CHARACTERIZATION OF FUNCTION AND ROLE OF FMTA IN *STAPHYLOCOCCUS AUREUS*

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ABSTRACT

Staphylococcus aureus is one of the major reasons for infectious mortality and morbidity. Due to extensive use of antibiotics, *S. aureus* has acquired resistance to almost all antibiotics. FmtA is one of the additional factors for methicillin resistance in *S. aureus* which interacts with wall teichoic acid (WTA) and localizes in the cell division septum. Previous studies showed that FmtA has very weak D-Ala-D-Ala carboxypeptidase (Cpase) activity. In lieu of these findings, we hypothesized that FmtA may require WTA as an activator ligand or protein-protein interactions to become fully functional as Cpase in the division septum. Here we show that WTA is not an activator ligand for Cpase activity of FmtA, but FmtA has an esterase activity on WTA. FmtA can remove D-Ala from D-alanyl ester in WTA. Additionally, we show serine and lysine from sequence motif Ser^{127}-X-X-Lys^{130} (conserved among penicillin binding proteins (PBPs), β-lactamase and family VIII esterase) of FmtA are involved in this catalytic activity. Mutation studies suggest that both Ser-X-X-Lys motif in FmtA, located at the position S63 and S127 are involved in catalysis. Our results suggest that FmtA's esterase enzymatic activity depends on both enzyme and substrate concentrations and high concentration of substrate (10 mg/mL WTA) can inhibit the reaction. Beside WTA, FmtA can also remove D-Ala from lipoteichoic acid (LTA). Results from *in-vivo* studies of WTA from *fmtA* deletion and *fmtA-CM* (conditionally over expressed) in *S. aureus* were in agreement with *in-vitro* esterase activity. In conclusion, WTA is not an activator ligand

for FmtA and that FmtA shows esterase activity towards D-alanyl ester of WTA and LTA. Both SXXK motifs in FmtA are involved in catalysis and Serine and lysine from SXXK motif are important for esterase catalysis. To date, biological function of most esterase is unknown. To our knowledge, this is the first study to report esterase from *S. aureus* that has sequence similarity to PBPs and β-lactamase and very selective to its biological substrate WTA and LTA compare to synthetic substrate.

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"*Imagination is more important than knowledge. For knowledge is limited to all we now know and understand, while imagination embraces the entire world, and all there ever will be to know and understand*."

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LIST OF ABBREVIATIONS

Ala – Alanine
Asp – Aspartic acid
Da – Daltons
DTT – Dithiothreitol
FPLC – Fast protein liquid chromatography
GlcNAc – N-Acetylglucosamine
His – Histidine
HPLC- High performance liquid chromatography
IPTG –Isopropyl ß-D-thiogalactopyranoside
LB– Luria Broth
LTA– Lipoteichoic acid
MES – 2-(N-morpholino)ethanesulfonic acid
MIC – Minimum inhibitory concentration
Ni-NTA – Nickel-nitrilotriacetic acid
NMR – Nuclear magnetic resonance
PBS – Phosphate buffered saline
PCR – Polymerase chain reaction
PG – Peptidoglycan
poly-RboP – polyribitol phosphate
polyGroP– polyglycerol phosphate
RPM – rotations per minute
SDS – Sodium dodecyl sulfate
SDS-PAGE – Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TA – Teichoic acid
TB–Terrific broth
TCA – Trichloroacetic acid
TCS – Two-component system
TSB – Tryptic soy broth
WT – Wild type
WTA – Wall teichoic acid

CHAPTER ONE

INTRODUCTION

1.1 *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive facultative anaerobic bacterium that causes hospital and community acquired infections which can lead to death (Diekema et al., 2001). This opportunistic pathogen is the leading cause of bloodstream, lower respiratory tract and skin/soft tissues infections. It can also cause central venous catheter-associated bacteremia, ventilator-assisted pneumonia and serious deep-seated infections, such as endocarditis and osteomyelitis (Diekema et al., 2001; Schito, 2006). *S. aureus* is often responsible for toxin-mediated diseases, such as toxic shock syndrome, scalded skin syndrome and staphylococcal foodborne diseases (SFD). In addition to its ability to cause life-threatening infections in human and animal pathogen, it has a remarkable potential to develop antimicrobial resistance (Lindsay & Holden, 2004).

