysis (Browder et al., 1965).

My data suggest that certain variants, specifically Y282A and D213AH173A, exhibit the highest susceptibility towards lysostaphin in the experiment, as, unlike other strains, these variants undergo lysis within 120 minutes of lysostaphin exposure. Among the strains that survived 200-minute lysostaphin exposure, the Y282AF346A (24 ± 2.6 CFU/ml) variant exhibited the highest susceptibility, showing a 95% increase compared to the wild type (486.7 ± 6.1 CFU/ml). This was followed by the F346A (110.33 ± 13.6 CFU/ml) and N343A (113.7 ± 85.2 CFU/ml) variants, each displaying 75% greater susceptibility, and the L342H (264.7 ± 8.02 CFU/ml) variant, which demonstrated a 45% increase in susceptibility relative to the wild type. This indicates that lysostaphin can more effectively access and cleave the pentaglycine peptide chain in these mutants, making them more vulnerable to its bacteriolytic action. This increased susceptibility correlates with the reduced D-Ala/Pi ratio observed in these variants compared to the wild type, except for the N343A strain. A lower D-Ala/Pi ratio recorded in these variants compared to the wild type suggests that these variants carry a higher net negative charge in their WTA, which likely enhances electrostatic interactions with lysostaphin, leading to greater lysis. A similar trend was also observed in the lysozyme susceptibility experiment for these variants, which further supports this hypothesis. While the N343A ($14.1 \times 10^3 \pm 8.3 \times 10^2$ CFU/ml) strain initially exhibited wild-type ($21.8 \times 10^3 \pm 1.6 \times 10^3$ CFU/ml) levels of resistance up to 160 minutes of lysostaphin exposure, its susceptibility increased beyond this point likely due to the effects of the mutation.

In their study (Qamar & Golemi-Kotra, 2012) demonstrated the localization of FmtA at the division septum in *S. aureus*, suggesting a role in cell division. Similarly, Santa Maria et al., (2014) explored the function of WTA in *S. aureus* and reported on WTA's role in the bacterial cell

division process. In my analysis of asymmetrically dividing cells and the cells forming multiple-division septa in the variants, I observed a trend of a statistically significant increase in the number of cells forming multiple-division septa in the variants (except *ΔfmtA*) compared to the wild type, where such cells were non-existent. This observed trend suggests that the appearance of multiple septa-forming cells is a result of mutations affecting the proper cell division process within the cells.

While some variations were observed in the number of asymmetrically dividing cells among the variants, with heightened frequencies in *ΔfmtA*, Y211F, Y282AF346A, and Y211AY282A variants and decrease in the others, these changes did not reach statistical significance, suggesting a minimal impact of the mutations on asymmetrical divisions observed in these strains.

In conclusion, my results indicate that while the variants under investigation exhibit characteristics of inactive enzymes *in vitro*, they do not uniformly manifest a complete loss of function *in vivo*. Instead, most variants demonstrate at least a partial deficiency in function. Furthermore, my research confirms the role of the *fmtA* gene in biofilm formation, resistance to lysozyme and lysostaphin, and cell division. I also demonstrate that variants exhibiting the more pronounced decrease in the D-Ala/Pi ratio are more susceptible to lysozyme and lysostaphin and show atypical multiple-division septa formation under electron microscopy. These functional traits are most likely attributed to the dependence of these genes on the D-Alanylation levels of WTA for proper cellular function.

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Chapter Four

Effects of BPEI in FmtA-WTA Interaction

4.1 Introduction

The primary strategy for addressing antibiotic resistance in *Staphylococcus aureus* has been synthesizing novel antimicrobial agents (Murugaiyan et al., 2022). Advanced antibiotics such as fifth-generation cephalosporins (e.g., ceftaroline) and lipoglycopeptides (e.g., telavancin) have demonstrated effectiveness against MRSA (Bal et al., 2017). However, the rapid emergence of bacterial resistance to these new drugs poses a significant challenge. The indiscriminate and excessive use of antibiotics in both clinical and agricultural settings accelerates this resistance development, thereby diminishing the therapeutic lifespan of newly introduced antibiotics (Muteeb et al., 2023). An alternative strategy to combat antibiotic resistance has been the implementation of combination therapies, which involve the concurrent administration of multiple antibiotics. This approach aims to target various bacterial pathways simultaneously, thereby reducing the likelihood of resistance development (Tyers & Wright, 2019). However, bacteria can still concurrently evolve resistance mechanisms to multiple drugs, particularly when combination therapies are overused or improperly prescribed (Marimani, 2020).

4.1.1 Approaches employed to combat antibiotic resistance in *Staphylococcus aureus*

Among the alternatives to traditional antibiotics, several innovative approaches are under investigation, such as bacteriophage therapy, anti-virulence strategies or immunotherapy (Theuretzbacher & Piddock, 2019). Bacteriophage therapy has gained attention in recent years, particularly against antibiotic-resistant pathogens like *S. aureus*. It employs viruses that specifically infect and lyse bacteria, providing a highly targeted approach to bacterial eradication

(Nikolich & Filippov, 2020). They target bacteria by binding to specific receptors on the bacterial surface, injecting their genetic material, and hijacking the bacterial machinery to produce more phages (Hommes & Surewaard, 2022). This process eventually results in bacterial cell lysis, releasing new phages that can infect additional bacteria (Nikolich & Filippov, 2020). For *S. aureus*, phages can be tailored to target specific strains, making them highly selective in comparison to broad-spectrum antibiotics (Kolenda et al., 2022). This selectivity reduces the risk of disrupting the body's normal microbiota, minimizing the side effects commonly associated with antibiotic use (Kolenda et al., 2022). Bacteriophage therapy has shown efficacy in targeting multi-drug resistant *S. aureus* strains, including MRSA and VRSA (Atshan et al., 2023). Since phages have a different mechanism of action than antibiotics, they can kill bacteria that have developed resistance to traditional treatments (Dickey & Perrot, 2019). In *S. aureus* some bacteriophages have also been shown to penetrate and disrupt biofilms, enhancing their effectiveness where antibiotics often fail (Dickey & Perrot, 2019). One of the key advantages of bacteriophage therapy is that phages only replicate in the presence of their specific bacterial hosts (Atshan et al., 2023). As the bacterial population diminishes, the phage numbers naturally decrease as well, minimizing the risk of unintended side effects and preventing the over-proliferation of the phages in the absence of their targets (Dickey & Perrot, 2019).

While phages' high specificity is an advantage, it can also be a limitation. A single phage may only target specific *S. aureus* strains, requiring careful selection of phages or the use of phage cocktails to ensure broad efficacy against diverse bacterial populations (Atshan et al., 2023). Moreover, the human immune system can recognize and neutralize bacteriophages before they reach their bacterial targets (Popescu et al., 2024). This immune response may reduce the efficacy of phage therapy, especially if multiple doses are required (Popescu et al., 2024). Research into

encapsulating phages or using immune-suppressing strategies is ongoing to address this issue (W. Z. Wang et al., 2024).

Anti-virulence strategies offer an innovative approach to combating *S. aureus* infections by focusing on neutralizing the bacterium's virulence factors rather than killing the pathogen outright (Hotinger et al., 2021). Unlike traditional antibiotics, which aim to eliminate bacterial cells and often contribute to the development of resistance, these strategies disarm the pathogen without exerting selective pressure for resistance (Hotinger et al., 2021). By mitigating pathogenicity instead of promoting bacterial death, anti-virulence approaches provide a more sustainable and effective means of managing infections, reducing the likelihood of resistant strains emerging (Mühlen and Dersch, 2016). Toxin inhibitors are one of the strategies that target the production or function of *S. aureus* toxins, neutralizing their effects on host cells (Ahmad-Mansour et al., 2021). For instance, α -hemolysin, a pore-forming toxin, damages host cell membranes, contributing to tissue injury (Ford et al., 2021). Small-molecule inhibitors or neutralizing antibodies that block its interaction with host cells have shown potential in reducing both tissue damage and inflammation during infections (Ford et al., 2021). Similarly, leukocidins, such as Pantone-Valentine leukocidin (PVL), target and kill immune cells like neutrophils (Chan et al., 2019). Anti-leukocidin antibodies or inhibitors can neutralize this toxin, thereby preserving immune function and enhancing the host's ability to combat the infection (Chan et al., 2019). Additionally, these strategies include compounds that inhibit bacterial surface proteins involved in adhesion, such as clumping factor A (ClfA) and fibronectin-binding proteins, preventing *S. aureus* from attaching to host tissues (Chan et al., 2019). This reduces colonization and hampers the initiation of infection (Chan et al., 2019). Anti-biofilm approaches are also key strategies that utilizing enzymes like DNases or dispersin B to degrade the biofilm matrix, or molecules that

disrupt biofilm formation, such as quorum-sensing inhibitors (Lister & Horswill, 2014). By targeting biofilms, these strategies enhance the effectiveness of both the host immune response and antibiotic treatments, improving overall infection control (Lister & Horswill, 2014).

Despite the promise of anti-virulence strategies, several challenges remain. Many anti-virulence therapies are highly specific to particular bacterial strains or virulence mechanisms, which could limit their broad application across diverse *S. aureus* populations (Ford et al., 2021). Also, while the risk of resistance is lower, *S. aureus* can still potentially evolve mechanisms to overcome virulence inhibitors, particularly if they are used as monotherapies (Ahmad-Mansour et al., 2021). Moreover, anti-virulence therapies are still largely in preclinical or early clinical stages, and their efficacy, safety, and regulatory pathways are not as well-established as traditional antibiotics (Chan et al., 2019).

Finally, immunotherapy against *S. aureus* represents a promising alternative to traditional antibiotic treatments, especially in the context of increasing antibiotic resistance (Abebe & Birhanu, 2023). This approach leverages the host's immune system to target and eliminate *S. aureus* infections more effectively (Abebe & Birhanu, 2023). Immunotherapy aims to augment the host's immune response, thereby enhancing the ability to combat infections more effectively (Wallis et al., 2023). One immunotherapeutic strategy involves the use of vaccines designed to stimulate the immune system to recognize and neutralize *S. aureus*. For instance, vaccines targeting components like clumping factor A (ClfA), capsular polysaccharides, or toxins such as α -hemolysin aim to enhance the body's natural immune response to prevent infection or reduce its severity (Jahantigh et al., 2022). Monoclonal antibodies (mAbs) also play a crucial role in *S. aureus* immunotherapy (Otto, 2010). These engineered antibodies can specifically target bacterial toxins,

surface proteins, or virulence factors, neutralizing them and aiding in bacterial clearance (Otto, 2010). For example, mAbs against α -hemolysin and PVL can block their toxic effects, protecting host tissues and immune cells during infection (Otto, 2010). Passive immunization through these antibodies provides immediate, though temporary, protection in patients at high risk of infection, such as those undergoing surgery or with weakened immune systems (Schaffer & Lee, 2008). Another innovative strategy involves immune system modulators that enhance the body's ability to fight *S. aureus* (Seebach & Kubatzky, 2019). These modulators may include cytokines or immune checkpoint inhibitors that boost immune cell activity, improving the response to infection (Seebach & Kubatzky, 2019).

Overall, Immunotherapy against *S. aureus* presents a multifaceted approach that includes enhancing host immunity through vaccines, providing passive protection with monoclonal antibodies, and modulating immune responses (Abebe & Birhanu, 2023). This strategy is particularly crucial in addressing antibiotic-resistant *S. aureus* infections. While no vaccine has yet been approved for clinical use, several candidates targeting *S. aureus* surface proteins, toxins, and other virulence factors have shown promise in preclinical studies, highlighting the potential of immunotherapy as a critical tool in the fight against these challenging infections (Abebe & Birhanu, 2023). Additionally, advanced drug-delivery systems are being developed to release antibiotics in response to specific environmental triggers at the infection site, thereby optimizing therapeutic efficacy (Canaparo et al., 2019).

4.1.2 Wall teichoic acid as a key target in anti-virulence strategies and an introduction to BPEI as a potential inhibitor

WTAs are integral in maintaining the structural integrity of the *S. aureus* cell wall (R. Sobral & Tomasz, 2019). They enhance cell wall rigidity and protect against osmotic stress by

reinforcing the peptidoglycan matrix (Swoboda et al., 2010). WTAs also play a crucial role in regulating autolytic enzymes necessary for cell wall remodelling and turnover (M. Wang et al., 2022). In terms of pathogenicity, WTAs are pivotal in mediating *S. aureus* interactions with the host immune system (van Dalen et al., 2020). They facilitate immune evasion by masking peptidoglycan from recognition by host pattern recognition receptors, such as Toll-like receptors (TLRs) (Weidenmaier & Peschel, 2008). Additionally, WTAs can modulate the host immune response by influencing the production of pro-inflammatory cytokines, thereby affecting infection outcomes (Bron et al., 2012). WTAs also contribute to the intrinsic resistance of *S. aureus* to specific antibiotics (Swoboda et al., 2009). D-alanylation provides an overall positive charge to WTA and interferes with the action of cationic antimicrobial peptides (CAMPs) by repelling these positively charged molecules, thus preventing their binding to the bacterial cell surface (Malanovic & Lohner, 2016).

Branched polyethyleneimine (BPEI), a cationic branched amine, electrostatically binds to WTAs in *S. aureus*, leading to modifications in its properties and structure (Foxley et al., 2016). The combination of low-molecular-weight (600 Da) BPEI with β -lactam antibiotics, such as ampicillin, has demonstrated significant potential in overcoming β -lactam resistance in *S. aureus*, an effect that ampicillin alone cannot achieve (Foxley et al., 2016). Furthermore, BPEI's binding to WTAs induces steric hindrance blocking access to WTA, leading to re-sensitizing MRSA strains to various β -lactam antibiotics, including amoxicillin, methicillin, nafcillin, and ceftizoxime (Foxley et al., 2016; Hill et al., 2019).

BPEI's ability to bind WTA in wild-type MRSA renders WTA inaccessible to PBP4, a key enzyme involved in peptidoglycan crosslinking (Hill et al., 2019). This interference causes the delocalization of PBP4, disrupting its role in maintaining the structural integrity of the bacterial

cell wall (Hill et al., 2019). In *S. aureus*, however, the application of BPEI was found to have no impact on critical cell division enzymes, such as PBP2a and FtsZ, indicating its specific effect on PBP4 without disrupting the broader cell division machinery (Hill et al., 2019). However, in *Staphylococcus epidermidis*, it was found that BPEI renders PBP2a ineffective by binding to WTA, making the bacteria susceptible to β -lactam antibiotics (Lam et al., 2018). Although different MRSA strains require varying BPEI concentrations to impair resistance, BPEI's high biocompatibility and low cytotoxicity underscore its potential as a lead compound for drug development (Foxley et al., 2017). In contrast to high-molecular-weight BPEIs (>25,000 Da), which are toxic, the 600 Da BPEI does not induce nephrotoxicity and shows promise as a β -lactam antibiotic potentiator (Hill et al., 2019). Additionally, the synergistic effect of BPEI and β -lactam antibiotics is specific to *Staphylococcus* strains (Foxley et al., 2017; Lam et al., 2018). This combination does not exhibit synergy against other bacteria, such as *Escherichia coli* or *Bacillus subtilis* (Foxley et al., 2017).

4.1.3 Objectives of Chapter 4

In this chapter, through *in vitro* and *in vivo* experimentation, I explore the use of BPEI to probe the D-alanine esterase activity of FmtA, hypothesizing that when BPEI interacts with WTA as reported by Hill et al. (2019), Foxley et al. (2016, 2017) and Lam et al., (2018) it should theoretically prevent FmtA from binding to WTA. This could potentially yield results akin to those reported by Hill et al. (2019) and Lam et al. (2018) for PBP4. Their studies showed that BPEI induces the delocalization of PBP4 in *S. aureus* and inhibits PBP2a from binding to WTA in *S. epidermidis*, thereby increasing the susceptibility of these strains to β -lactam antibiotics.

4.2 Materials and Methods

4.2.1 Materials and Chemicals

The growth media, glucose, HEPES buffer, D-Amino oxidase (DAO), Flavin adenine dinucleotide (FAD), Horseradish peroxidase (HRP) and amplex red for the enzyme coupled assay were procured from Sigma-Aldrich (Canada) while BPEI (600 Da) was purchased from Thermo fisher scientific.

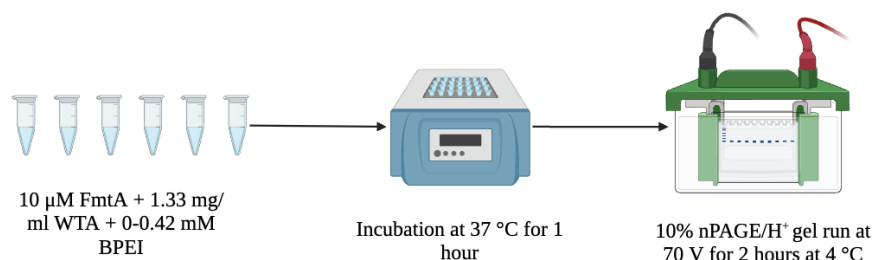
4.2.2 Investigation of the binding interaction between FmtA^{Δ27} and WTA in the presence of BPEI using nPAGE/H⁺ analysis

In Chapter 2 of this thesis, I examined the varying degrees of binding between FmtA^{Δ27} and its variants with WTA, using nPAGE/H⁺ for analysis. Building on this foundation, Here I designed a similar experiment to investigate the effects of BPEI on the binding between FmtA^{Δ27} and WTA under optimized conditions. The optimum binding condition was carefully selected to ensure complete binding between FmtA^{Δ27} and WTA, which results in the protein not appearing on an nPAGE/H⁺ gel.

The experimental parameters involved reaction mixtures containing 10 μM FmtA^{Δ27} and 1.33 mg/ml WTA (67 μM) (optimum binding condition), which were incubated with varying concentrations of BPEI (ranging from 0 to 0.42 mM) for one hour at 37°C. To prioritize binding with WTA, BPEI was introduced into the reaction mixture 30 minutes prior to the addition of FmtA^{Δ27}. Subsequently, acidic native gel (nPAGE/H⁺) electrophoresis was conducted with the samples at 4°C, followed by quantification of the Coomassie-stained bands using NIH ImageJ V1.53 software. The reaction mixture, totaling to 15 μl, was prepared in 50 mM sodium phosphate buffer (pH 7.2).

In addition to the main experiments, two control experiments were also conducted. Control 1 (C₁) involved running the FmtA^{Δ27} protein alone, allowing us to establish the baseline of the

migration pattern of FmtA^{Δ27} in the absence of any binding partners. Control 2 (C₂) was designed to represent a scenario where FmtA^{Δ27} was exposed to half the concentration of WTA required for complete binding (0.3 mg/ml or 17 μM of WTA). These controls were essential for providing reference points against which the experimental results could be compared (Fig. 4.2.2).



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Figure 4.2.2. Illustration representing the experimental parameters adapted to examine the binding between FmtA^{Δ27} and WTA in the presence of BPEI using acidic native gel (n/PAGE/H⁺) electrophoresis. 10 μM FmtA^{Δ27} and 1.3 mg/ml WTA (67 μM) samples were incubated with varying concentrations of BPEI (ranging between 0-0.42 mM) for one hour at 37°C before electrophoresis.

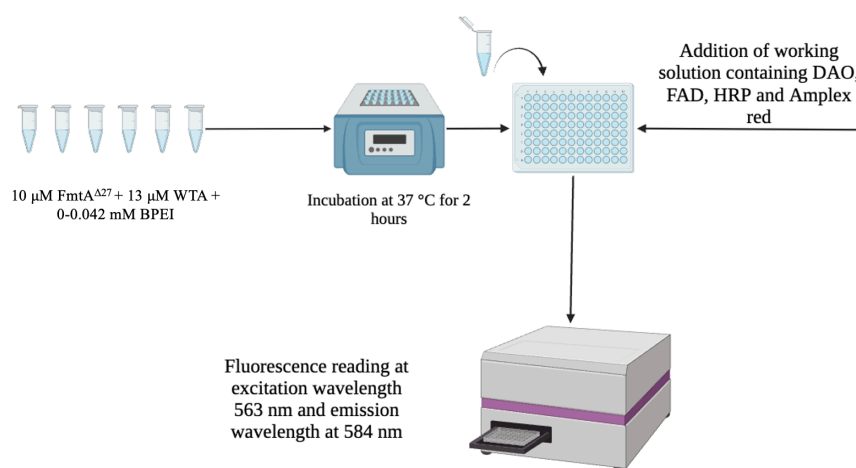
4.2.3 Investigation of the enzymatic activity of FmtA^{Δ27} against WTA in the presence of BPEI using FBEC assay

Drawing upon a similar experiment outlined in Chapter 2 of this thesis, which examined the variance in the enzymatic activity of the FmtA^{Δ27} variants compared to the wild type against WTA, using a fluorescence-based enzyme-coupled assay, this study delves into probing the esterase activity of FmtA^{Δ27} against WTA in the presence of BPEI, employing a similar fluorescence-based approach adopted from Gutheil et al. (2000). BPEI was introduced to the

experiment at an optimum condition (determined from a similar experiment outlined in Chapter 2 of this thesis) of 10 μM protein incubated with 13 μM (0.25 mg/ml) WTA where the maximum D-Ala yield was recorded.

In this assay, each 100 μl reaction mixture comprising of 10 μM protein and 13 μM (0.25 mg/ml) WTA, were incubated with varying concentrations of BPEI (ranging from 0 to 0.42 mM) in 0.1 M Tris (pH 8.5) buffer. BPEI was introduced 30 minutes prior to FmtA $^{\Delta 27}$ to ensure binding with WTA before FmtA $^{\Delta 27}$ competed for binding. The reaction mixtures were then incubated at 37°C for 2 hours.

Subsequently, the reaction mixtures were incubated in a 96-well plate with a detection solution containing DAO (0.05 units), FAD (2.5 $\mu\text{g}/\text{ml}$) HRP (0.75 units), and Amplex Red (20 μM) (Sigma-Aldrich Canada) at room temperature in the dark for one hour in a 1:1 ratio, the final volume being 40 μl . The resulting fluorescence was then measured using the Synergy H4 Hybrid Reader at an excitation wavelength of 563 nm and an emission wavelength of 584 nm (Fig. 4.2.3).



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Figure 4.2.3. Illustration representing the experimental parameters adapted for the fluorescence-based enzyme coupled assay (FBEC) experiment performed with 10 μM FmtA $^{\Delta 27}$ and 13 μM

(0.25 mg/ml) WTA incubated with different concentrations of (0-0.42 mM) BPEI for 2 hours at 37 °C before coupling with the working solution composed of DAO, FAD, HRP and Amplex red.

4.2.4 Investigating the impact of BPEI on biofilm formation in the RN4220 strain

In the 3rd chapter of this thesis, I investigated the role of endogenous FmtA in the RN4220 strain, focusing on the impact of *fmtA* gene deletion and its complementation with wild-type or mutant genes on biofilm formation. My findings demonstrated a reduced biofilm formation when the *fmtA* gene was deleted and in the case of certain mutations. These results highlighted the importance of the *fmtA* gene in regulating biofilm formation by the RN4220 strain.

As BPEI is expected to bind to WTA, the substrate for FmtA, this experiment aimed to assess how the gradual introduction of BPEI during the initiation of the biofilm formation process by the RN4220 strain would influence biofilm development. By interfering in the WTA-FmtA interaction, this study sought to evaluate the potential impact of BPEI on the biofilm formation process. The RN4220 strain cells were cultured in TSB media (without any supplements) until reaching an O.D.₆₀₀ of 1 from an overnight culture. The bacterial culture was diluted 1:50 (to a final volume of 25 ml) in TSB media supplemented with 0.2% glucose, where the final O.D.₆₀₀ = 0.02. Aliquots of 200 µl were dispensed into sterile, flat-bottom tissue culture plates (Corning Incorporated). BPEI was subsequently introduced in a gradient of increasing concentrations (0–0.42 mM). Finally, the plates were incubated at 37°C for 24 hours.