1.2 Antibiotics and Multi drug resistance

Penicillin G (benzylpenicillin), a β-lactam antibiotic was discovered by Sir Alexander Fleming, a Scottish microbiologist in 1928. Penicillin was first introduced into clinical practice in the early 1940s; however, resistance against penicillin in staphylococci was discovered in 1942. In next two decades, almost 80% of staphylococcal isolates from hospital and community were resistant to penicillin (Lowy, 2003; Klein et al., 2007).

Typically, these resistant strains produce an enzyme, known as β-lactamase which is responsible for inactivation of β-lactams.

In 1959 a semisynthetic, narrow spectrum, antibiotic named methicillin was introduced to treat penicillin-resistant *S. aureus*. Unfortunately, methicillin-resistant *S. aureus* (MRSA) was identified immediately upon the introduction of methicillin into clinical practice (Jevons et al., 1963). Soon after the isolation of MRSA strain, several other countries also reported the resistance to this antibiotic where methicillin or other penicillinase-resistant penicillins were not yet available (Ayliffe, 1997). Resistance in MRSA strain was not associated with β-lactamase production but due to the expression of an additional penicillin-binding protein (proteins that are involve in the final stage of peptidiglycan synthesis), known as PBP2a or PBP2', which is imported from another species *S. fleurettii* (Tsubakishita et al., 2010). In MRSA strain, PBP2a was encoded by *mecA* gene which is a part of cassette chromosome mec (SCCmec). This protein shows low affinity for all β-lactam antibiotics.

Although *S. aureus* strains harboring *mecA* are referred to MRSA, in reality, they are resistant to all β-lactam antibiotics (Berger-Bachi, 1994). It has been observed that in heterogeneous population of MRSA, expression level of PBP2a doesn't correlate with the level of resistance. As a result, other genes known as *fem* (factors essential for methicillin resistance) or *aux* (auxiliary factors) are proposed and identified as high level resistance in MRSA (Fuda et al, 2005). Over the year, extensive use of different types of antibiotics has led to emergence of multidrug-resistant MRSA strains which are also referred as multidrug resistant strains (MDR). MDRs result from mutations in genes encoding for

drug target proteins and through acquisition and accumulation of many other determinants of resistance into the sequence of SCCmec (staphylococcal cassette chromosome mec, responsible for PBP2a) such as plasmids, transposons, and insertion sequences (Stefani and Goglio, 2010).

Initially, MRSA strains were limited to hospital settings and are referred to Hospital Acquired MRSA strains (HA-MRSA), later, a new group of MRSA strain was identified in community called community-acquired MRSA(CA-MRSA) and livestock associated MRSA (LA-MRSA) (Otter et al., 2012). HA-MRSA often lead to severe and life-threatening infections; however, CA-MRSA is responsible for superficial skin infections (Chambers and DeLeo 2009). According to recent data, more than 50% infections in the hospitals are caused by MRSA and this has resulted increased mortality and morbidity in patients with bacterial infections (Taiwo, 2009). Currently, HA –MRSA and CA-MRSA have outspread all over the world and are a great concern in clinical settings. In 2005, there were aproximately 11,406 *S. aureus*–related deaths reported, of which 6,639 were MRSA-related (Klein et al., 2007). In the recent years from 1999 to 2005, hospitalization due *S. aureus* increased by 62 % and mortality related to these infections is very significant (Klein et al., 2007). Due to rapid increase of staphylococci resistant to many antibiotics, it is very important to understand the factors and signal mechanism related to antibiotic resistance.