After incubation, the solutions from the wells were carefully aspirated using a pipette. The wells were then washed four times with sterile 1x Phosphate Buffered Saline (PBS), which contains 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄, (Purchased from the University science store) by gently aspirating the washing solution with a pipette. The adherent

biofilms in the wells were fixed with Bouin's fixative (Purchased from Sigma) for 4 hours and stained with 0.1% safranin for 10 minutes. Afterward, the wells were rinsed in running tap water, and photographs were taken using a phone camera. The remaining biofilms were dissolved in 30% glacial acetic acid for 15 minutes, and the relative biofilm formation was quantified by measuring absorbance at 600 nm wavelength using a Hybrid H₄ plate reader ensuring the recorded O.D.₆₀₀ ranges between a reliable reading range (0-2 O.D.₆₀₀) (Fig. 4.3.4.). Each assay was conducted in triplicate.

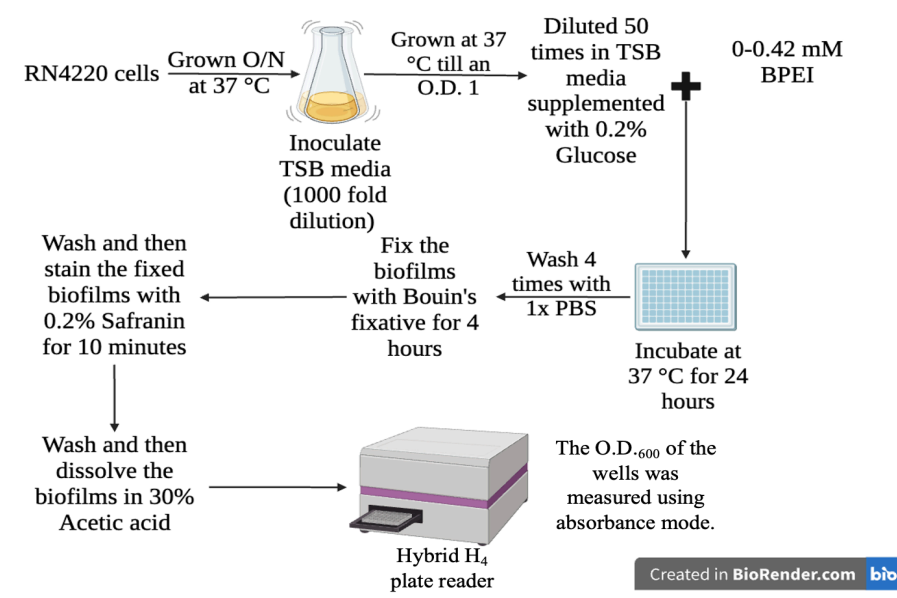


Figure 4.3.4. Illustration depicting the experimental parameters of the biofilm formation assay conducted with RN4220 cells of O.D.₆₀₀ = 0.02 supplemented with 0.2% glucose, incubated with 0-0.42 mM BPEI for 24 hours at 37 °C in a 95-well flat bottom plate for biofilms to form.

4.2.5 Investigating the impact of BPEI on biofilm formation in the RN4220 strain in the presence of exogenous FmtA^{Δ27}

In Chapter 3 of the thesis, I demonstrated that the presence of exogenous FmtA^{Δ27} led to an increase in biofilm formation by the *S. aureus* RN4220 strain up to a concentration of [FmtA^{Δ27}] ≤ 0.2 μM, corroborating the findings of a similar study conducted by Qamar & Golemi-Kotra,

(2012). Here, the underlying hypothesis is that BPEI would bind to WTA, thereby preventing FmtA^{Δ27} from accessing WTA. This phenomenon is hypothesized to result in a measurable reduction in the enhancement of biofilm formation, which typically occurs when FmtA^{Δ27} is applied externally. In this study, my objective was to assess the impact of BPEI at varying concentrations on biofilm formation in the presence of FmtA^{Δ27}. By monitoring any reduction in biofilm development, I aimed to evaluate BPEI's effectiveness in inhibiting FmtA^{Δ27} activity against WTA.

For this assay, RN4220 cells were cultured in TSB media (without any supplements) until reaching an O.D.₆₀₀ of 1 from an overnight culture. The bacterial culture was diluted 1:50, (to a final volume of 25 ml), in TSB media supplemented with 0.2% glucose where the final O.D.₆₀₀ = 0.02. Aliquots of 200 μl were dispensed into sterile, flat-bottom tissue culture plates (Corning Incorporated). Before incubation, varying concentrations of BPEI (ranging from 0 to 0.42 mM) were introduced along with 0.2 μM FmtA^{Δ27}. BPEI was added 15 minutes before FmtA^{Δ27} to facilitate its binding to WTA. Additionally, two controls were incorporated into the experiment: C₁ and C₂. C₁ was the negative control, consisting of 200 μl aliquots of bacterial culture (O.D.₆₀₀ = 0.02) without any treatment. C₂ was a control, comprising 200 μl aliquots of bacterial culture (O.D.₆₀₀ = 0.02) supplemented with 0.42 mM BPEI.

After incubation, the solutions from the wells were carefully aspirated using a pipette. The wells were then washed four times with sterile 1x PBS, (Purchased from the University science store) by gently aspirating the washing solution with a pipette. The adherent biofilms in the wells were fixed with Bouin's fixative (Purchased from Sigma) for 4 hours and stained with 0.1% safranin for 10 minutes. Afterward, the wells were rinsed in running tap water, and photographs were taken using a phone camera. The remaining biofilms were dissolved in 30% glacial acetic

acid for 15 minutes, and the relative biofilm formation was quantified by measuring absorbance at 600 nm wavelength using a Hybrid H₄ plate reader ensuring the recorded O.D.₆₀₀ ranges between a reliable reading range (0-2 O.D.₆₀₀) (Fig. 4.2.5). Each assay was conducted in triplicate.

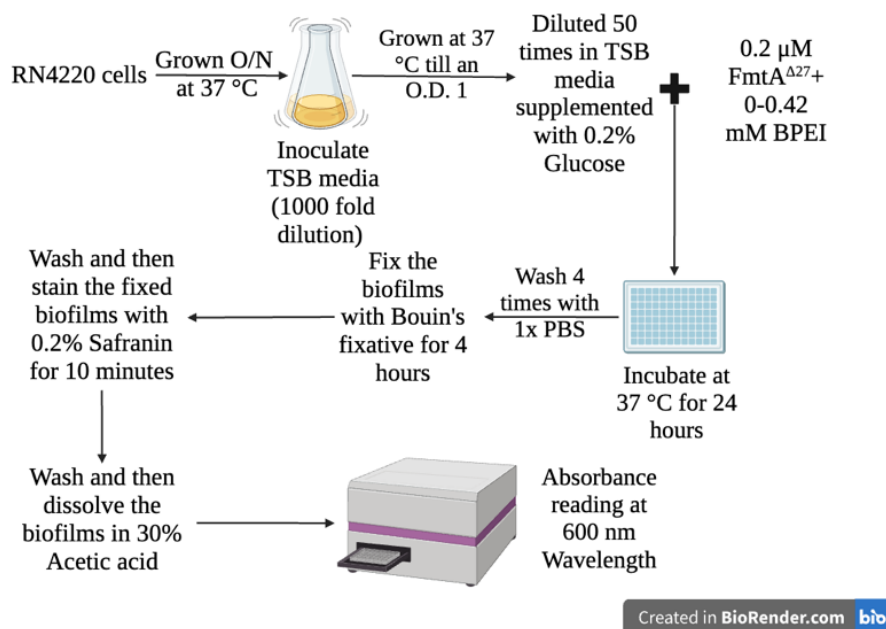
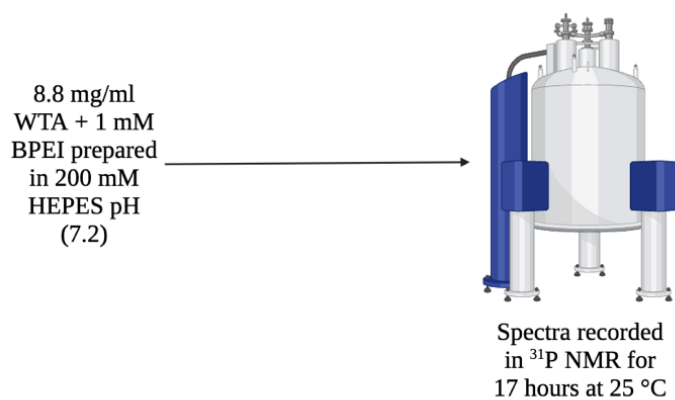


Figure 4.2.5. Illustration representing the experimental parameters followed during the biofilm formation assay performed with RN4220 cells of O.D.₆₀₀ = 0.02 supplemented with 0.2% glucose, incubated with 0.2 μM exogenous FmtA^{Δ27} and 0-0.42 mM BPEI for 24-hours at 37 °C in a 95-well flat bottom plate for biofilms to form.

4.2.6 Analysis of BPEI-WTA binding by ³¹P-NMR

Numerous studies have investigated the electrostatic interactions between BPEI and WTA (Foxley et al., 2016, 2017; Hill et al., 2019) where Foxley et al. (2016) observed alterations in WTA when mixed with low-MW BPEI using ³¹P NMR spectroscopy. The binding mechanism is believed to involve electrostatic attraction between the multiple cationic primary amines of BPEI and the anionic phosphate groups of WTA (Foxley et al., 2016). This interaction is thought to alter the properties of WTA by modifying its molecular structure, creating steric hindrance due to the branched nature of BPEI.

To investigate the impact of BPEI on the enzymatic activity of FmtA^{Δ27} against WTA, I initially sought to identify the concentration of BPEI that would effectively bind to WTA and inhibit the enzymatic function of FmtA^{Δ27}. To build on existing data, a BPEI concentration of 1 mM was added to an 8.8 mg/mL (457.6 μM) WTA solution prepared in 200 mM HEPES buffer (pH 7.2). HEPES was chosen over 50 mM sodium phosphate to avoid the introduction of additional phosphate from the buffer. The ³¹P-NMR spectra were recorded for 17 hours, where the ³¹P (¹H decoupled) NMR spectra were collected at 242.8 MHz on a Bruker DRX 600 NMR spectrometer equipped with a 5 mm, ¹H/broadband probe. Each data set was acquired with 65,536 data points, a spectral width of 100 ppm, with a 0.6-second relaxation delay and approximately 10,000 scans. The data was processed using a 10 Hz line broadening factor and was analyzed with Topspin 4.3.0 software (Bruker Ltd.) (Fig. 4.2.6).



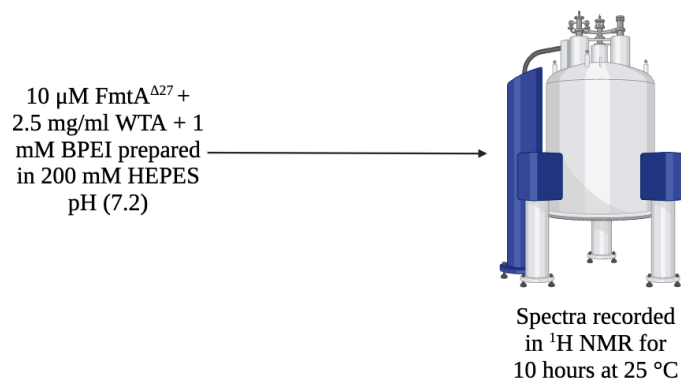
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Figure 4.2.6. Illustration showcasing the experimental parameters involved in the analysis of BPEI-WTA binding by ³¹P-NMR

4.2.7 Probing the enzyme kinetics between FmtA^{Δ27} and WTA in the presence of BPEI

Rahman et al. (2016) established a method for quantifying the degree of D-Ala removal by FmtA^{Δ27}, utilizing pseudo-first-order rate kinetics measured through ¹H-NMR. Building on this foundation, I investigated the impact of BPEI on the enzyme kinetics of the FmtA^{Δ27}-WTA interaction, similar to the research conducted by Qamar & Golemi-Kotra, (2012), which explored the enzymatic activity of FmtA^{Δ27} on WTA.

10 μM of FmtA^{Δ27} protein was prepared with 2.5 mg/ml WTA (130 μM) and 1 mM BPEI in 200 mM HEPES (pH 7.2) buffer (final volume being 300 μl) in a 3 mm NMR tube and the relative amount of D-Ala released was monitored by 700 MHz ¹H-NMR over 10 hours at 25 °C. 3 control experiments were performed alongside, one with 2.5 mg/ml WTA alone, then 2.5 mg/ml WTA in 1 mM BPEI and finally 2.5 mg/ml WTA with 10 μM of FmtA^{Δ27} protein (positive control). The ¹H NMR spectra were collected on a Bruker AV III 700 NMR spectrometer equipped with a 5 mm, liquid nitrogen-cooled H/C/N Prodigy probe, where the 1D spectra were acquired using the standard “zgesgp” excitation sculpting pulse program, which used 32,768 data points, a spectral width of 16 ppm, with a 7-second relaxation delay and 64 scans. The kinetic data was acquired using a Bruker automation program, which allows the acquisition of sequential data sets on a sample using identical parameters. The resulting data were then analyzed with Topspin 4.3.0 and Dynamic Center 2.8.4 software (Bruker Ltd.) (Fig. 4.2.7). The function used to calculate the rate constant of the appearance of free D-Ala was $f(t) = I_0 * e^{(-kt)}$ where I_0 represents the initial value, k is the rate constant, and t is the time.



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Figure 4.2.7. Illustration showcasing the experimental parameters involved in probing the enzyme kinetics between FmtA $^{\Delta 27}$ and WTA in the presence of BPEI by ^1H -NMR

4.3 Results

4.3.1 Analysis of the effects of BPEI on the binding interactions between WTA and FmtA $^{\Delta 27}$

Here, my findings demonstrated a concentration-dependent influence of BPEI on the FmtA $^{\Delta 27}$ -WTA binding when analyzed by an nPAGE/H+ gel. As the concentration of BPEI increased, a corresponding rise in the amount of free FmtA $^{\Delta 27}$ was observed in the nPAGE/H+ gel. This indicates that higher BPEI levels disrupt the FmtA $^{\Delta 27}$ -WTA binding, as evidenced by the inverse relationship between the protein's gel appearance and its binding to WTA. This trend reached saturation at $[\text{BPEI}] \leq 0.23$ mM, at which approximately 57% of the free FmtA $^{\Delta 27}$ protein was detectable in the gel (Fig. 4.3.1).

The data were fitted to the equation $Y = B_{\text{max}} * X / (K_d + X)$ to estimate the dissociation constant (K_d). Y in this equation denotes the free FmtA, while X represents the BPEI concentration in mM. The parameter B_{max} corresponds to the maximum specific binding capacity in this equation.

The curve was fitted using GraphPad Prism 10 software, yielding a K_d of 0.043 mM from the equation.

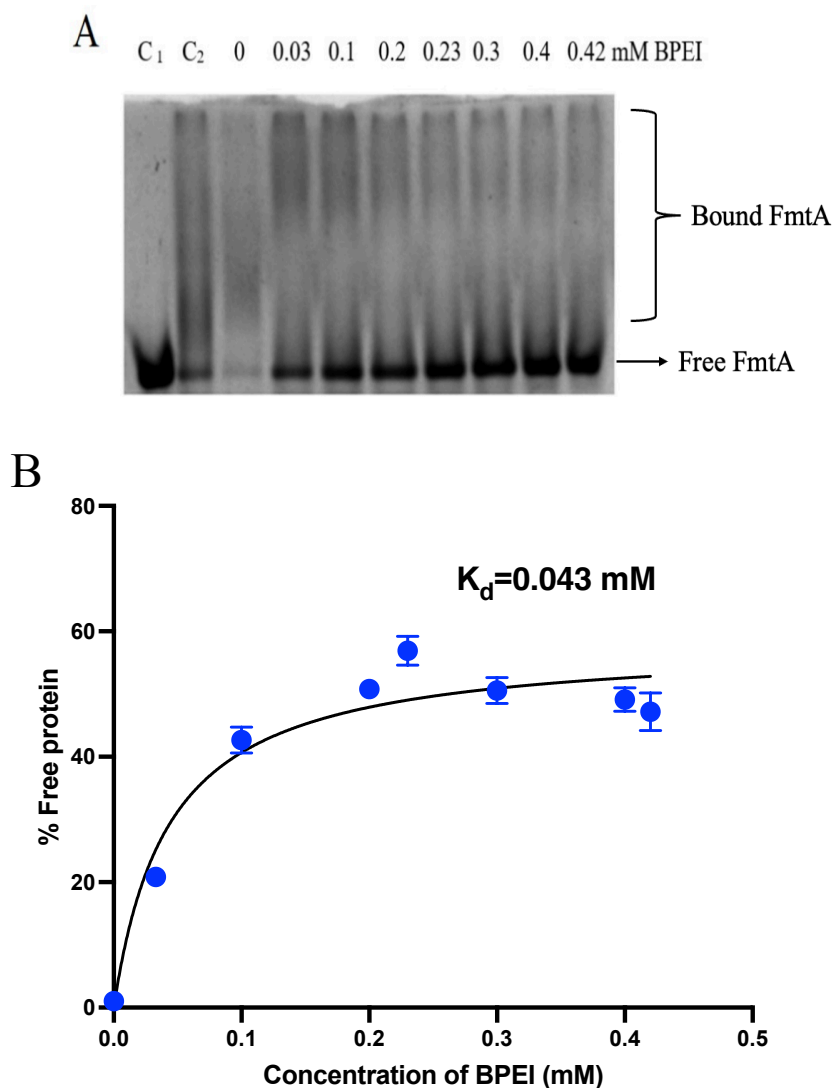


Figure 4.3.1: Analysis of interaction between FmtA^{Δ27} and WTA in the presence of BPEI by acidic native polyacrylamide gel electrophoresis (nPAGE/H⁺): **A.** 10% nPAGE/H⁺ gel run of the reaction mixtures consisting of 10 μM FmtA^{Δ27} alone (C₁), 10 μM FmtA^{Δ27} with 0.33 mg/ml (17 μM) WTA (C₂) and 10 μM FmtA^{Δ27} and 1.33 mg/ml WTA (67 μM) with 0-0.42 mM BPEI. The reactions were carried out in 50 mM Sodium phosphate buffer pH 7.2. 15 μl reaction mixtures were incubated at 37 °C for 1 hour before electrophoresis. **B.** Graphical representation of the % free protein in the gel. Error bars represent the standard deviation calculated from three independent trials. The dissociation constant (K_d) was determined by fitting the data to a one-site specific binding model using Prism 10 software.

4.3.2 Analysis of the effects of BPEI on the enzymatic activity of FmtA^{Δ27} against WTA

The enzyme-coupled assay revealed a concentration-dependent inhibition by BPEI of the enzymatic removal of D-Ala from WTA by FmtA^{Δ27}. The inhibitory effect increased with higher concentrations of BPEI, although a complete inhibition of FmtA^{Δ27} activity was not observed in this assay. The optimal concentration of BPEI for maximum inhibition was identified to be at 0.3 mM, resulting in an approximately 85% reduction in D-Ala release compared to a control experiment without BPEI. Beyond this concentration, the inhibitory effect of BPEI plateaued, indicating a saturation point in the competition between BPEI and FmtA^{Δ27} to access WTA (Table 4.3.2 and Fig. 4.3.2).

The data were fitted to the equation $Y = \text{Min} + (\text{Max} - \text{Min}) / (1 + 10^{((\text{LogIC}_{50} - X) * (-1))})$ to estimate the half-maximal inhibitory concentration (IC₅₀). Y in this equation denotes the D-Ala released, while X represents the BPEI concentration in mM. The parameters Max and Min values represent the two extremities of the curve. The curve was fitted using GraphPad Prism 10 software, yielding an IC₅₀ value of 0.103 mM from the equation.

Additionally, the impact of pH changes on D-Ala hydrolysis due to BPEI addition was investigated. At the highest BPEI concentration used (0.42 mM), the pH of the solution increased from 7.2 to 7.22 units, which is negligible and does not significantly influence the hydrolysis process. Therefore, the observed reduction in D-Ala release is attributed solely to the enzymatic activity of FmtA^{Δ27}, independent of pH alterations.

Table 4.3.2. Table showing the release of D-Ala (pM) with increasing BPEI concentrations (mM)

Concentration of BPEI (mM)	D-Ala released (pM)
0	123.1±0.9
0.041	94.01±4.4
0.08	74.02±0.5
0.2	56.3±1.4
0.25	31.5±0.4
0.3	18.6±1.5
0.33	19.1±0.4
0.4	23.9±2.4
0.42	19.3±0.6

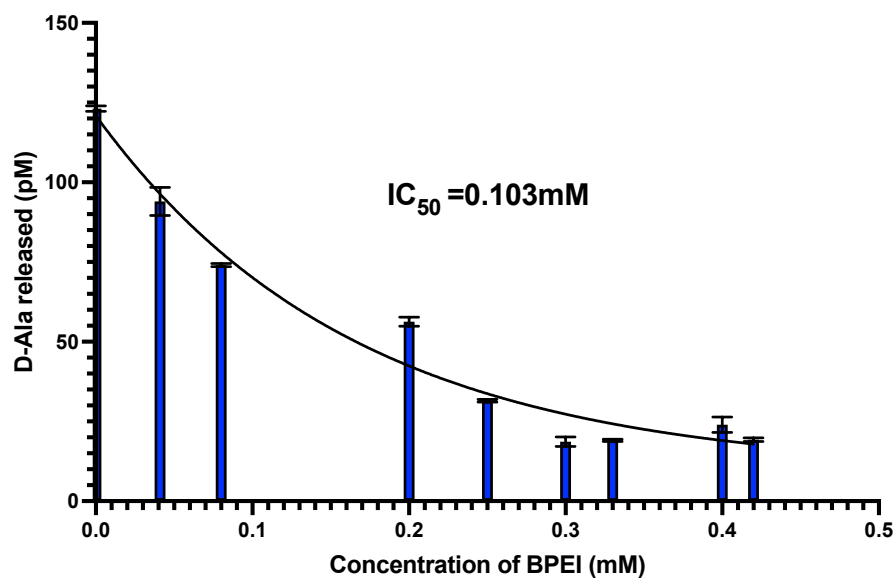


Figure 4.3.2. Effects of BPEI in the enzymatic activity of FmtA^{Δ27} against WTA assessed by Fluorescence-based enzyme coupled assay: 10 μM FmtA^{Δ27} was incubated with 13 μM (0.25 mg/ml) WTA and 0-0.42 mM BPEI prepared in 50 mM sodium phosphate buffer (pH 7.2) for 2 hours at 37 °C. Samples were prepared in triplets in a flat bottom 96 well plate and the fluorescence was developed by incubating the samples with the enzyme cocktail consisting of DAO, FAD, HRP, and Amplex red. Samples were incubated for another hour at room

temperature in the dark and finally, fluorescence was measured in a Synergy H4 Hybrid plate reader (emission wavelength=563 nm; extinction wavelength=584 nm). Control 1 represents 10 μ M FmtA $^{\Delta 27}$ protein without BPEI and WTA and Control 2 represents 10 μ M FmtA $^{\Delta 27}$ protein with 0.42 mM BPEI without WTA where their average values were subtracted from the experimental values to adjust for background. Error bars represent the standard deviation calculated from three independent trials performed in triplicate. The half-maximal inhibitory concentration (IC₅₀) was determined by fitting the data to a four-parameter logistic equation model using Prism 10 software.