1.3 The cell walls

Cell wall of *Staphylococcus aureus* is a dynamic, semi-rigid structure (Scheffers et al., 2005). The composition of staphylococcal cell wall includes three major components: peptidoglycan, teichoic acids and surface proteins.

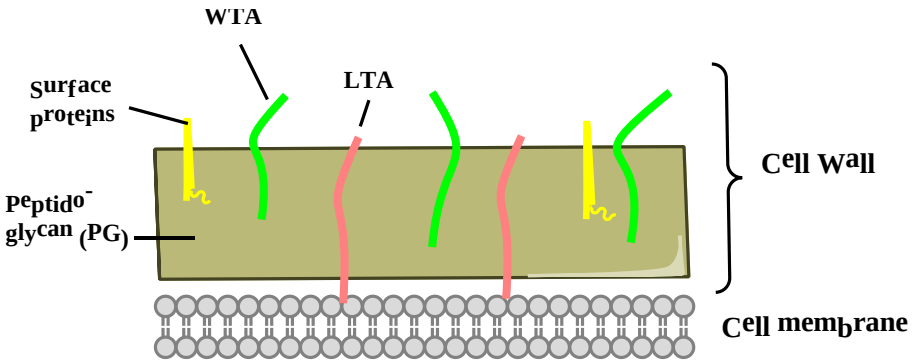

Figure 1.3 Schematic representation of *S aureus* cell envelope of gram positive bacteria.

1.3.1 Peptidoglycan

Peptidoglycan (PG), also known as murein, is an essential and elastic component of the bacterial cell wall found on the outside of the cytoplasmic membrane of almost all bacteria. Its main function is to resist lysis as a result of its high intracellular osmotic pressure. It also provides strength, relative rigidity, shape, and protection (Rogers et al., 1980). The main structural features of peptidoglycan are linear glycan strands cross-linked by short peptides. The glycan strands are made of repeating disaccharide units of N-acetylglucosamine and N-acetylmuramic acid (Rogers et al., 1980). These glycan chains vary in a length of 3–10 disaccharide units. A short stem peptide with the sequence

L-Ala-D-Glu-L-Lys-D-Ala-D-Ala is attached to the carboxyl group of each N-acetylmuramic acid (Scheffers & Pinho, 2005). One of the characteristic features of the *S. aureus* cell wall includes attachment of a series of five L-glycine residues to the L-lysine component of one stem peptide to the penultimate D-Alanine of a neighboring stem peptide in which glycan strands are cross-linked with short peptide regions.

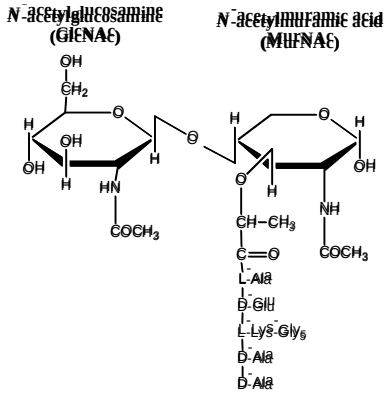

Figure 1.3.1.1 Chemical structure of *S. aureus* peptidoglycan
General peptidoglycan (PG) monomer composed of two sugars: N-acetylglucosamine and N-acetlymuramic acid. Polymers are linked via the pentaglycineinterbridge from $_{L}$-Lys to $_{D}$-Ala.

PG biosynthesis is a complex process which can be divided into three stages. Initially, water soluble precursor's formation starts in the cytoplasm where formation of UDP-Mur-L-Ala-D-Glu-L-Lys-D-Ala-D-Ala (UDP-MurNAc-Penta) through a series of steps starting from UDP-N acetylglucosamine (UDP-GlcNAc) catalyzed by Mur enzymes. The finished PG precursor is an insoluble polymer outside the cytoplasmic membrane. The water-soluble precursors must therefore cross the membrane. To do this, a carrier lipid is employed. In the second stage of synthesis, UDP-MurNAc-Penta is anchored to inner leaflet of the cell membrane catalyzed by MraY leading to formation of lipid I intermediate. In last step, the transfer of N-acetylglucosamine (NAM) to Lipid I to form