4.3.3 Analyzing the effects of BPEI on biofilm formation in the RN4220 strain

This study aimed to evaluate the impact of BPEI on the biofilm formation by the RN4220 strain, for which the cells were incubated with a gradient of BPEI (0-0.42 mM). The results demonstrate a noticeable alteration in biofilm formation by RN4220 in response to BPEI exposure. Increasing concentrations of BPEI led to a reduction in biofilm formation, with a 43% decline observed as BPEI concentration increased up to the 0.33-0.4 mM range. Beyond this concentration, an increase in biofilm formation of approximately 32% was observed, as the biofilm levels did not revert to the control conditions (Fig. 4.3.3).

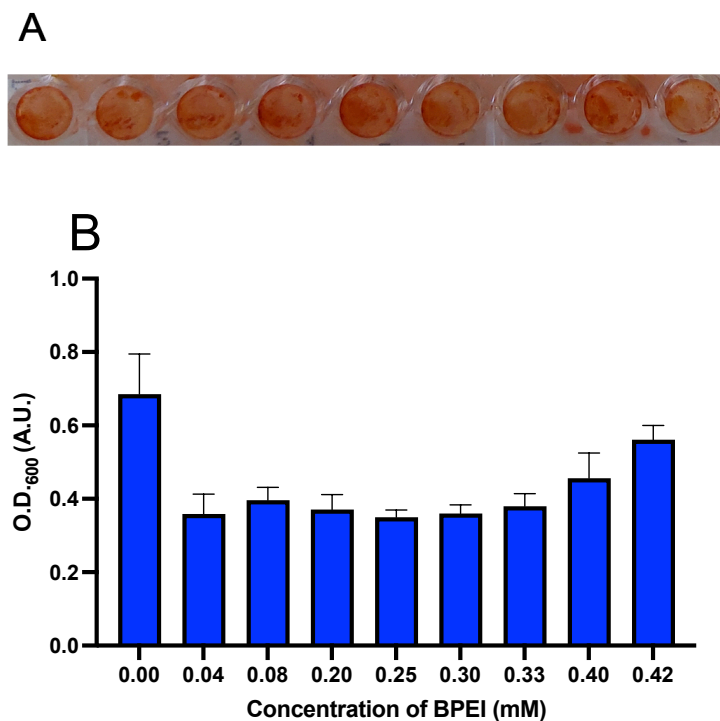


Figure 4.3.3. Biofilm formation in RN4220 by FmtA $^{\Delta 27}$ in the presence of BPEI: **A.** Biofilm formation in the presence of a gradient of BPEI. **B.** Graphical representation of the differences in

biofilm formation in the presence of a BPEI gradient. RN4220 cells of $O.D._{600} = 0.02$ were incubated with 0-0.42 mM BPEI and were allowed to form biofilms, which were subsequently quantified at $O.D._{600}$ in a Synergy H4 Hybrid plate reader.

4.3.4 Analysis of the impact of BPEI on biofilm formation in the RN4220 strain in the presence of exogenous FmtA^{Δ27}

This study aimed to evaluate the impact of BPEI on biofilm formation in RN4220 cells in the presence of exogenous FmtA^{Δ27}. Upon introducing BPEI, there was an initial reduction in biofilm formation up to a concentration of 0.2 mM, where the decrease was approximately 23%. Beyond 0.2 mM BPEI, a slight increase in biofilm formation was observed, with the maximum increase (~13%) at 0.3 mM BPEI. However, this increase did not fully restore biofilm formation to the levels observed under optimal conditions without BPEI. Instead, it indicated a saturation point (Fig 4.3.4).

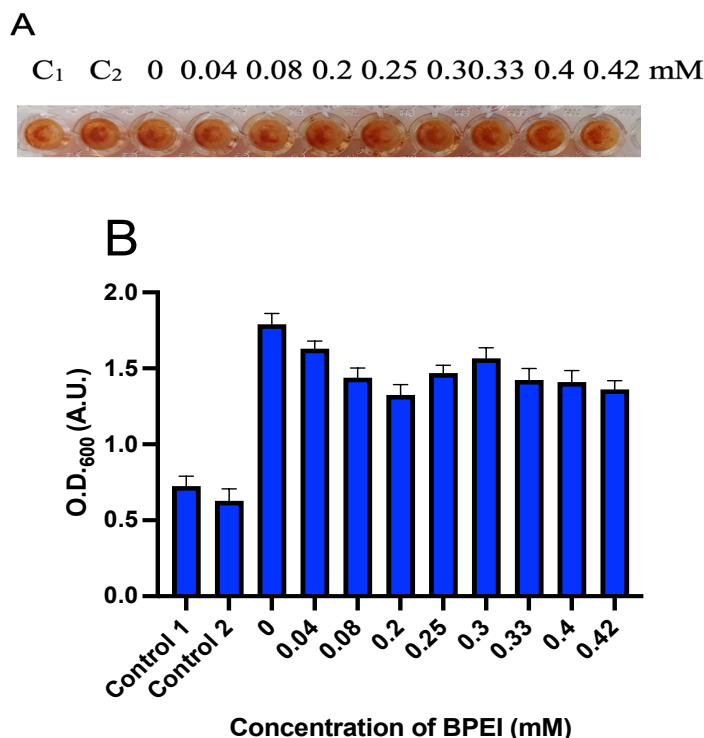


Figure 4.3.4 : Stimulation of biofilm formation in RN4220 by FmtA^{Δ27} in the presence of BPEI: **A.** Biofilm formation in the presence of a gradient of BPEI at an optimum concentration of exogenous FmtA^{Δ27}. **B.** Graphical representation of the differences in biofilm formation in the

presence of a BPEI gradient at optimum FmtA^{Δ27} concentration. RN4220 of O.D.₆₀₀ = 0.02 were incubated with 0.2 μM FmtA^{Δ27} and a 0-0.42 mM BPEI gradient and were allowed to form biofilms which were subsequently quantified at O.D.₆₀₀ in a Synergy H4 Hybrid plate reader. Control 1 represents RN4220 without BPEI and FmtA^{Δ27} and Control 2 represents RN4220 with 0.42 mM BPEI where their average values were corrected for background. Error bars represent the standard deviation calculated from three independent trials.

4.3.5 Characterization of the BPEI -WTA binding by ³¹P-NMR

The primary goal of using ³¹P NMR was to determine the optimal concentration of BPEI required to bind WTA for subsequent kinetic experiments. This experiment is based on the study by Foxley et al. (2016), where they observed alterations in peak height and other spectral changes in the WTA signal when BPEI binds to WTA detected through ³¹P NMR.

The control NMR spectra displayed a doublet peak at 1 ppm, characteristic of WTA. In contrast, the experimental spectra with 1 and 2 mM BPEI revealed a more pronounced and elevated peak, differing significantly from the control. Notably, two distinct peaks emerged in the experimental spectra at approximately -0.75 ppm and 3.25 ppm, which were absent in the control. Given that BPEI is an amine solution, the addition of higher BPEI concentrations can potentially increase the solution's pH. The study found that introducing 1 or 2 mM BPEI shifted the solution's pH to 7.6 and 9.2, respectively from 7.2 (Fig. 4.3.5.1).

To determine whether the appearance of the two new peaks or the sharpening of the existing ones was a result of this pH shift or indeed a consequence of BPEI binding to WTA, I conducted a parallel experiment. Here, I used a 200 mM HEPES (pH 7.2) buffer instead of the 50 mM HEPES buffer (pH 7.2). The results revealed that, after the preparation of 1 and 2 mM BPEI in 200 mM HEPES buffer (pH 7.2), the pH change was minimal, measuring at 7.28 and 7.37, respectively.

In the control spectra featuring 8.8 mg/ml (457.6 μM) WTA within a 200 mM HEPES (pH 7.2) environment, a peak at (-0.75) ppm mirrors the characteristic of the experimental

spectra in the presence of 1 mM BPEI. Yet, the occurrence of the peak at 3.25 ppm was absent in the control spectra. This observation suggests that the emergence of the 3.25 ppm peak was indeed a consequence of the BPEI binding. Notably, the sharpness and increased intensity of the 1 ppm peak remained consistent across the trials, even when the buffers were altered (Fig. 4.3.5.2).

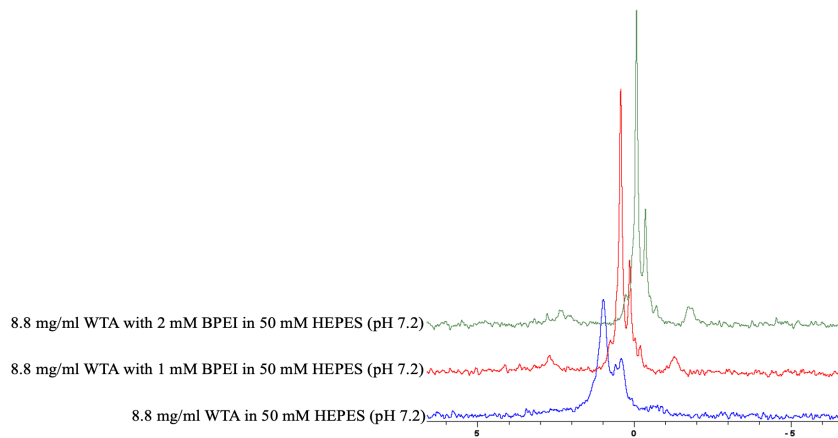


Figure 4.3.5.1 WTA-BPEI binding in 50 mM HEPES (pH 7.2) assessed by ^{31}P -NMR: 8 mg/ml WTA (457.6 μM) isolated from the *S. aureus* RN4220 strain was prepared in (Blue) 50 mM HEPES buffer (pH 7.2) and with either (Red) 1 or (Green) 2 mM BPEI. The spectra were recorded for 17 hours at 25 $^{\circ}\text{C}$.

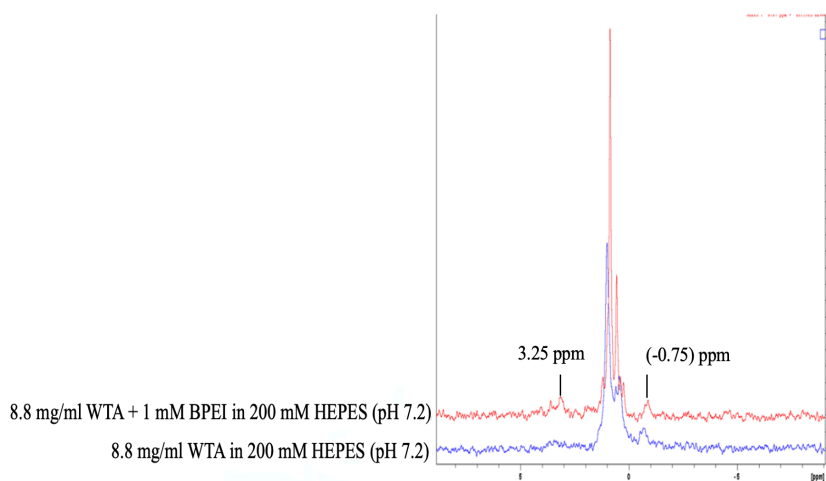


Figure 4.3.5.2 WTA-BPEI binding in 200 mM HEPES (pH 7.2) assessed by ^{31}P -NMR: 8 mg/ml (457.6 μM) WTA isolated from the *S. aureus* RN4220 strain was prepared in (Blue) 50 mM HEPES buffer and with (Red) 1 mM BPEI. The spectra were recorded for 17 hours.

4.3.6 Analyzing the inhibitory effects of BPEI in FmtA^{Δ27}- WTA enzyme kinetics

The investigation here focuses on assessing the impact of BPEI on the enzyme kinetics governing the interaction between WTA and FmtA^{Δ27}. To explore this, in the kinetic experiment between FmtA^{Δ27} and WTA, BPEI was introduced, and the appearance of free D-Ala was recorded over 10 hours, and the rate kinetics was analyzed and compared to the trials without BPEI.

My data show a relatively higher D-Ala in the presence of BPEI compared to control trials without BPEI. Specifically, the control trial exhibited a rate constant of 0.6 h⁻¹, while the trial with BPEI showed an increased rate constant of 0.9 h⁻¹ (Fig. 4.3.6). To investigate this unusual phenomenon, further, the WTA/BPEI ratios were examined in these trials, as they may provide clearer insights into the results. In the kinetic experiment, the WTA/BPEI ratio was determined to be 0.13, whereas in the native gel experiment (Fig 4.3.1), this ratio ranged between 0.2 to 2.1, with a 57% inhibition observed at a ratio of 0.3 before reaching saturation. In the FBEC assay, the WTA/BPEI ratio ranged from 0.03 to 0.31, with an 85% reduction in enzymatic activity noted at a ratio of 0.04.

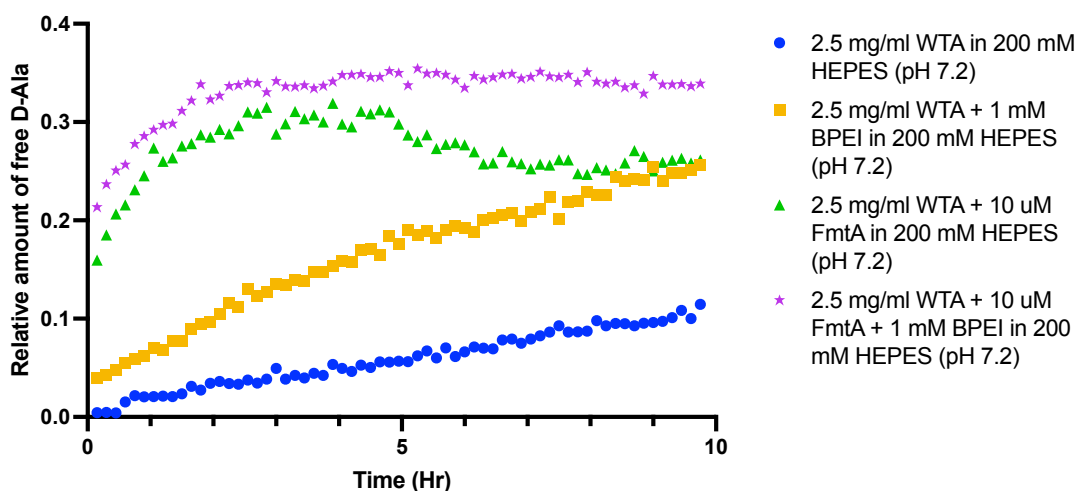


Figure 4.3.6. Enzyme kinetics between FmtA^{Δ27} and WTA in the presence of BPEI: 10 μM FmtA^{Δ27} was incubated with 2.5 mg/ml (130 μM) WTA prepared in 200 mM HEPES (pH 7.2)

with and without 1 mM BPEI. As controls, a WTA sample in 200 mM HEPES (pH 7.2) and a WTA sample in 200 mM HEPES (pH 7.2) with 1 mM BPEI were treated the same. The release of free D-Ala was monitored over time by ^1H -NMR at 25 °C.

4.4 Discussion

The defense mechanism of *S. aureus* against β -lactam antibiotics is fundamentally anchored in its extensively crosslinked peptidoglycan layer, which serves as a formidable barrier against external threats (Schneewind & Missiakas, 2019). WTA being an integral component of this structure, undergoes D-alanylation post-synthesis, a modification critically regulated by the enzyme FmtA $^{\Delta 27}$ (Rahman et al., 2016; van Dalen et al., 2020). The degree of D-alanylation of WTA is vital for *S. aureus* physiology, influencing essential processes such as cell division, virulence, host adhesion, and autolysis (Fabretti et al., 2006).

MRSA strains harbour PBPs such as PBP2a, which impart resistance to a broad spectrum of β -lactam antibiotics (Hill et al., 2019). In community-associated MRSA (CA-MRSA) strains, the low affinity of β -lactams for PBP4 contributes significantly to oxacillin resistance (Memmi et al., 2008). Although ceftiofur, a second-generation cephalosporin, targets PBP4 and augments the efficacy of β -lactams, the potential for developing resistance remains a critical concern (Memmi et al., 2008). PBPs primarily facilitate peptidoglycan crosslinking and are localized at the division septum, where this process is mediated by WTAs (Hill et al., 2019). Hill et al. (2019) further elucidate that BPEI electrostatically binds to WTA, inducing steric hindrance that disrupts PBP4 positioning. Additionally, Lam et al. (2018) demonstrated that BPEI-mediated inhibition of WTA renders it inaccessible to PBP2a, thereby inactivating PBP2a in *S. epidermidis*.

This investigation aimed to explore the hypothesis that BPEI binding to WTA induces steric hindrance, potentially affecting the binding and kinetic activity of FmtA $^{\Delta 27}$ towards WTA. The study focused on examining the influence of BPEI on the FmtA $^{\Delta 27}$ -WTA interaction. Results

indicate that increasing concentrations of BPEI lead to inhibition of the interaction of FmtA^{Δ27} with WTA, although complete inhibition of FmtA^{Δ27} binding to WTA was not achieved. This finding highlights the specific interaction of FmtA^{Δ27} with WTA.

The findings from the FBEC assay reveal a progressive decrease in the enzymatic activity of FmtA^{Δ27} against WTA with the incremental introduction of BPEI. This observation supports the hypothesis that BPEI interacts with WTA via the negatively charged backbone WTA, which is likely to hinder FmtA^{Δ27} from accessing D-Ala resulting in partial binding of FmtA^{Δ27} to WTA and subsequently leading to a reduction in its enzymatic activity.

Building on our group's prior research demonstrating a concentration-dependent increase in biofilm formation with exogenous FmtA^{Δ27} (Qamar & Golemi-Kotra, 2012), this study investigated the impact of BPEI on this process by gradually introducing BPEI. Interestingly, in the presence of both BPEI and exogenous FmtA^{Δ27}, biofilm formation decreased up to 0.2 mM BPEI, followed by an increase and subsequent plateau. Notably, this effect did not reach the level of biofilm formation observed without BPEI. Further analysis of the impact of BPEI alone on biofilm formation in RN4220 cells revealed that BPEI indeed influences biofilm formation. The observed increase in biofilm formation at concentrations exceeding 0.3 mM BPEI is likely due to the dissolution of biofilms, a phenomenon noted at high BPEI concentrations.

Research conducted by Foxley et al. (2016) utilized ³¹P NMR spectroscopy to elucidate the interaction between BPEI and WTA. The study revealed that BPEI binds to WTA through electrostatic interactions, specifically between the positively charged amine groups of BPEI and the negatively charged phosphate groups of WTA. Additionally, Foxley et al. (2016) demonstrated that BPEI induces alterations in the chemical milieu of teichoic acid, suggesting that BPEI's

binding to WTA modifies the latter's properties by influencing its molecular structure and introducing steric hindrance due to the branched nature of the polymer.

In my research, the binding interaction between WTA and BPEI was reaffirmed using ^{31}P NMR spectroscopy in a 200 mM HEPES buffer (pH 7.2), aligning with the observations reported by Foxley et al. (2016). The selection of 200 mM HEPES buffer over alternatives such as 50 mM sodium phosphate (pH 7.2) or water was crucial to mitigate pH changes induced by BPEI. Additionally, the emergence of a new peak at 3.25 ppm, along with an increase in peak intensity, further supported the binding interaction. Contrary to the initial hypothesis, where we suspect BPEI binding to WTA, would thereby block FmtA $^{\Delta 27}$'s access to WTA and subsequently reduce the protein's enzymatic activity against WTA, but the results indicated an unexpected outcome. Rather than inhibiting the enzymatic process, BPEI appeared to facilitate it, leading to an enhanced formation of free D-Ala.

In summary, this study elucidates the influence of BPEI on the interaction between FmtA $^{\Delta 27}$ and WTA. Although BPEI binds to WTA, its binding affinity is insufficient to fully displace FmtA $^{\Delta 27}$ from WTA. This observation implies that the enzymatic affinity of FmtA $^{\Delta 27}$ for WTA is greater than the electrostatic interactions between BPEI and WTA. Consequently, BPEI alone does not effectively inhibit FmtA $^{\Delta 27}$ activity, suggesting that additional factors are needed to disrupt the FmtA $^{\Delta 27}$ -WTA interaction comprehensively.

Nonetheless, BPEI demonstrates promise as a therapeutic agent by impeding WTA and thereby hindering the accessibility of crucial proteins such as PBP2a, PBP4, and FmtA $^{\Delta 27}$. This potential has been corroborated by studies from Foxley et al. (2016); Hill et al. (2019) and Lam et al. (2018). The inhibition of WTA likely interferes with the biosynthesis of highly cross-linked peptidoglycan, which is essential for bacterial resilience against β -lactam antibiotics. By disrupting

bacterial cell wall synthesis and altering the chemical environment, BPEI enhances the susceptibility of *S. aureus* to treatments, underscoring its potential as an effective tool in addressing antibiotic-resistant bacterial infections.

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Chapter Five

Summary and Future Directions

Research on the role of FmtA in *Staphylococcus aureus* is crucial in understanding the bacterium's role in antibiotic resistance. Earlier discoveries by Fan et al. (2007); Qamar & Golemi-Kotra, (2012) and Rahman et al., (2016) explain its biological function and suggest that FmtA plays a role in cell wall modification, biofilm formation, cell division and the regulation of autolysis. This ongoing research is vital for developing strategies to combat the growing threat of antibiotic resistance in *S. aureus*, which is a major cause of infections in both hospital and community settings. The pathogen is increasingly difficult to treat due to its ability to develop resistance to most antibiotics either through intrinsic mechanisms or by acquiring resistance traits. Notably, methicillin-resistant *Staphylococcus aureus* (MRSA) strains have developed resistance to all available β -lactams, prompting the use of alternative drugs like vancomycin. This shift has, in turn, led to the emergence of vancomycin-resistant *Staphylococcus aureus* (VRSA) strains. This escalating resistance has driven significant research efforts to uncover new targets for antibiotic development, aiming to combat infections caused by these resistant strains.

The resistance mechanisms in *S. aureus* are complex and multifaceted, involving various factors that contribute to antibiotic resistance. Among these factors, the gene *fntA* was initially identified through a Tn551 insertion mutant analysis of MRSA, which revealed a reduced level of methicillin resistance and rapid cell lysis in the presence of Triton X-100, a non-ionic surfactant (Komatsuzawa et al., 1997). The biological function of FmtA and its specific role in antibiotic resistance in *S. aureus* remained unclear for some time. However, investigations have now provided insights into the D-Amino esterase activity of FmtA and its potential role in antibiotic resistance. Data indicate that FmtA exhibits a low binding affinity for β -lactams, distinguishing it

from the other penicillin-binding proteins (PBPs) of *S. aureus*. Traditionally, *S. aureus*'s methicillin resistance has been attributed to the acquisition of PBP2a, a protein with low β -lactam binding affinity.

In hospital settings, *S. aureus* has established itself as the most common cause of infections among patients, underscoring its virulence and adaptability. The bacterium employs two primary strategies to combat β -lactam antibiotics: the expression of β -lactamase, an enzyme that deactivates β -lactams, and the production of PBP2a, which has a reduced affinity for β -lactams. However, the levels of β -lactamase and PBP2a expression do not always correlate with the degree of methicillin resistance observed in MRSA strains, suggesting the involvement of additional factors in this resistance mechanism (Lade & Kim, 2023). One of these factors is identified to be FmtA, which is part of the cell wall stimulon, a group of genes commonly induced in response to treatment with cell wall-active antibiotics (Qamar & Golemi-Kotra, 2012). Inactivation of the *fmtA* gene has been shown to result in decreased methicillin resistance, highlighting its potential role in the resistance mechanism (Komatsuzawa et al., 1999).