β-(1,4) linked NAG-NAM- pentapeptide (Lipid II) catalyzes by MurF enzyme. Furthermore, a pentaglycine peptide of PG precursor is synthesized in a sequential manner by the family of FemABX (Berger-Bachi & Tschierske, 1998; Rohrer et al., 2003). In the final step, polymerizations of PG occur with the help of enzymes glycosyltransferases that catalyze the formation of linear glycan chains and transpeptidases that catalyze the formation of peptide cross bridges. These polymerization steps are carried out by some enzymes known as Penicillin binding proteins. The glycan chain synthesis is catalyzed by glycosyltransferase domain of penicillin-binding proteins in which the pyrophosphate on the MurNAc moiety of one Lipid II subunit is displaced by a hydroxyl group on the GlcNAc moiety of a second Lipid II molecule. As a result, free undecaprenyl pyrophosphates are generated, which is flipped back to the cytoplasmic face of the cell membrane and recharged via hydrolysis to undecaprenyl phosphate (Macheboeuf et al., 2006). Whereas the Lipid II pentapeptides cross-linking reaction is catalyzed by the transpeptidation domain of penicillin-binding proteins (Van Heijenoort, 2001).The transpeptidation (TP) reaction results in the cleavage of the C-terminal D-Ala in the Lipid II pentapeptide, and the formation of an amide bond between the carbonyl group on the remaining alanine residue and a free amino group on the pentaglycine crossbridge (Rohrer et al., 2003). Some PBPs can cleave the terminal D-alanine from the pentapeptide using water as an electron acceptor, thereby preventing cross-linking which is known as carboxypeptidase activity (Van Heijenoort, 2001).