FmtA's enzymatic activity and interaction with WTA and LTA provide important insights into its role in *S. aureus*. Traditionally, PBPs have been regarded as relatively uniform in function, primarily involved in cell wall synthesis and maintenance, and considered the main targets of β -lactam antibiotics. However, PBPs play a significant role in *S. aureus* antibiotic resistance by enabling the bacterium to maintain peptidoglycan integrity even under antibiotic pressure, effectively mitigating the stress imposed by these drugs. The ability of FmtA to function as an esterase rather than a traditional PBP challenges the conventional understanding of PBPs and their involvement in antibiotic resistance. This esterase activity, particularly against WTA and LTA,

may contribute to the bacterium's ability to modify its cell wall in response to antibiotic stress, thereby enhancing its survival in hostile environments.

Building upon the structural analysis of FmtA conducted by Dalal et al., (2019), my research further elucidates the critical components of the protein's core structure. This study through mutagenesis reveals how FmtA, originally adapted from PBPs, has evolved to accommodate WTA as a substrate. Additionally, it demonstrates the protein's D-amino esterase activity against WTA, highlighting its specialized functional adaptation. *In vitro* studies suggest that the mutations of residues Y282, F346, L342, and D213, along with the concurrent mutations of H173 and Y211 with Y282, result in a substantial reduction in enzymatic activity. The impact of the variants mentioned here seems to be so profound that the protein's activity is nearly indistinguishable from that of the enzymatically inactive mutant.

My study further explored the *in vivo* effects of mutating specific residues within *S. aureus*, revealing significant impacts on several crucial cellular functions. Mutations at residues Y282, F346, L342, and D213, as well as combined mutations involving H173 and Y211 with Y282, resulted in notable variability in biofilm formation and altered resistance profiles against Triton X-100, lysozyme, and lysostaphin. Additionally, these mutations led to defective septum formation during cell division, indicating disrupted cellular division processes. These observations suggest that these residues indeed play a critical role in the active site of FmtA, and their alteration substantially hampers the protein's proper function. The pronounced differences between strains expressing the wild-type gene and those with mutated versions underscore the importance of these residues in maintaining the essential activities mediated by FmtA in *S. aureus*.

Future research directions include exploring the application of external FmtA on *S. aureus* RN4220 cells *in vivo* to assess its impact on antibiotic resistance profiles, particularly against

oxacillin or ampicillin. Expanding this study to other strains, such as Mu50, SA1112, SA113 and the SA113 *fmtA*-deleted version, would provide insights into the strain-specific effects and generalizability of FmtA's role in antibiotic resistance modulation. Furthermore, investigating the phosphorylation of the *fmtA* gene in the presence of the *Stk1* and *Stp1* genes could provide deeper insights into the regulatory mechanisms affecting FmtA activity. Stk1 phosphorylates key substrates involved in cell wall synthesis, supporting MRSA's cell wall integrity under stress. Through its regulation of cell wall dynamics and cellular processes, Stk1 enhances MRSA's resistance to β -lactam antibiotics and influences biofilm formation. Given that FmtA is known to be phosphorylated by Stk1, this investigation could shed light on additional regulatory pathways. These experiments are currently underway in our lab, aiming to deepen our understanding of the regulation of FmtA and its potential as a target in anti-virulence strategies.

In summary, this investigation extends prior structural studies by providing new insights into the intricate architecture of FmtA and its evolution. The research demonstrates how specific amino acid changes critically affect the enzyme's functionality, particularly its interaction with WTA. Experimental evidence points to a significant decline in enzyme performance when certain residues are altered, which suggests their pivotal role in maintaining activity. Further analysis reveals that these modifications have far-reaching consequences on cell behaviour, including growth patterns, resistance mechanisms, and cell division. These findings emphasize the importance of these key amino acids in preserving the integrity and function of FmtA in bacterial cells.

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Appendix A

Table A: Table showing the raw data for the assays performed to determine the D-Ala/Pi ratios. [WTA] indicates the concentration of WTA in the assay (300 μ L). The Pi concentration in each WTA solution, listed under the “[Pi]” column, was calculated using varying amounts of WTA. The D-Ala concentration in each WTA solution, listed under the “[D-Ala]” column, was calculated using 200 μ g and 300 μ g WTA samples.

Variants	Cell pellet isolated	[WTA]	[D-Ala]	[Pi]	D-Ala/Pi	D-Ala/GlucNAc
	(g)	(μ g/ μ l)	(mM)	(mM)		
Wild type	1.2-1.4	1.82	0.28	1.16	0.24	0.35
RN4220 <i>ΔfntA</i>	1.2-1.4	5.28	6.042	39.09	0.15	3.1
RN4220 <i>ΔfntA</i> pMK4:: <i>fntA</i>	1.2-1.4	2.5	0.21	0.74	0.28	0.42
RN4220 <i>ΔfntA</i> pMK4:: <i>fntA</i> L342H	1.2-1.4	2.5	0.47	4.09	0.12	1.94
RN4220 <i>ΔfntA</i> pMK4:: <i>fntA</i> Y282A	1.2-1.4	2.5	0.81	4.89	0.16	2.85
RN4220 <i>ΔfntA</i> pMK4:: <i>fntA</i> F346A	1.2-1.4	2.5	0.23	3.4	0.07	0.43
RN4220 <i>ΔfntA</i> pMK4:: <i>fntA</i> D213AH173A	1.2-1.4	2.5	0.15	1.17	0.13	0.48
RN4220 <i>ΔfntA</i> pMK4:: <i>fntA</i> Y282AF346A	1.2-1.4	2.5	0.19	2.1	0.09	0.5
RN4220 <i>ΔfntA</i> pMK4:: <i>fntA</i> Y211AY282A	1.2-1.4	2.5	0.56	4.67	0.12	0.46
RN4220 <i>ΔfntA</i> pMK4:: <i>fntA</i> N343A	1.2-1.4	2.5	0.57	1.98	0.29	0.6

Appendix B

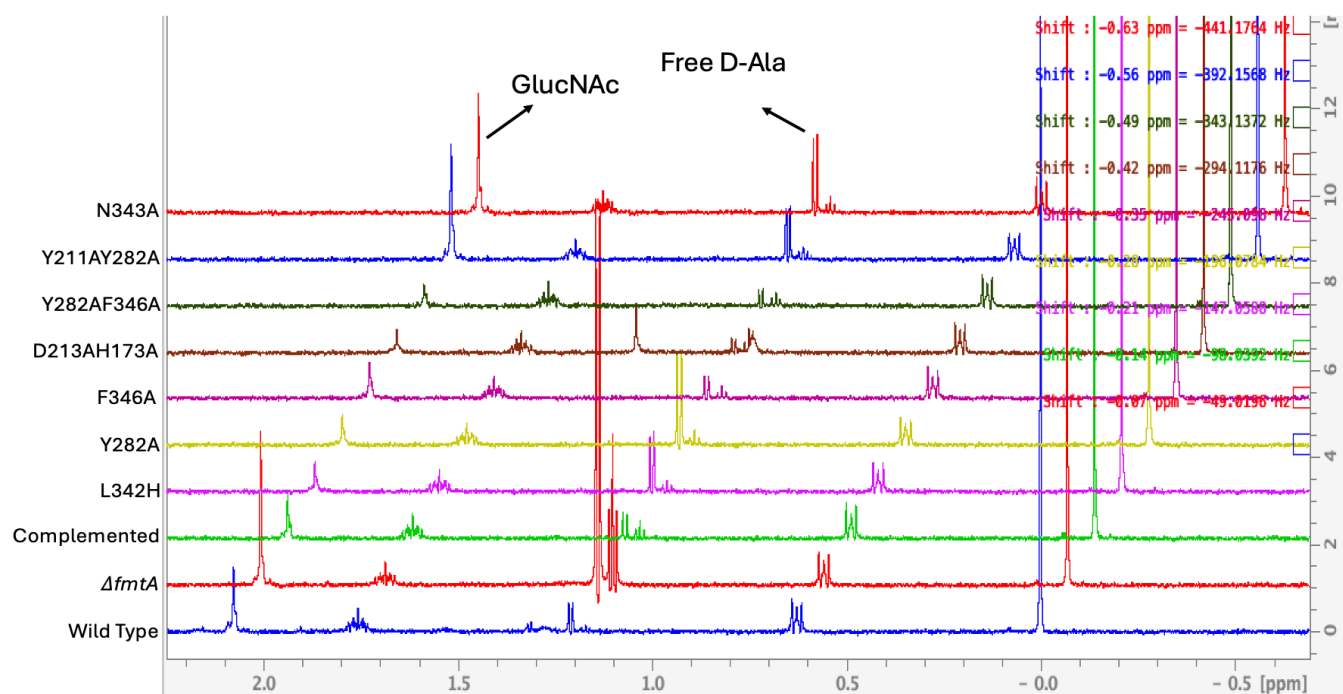


Fig B₁: Multiple displays presenting ¹H-NMR data for the wild-type and variant strains from the hydrolysis assay, showcasing 200 µg WTA samples.

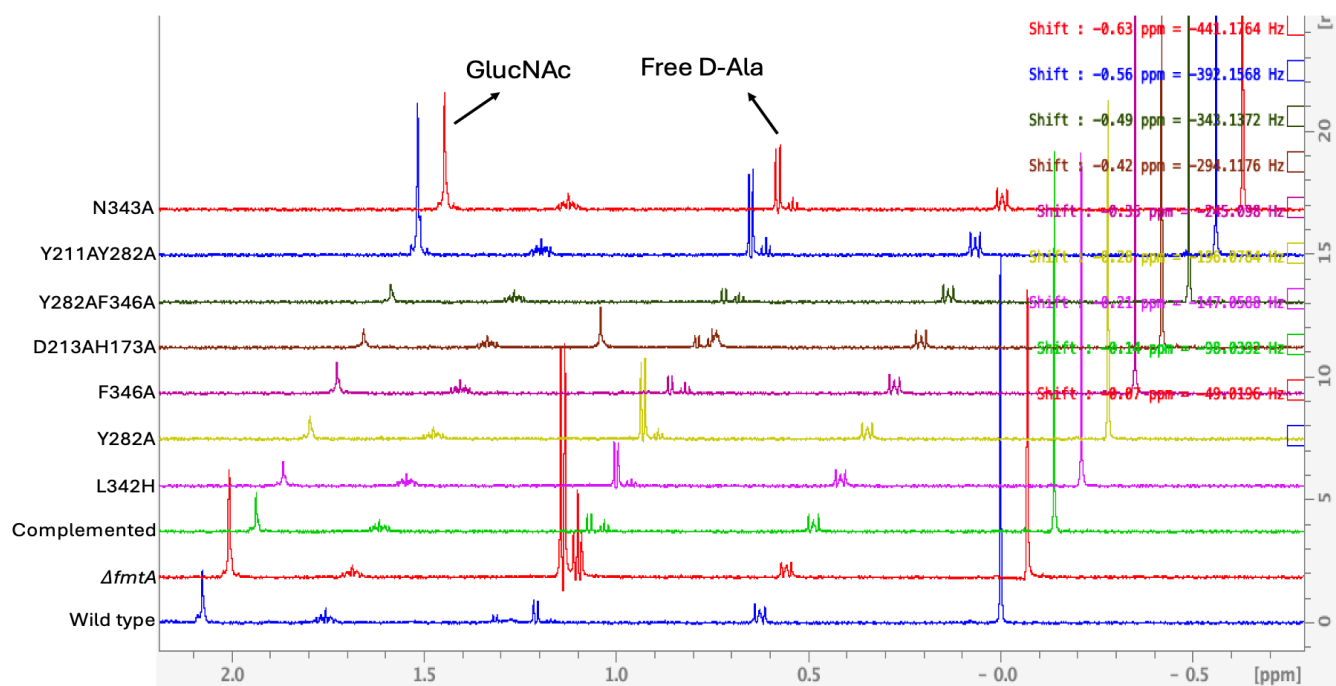


Fig B₂: Multiple displays presenting ¹H-NMR data for the wild-type and variant strains from the hydrolysis assay, showcasing 300 µg WTA samples.

Appendix C

Table C: Table showing the raw data for the lysis assay performed with lysozyme

Sample	0- Hour	1- Hour	2- Hour	3- Hour	4- Hour	5- Hour	6- Hour	7- Hour	8- Hour
Control	100	86.23	82.03	78.38	75.76	74.70	74.10	73.13	71.96
RN4220	100	81.75	79.43	77.06	73.81	72.52	70.70	69.22	68.31
RN4220 Δ <i>fmtA</i>	100	35.25	32.28	30.66	25.70	21.13	19.20	18.83	17.57
RN4220 Δ <i>fmtA</i> pMK4:: <i>fmtA</i>	100	74.17	71.22	69.24	66.08	64.78	62.56	62.30	59.05
RN4220 Δ <i>fmtA</i> pMK4:: <i>fmtA</i> Y211F	100	45.51	38.88	36.70	29.61	24.56	23.07	22.94	20.39
RN4220 Δ <i>fmtA</i> pMK4:: <i>fmtA</i> L342H	100	34.35	28.00	24.07	22.37	21.00	17.77	17.45	16.25
RN4220 Δ <i>fmtA</i> pMK4:: <i>fmtA</i> Y282A	100	34.36	32.00	27.36	20.21	18.63	18.32	18.23	18.09
RN4220 Δ <i>fmtA</i> pMK4:: <i>fmtA</i> F346A	100	63.40	24.14	21.01	19.38	18.14	16.95	16.10	16.38
RN4220 Δ <i>fmtA</i> pMK4:: <i>fmtA</i> D213AH173A	100	30.39	26.62	23.62	21.70	21.22	17.66	17.91	16.87
RN4220 Δ <i>fmtA</i> pMK4:: <i>fmtA</i> Y282AF346A	100	33.28	24.85	21.55	20.20	19.16	15.98	13.66	14.14
RN4220 Δ <i>fmtA</i> pMK4:: <i>fmtA</i> Y211AY282A	100	66.55	47.64	40.22	31.89	27.63	23.33	22.76	21.57
Standard deviation	0- Hour	1- Hour	2- Hour	3- Hour	4- Hour	5- Hour	6- Hour	7- Hour	8- Hour
Control	0.00	1.21	1.29	2.26	3.21	4.31	4.77	4.37	5.87
RN4220	0.00	1.21	0.38	0.84	2.94	4.37	4.99	4.76	6.05
RN4220 Δ <i>fmtA</i>	0.00	4.60	1.25	1.04	2.29	7.29	5.72	5.38	6.31

RN4220 $\Delta fmtA$ pMK4:: <i>fmtA</i>	0.00	0.15	0.02	2.66	3.14	4.17	3.82	3.02	2.10
RN4220 $\Delta fmtA$ pMK4:: <i>fmtA</i> Y211F	0.00	1.04	0.76	3.18	6.75	7.89	7.88	7.72	7.88
RN4220 $\Delta fmtA$ pMK4:: <i>fmtA</i> L342H	0.00	3.03	1.36	1.53	1.50	2.21	1.31	1.85	0.51
RN4220 $\Delta fmtA$ pMK4:: <i>fmtA</i> Y282A	0.00	0.01	2.10	6.33	7.19	6.15	5.85	5.79	5.81
RN4220 $\Delta fmtA$ pMK4:: <i>fmtA</i> F346A	0.00	2.90	0.46	1.12	1.38	1.35	1.61	0.47	1.29
RN4220 $\Delta fmtA$ pMK4:: <i>fmtA</i> D213AH173A	0.00	0.39	4.14	4.70	5.41	5.89	1.35	1.20	2.57
RN4220 $\Delta fmtA$ pMK4:: <i>fmtA</i> Y282AF346A	0.00	2.53	5.25	5.32	4.46	4.66	0.76	1.14	0.03
RN4220 $\Delta fmtA$ pMK4:: <i>fmtA</i> Y211AY282A	0.00	1.21	1.29	2.26	3.21	4.31	4.77	4.37	5.87

Appendix D

Table D: Table showing the raw data for the binding experiment between 10 μM FmtA ^{Δ 27} and 67 μM WTA in the presence of 0-0.42 mM BPEI.

BPEI concentration (mM)	Average value	Standard deviation
Control 1	100.00	0.00
Control 2	11.50	0.59
0	1.04	0.13
0.033	20.83	1.16
0.1	42.68	2.09
0.2	50.80	0.61
0.23	56.91	2.30
0.3	50.56	2.04
0.4	49.13	1.89
0.42	47.18	2.99

Appendix E

Table E: Table showing the raw data for the Fluorescence-based enzyme coupled assay with 10 μM FmtA ^{$\Delta 27$} and 15 μM WTA in the presence of 0-0.42 mM BPEI

BPEI concentration (mM)	Average value	Standard deviation
Control 1	0.00	0.00
Control 2	0.00	0.00
0	123.12	0.87
0.041	94.01	4.39
0.08	74.02	0.52
0.2	56.27	1.43
0.25	31.51	0.44
0.3	18.63	1.49
0.33	19.06	0.38
0.4	23.96	2.39
0.42	19.25	0.61

Appendix F

Table F: Table showing the raw data for the Biofilm formation by RN4220 in the presence of 0-0.42 mM BPEI and 0.2 μ M FmtA ^{Δ 27}

BPEI concentration (mM)	Average value	Standard deviation
Control 1	0.72	0.07
Control 2	0.63	0.08
0	1.79	0.07
0.04	1.63	0.05
0.08	1.44	0.06
0.2	1.33	0.07
0.25	1.47	0.05
0.3	1.57	0.07
0.33	1.42	0.08
0.4	1.41	0.08
0.42	1.36	0.06

Appendix G

Table G: Table showing the raw data for the Biofilm formation by RN4220 in the presence of 0-0.42 mM BPEI

BPEI concentration (mM)	Average value	Standard deviation
0	0.7	0.11
0.04	0.4	0.05
0.08	0.4	0.04
0.2	0.4	0.04
0.25	0.34	0.02
0.3	0.4	0.02
0.33	0.4	0.03
0.4	0.5	0.07
0.42	0.6	0.04

Appendix H

Table H: Table presenting the raw data from the TEM analysis of *fntA* variants.

Variants	Number of dividing cells		Asymmetrical cell division		Multiple division septa formation		Asymmetrical cell division (% frequency)				Multiple division septa formation (% frequency)			
	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2	Average	Standard deviation	Trial 1	Trial 2	Average	Standard deviation
Wild type	98	102	9	5	0	0	9.18	4.90	7.04	3.03	0.00	0.00	0.00	0.00
RN4220 $\Delta fntA$	100	106	6	10	4	6	6.00	9.43	7.72	2.43	4.00	5.66	4.83	1.17
RN4220 $\Delta fntA$ pMK4:: <i>fntA</i>	107	99	4	5	14	9	3.74	5.05	4.39	0.93	13.08	9.09	11.09	2.82
RN4220 $\Delta fntA$ pMK4:: <i>fntA</i> Y211F	101	104	9	10	12	9	8.91	9.62	9.26	0.50	11.88	8.65	10.27	2.28
RN4220 $\Delta fntA$ pMK4:: <i>fntA</i> L342H	102	98	7	5	17	10	6.86	5.10	5.98	1.25	16.67	10.20	13.44	4.57
RN4220 $\Delta fntA$ pMK4:: <i>fntA</i> Y282A	101	104	5	5	9	11	4.95	4.81	4.88	0.10	8.91	10.58	9.74	1.18
RN4220 $\Delta fntA$ pMK4:: <i>fntA</i> F346A	99	100	6	5	8	9	6.06	5.00	5.53	0.75	8.08	9.00	8.54	0.65
RN4220 $\Delta fntA$ pMK4:: <i>fntA</i> D213AH173A	115	93	5	5	10	8	4.35	5.38	4.86	0.73	8.70	8.60	8.65	0.07
RN4220 $\Delta fntA$ pMK4:: <i>fntA</i> Y282AF346A	98	100	8	7	9	12	8.16	7.00	7.58	0.82	9.18	12.00	10.59	1.99
RN4220 $\Delta fntA$ pMK4:: <i>fntA</i> Y211AY282A	104	102	9	8	7	7	8.65	7.84	8.25	0.57	6.73	6.86	6.80	0.09

Appendix I

Table I. Summary of experimental results for FmtA variants, consolidating key findings from all the assays performed.

Experiments	Wild Type	RN4220 <i>ΔfmtA</i>	RN4220 <i>ΔfmtA</i> pMK4 <i>fmtA</i>	Y211F	D213N	Y282F	Y329F	H173A	F346A	Y282A	N343A	N343G	L342H	Y282A F346A	D213A Y329F	Y282F Y329F	D213A H173A	Y211A Y282A	Y211A D213A
Thermal Stability (°C)	53	NA	NA	56	57	51	58	62	56	59	55	53	58	59	64	62	66	57	58
Chemical Stability (M)	2.63	NA	NA	2.63	2.63	3.13	2.63	2.3	2.63	2.9	2.8	3.13	2.63	2.9	2.63	2.63	2	2.13	2.4
Conc.-Dependent Bocillin Binding (%)	7.9	NA	NA	9.11	21.6	12.9	7.9	9.6	7.4	13.5	3.5	14.7	19.5	61.5	24.1	22.1	31.1	14.3	26.4
Time-Dependent Bocillin Binding (min ⁻¹)	0.13	NA	NA	0.2	0.5	0.3	0.2	0.31	0.1	0.6	0.3	0.03	0.24	0.8	0.84	0.34	0.7	0.3	0.4
WTA Binding (%)	100	NA	NA	97.1	94.3	94.4	92.1	91.9	100	97.9	94.8	73.1	75.03	74.8	85.9	93.7	97.4	86.8	86.3
FBEC Assay (WTA) (pM)	106.6	NA	NA	77.7	111.4	106.9	110.5	81.9	104.8	99.6	99.8	70	59.1	46.1	83.7	82.9	81.9	85.9	87.1
Activity (%)	100	NA	NA	38	20	33	23	38	17	7	67	39	16	7	27	27	10	7	23
Biofilm (Exogenous FmtA) (A.U.)	1.85	NA	NA	NA	NA	NA	NA	NA	1.1	1.1	NA	NA	1.75	1.2	NA	NA	1.2	0.71	NA
Biofilm Formation (%)	100	66	100	NA	NA	NA	NA	NA	60	84	NA	NA	90	75	NA	NA	52	69	NA
D-Ala/Pi	0.24	0.15	0.28	NA	NA	NA	NA	NA	0.07	0.16	0.29	NA	0.12	0.09	NA	NA	0.13	0.12	NA
Triton X Susceptibility (% initial O.D A.U.)	41.9	22.9	44.1	NA	NA	NA	NA	NA	23.8	27.6	NA	NA	22.4	26.3	NA	NA	26.4	22.3	NA
Lysozyme Susceptibility (% initial O.D A.U.)	68.3	17.6	59.1	NA	NA	NA	NA	NA	61.4	18.1	NA	NA	16.3	14.1	NA	NA	16.9	21.6	NA
Lysostaphin Susceptibility (Log ₁₀ CFU/ml at 200 min)	486.7	1157.3	792	NA	NA	NA	NA	NA	110.3	0	113.7	NA	264.7	0	NA	NA	24	704	NA
Multi-Division Septa Formation (% frequency)	0	4.8	11.1	NA	NA	NA	NA	NA	8.5	9.7	NA	NA	13.4	10.6	NA	NA	8.6	6.8	NA

NA = Not Applicable (Experiment not performed)