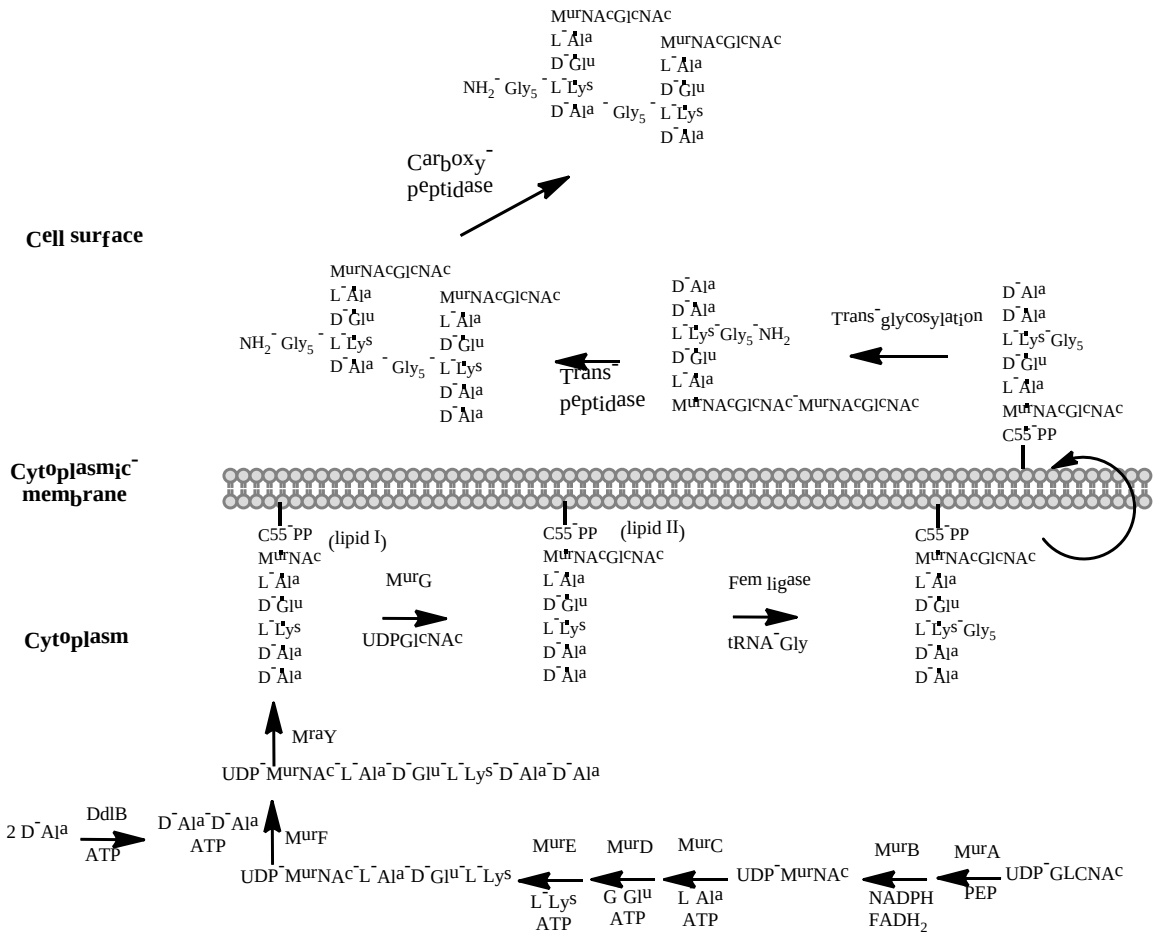

Figure 1.3.1.2 Diagram of the biosynthetic pathway of cell wall assembly
Generation of cell wall precursors begins in the cytoplasm, resulting in the synthesis of UDP-MurNAc–L-Ala–D-iGlu–L-Lys–D-Ala–D-Ala. The PG precursor subunit is transferred to a lipid carrier in the membrane to generate lipid I. After further modification, the lipid-anchored PG precursor is translocated to the extracellular face of the cytoplasmic membrane. The PG precursor is subsequently incorporated into the cell wall by transpeptidation and transglycosylation reactions with the concomitant displacement of the lipid carrier. The terminal D-alanine of the wall pentapeptide is subject to substitution by cross-linking to other wall subunits or can be removed by the action of a D-alanyl–D-alanine carboxypeptidase. PEP, phosphoenolpyruvate; MN-GN, MurNAc-GlcNAc.

1.3.2 Penicillin-binding proteins

Penicillin-binding proteins (PBPs) are ubiquitous among Gram-positive and Gram-negative bacteria. They are involved in the latter steps of PG biosynthesis where they recognize the terminal D-Ala-D-Ala unit (Van Heijenoort, 2001). Based on the molecular weight, PBPs are classified into two classes: high molecular mass (HMM) PBPs (20-120 kDa) and low molecular mass (LMM) PBPs (20-50 kDa). Both HMM and LMM PBPs are further categorized based on their catalytic activity. HMM PBPs are broadly divided into Class A (bifunctional, having both transglycosylase and peptidase activity) or Class B (monofunctional, only transpeptidase PBPs); whereas, LMM PBPs are generally DD- carboxypeptidases (monofunctional) (Ghuysen, 1991).

HMM PBPs play a vital role in cell elongation, cell morphology, and cell division, thus are essential for *S. aureus* viability; as a result they are the lethal targets for β- lactam antibiotics. On the other hand, LMM PBPs are nonessential for cell viability; they often play important roles in *S. aureus* cell morphology (Macheboeuf et al., 2006). Inhibitors of transpeptidase (TP) interfere with cell wall synthesis by weakening the cell wall and eventually lead to cell death. Currently, TP inhibitors (e.g. β-lactams and glycopeptides) are in use as chemo-therapeutics due to low toxicity.

All PBPs contain a common domain which uses the D-alanyl-D-alanine dipeptide on the C-terminal end of the Lipid II pentapeptide as a substrate for peptidation reactions. They harbor three highly conserved active site sequence motifs (SXXK, S/YDN, and K(T/S)G) (where X resembles any variable amino acid) (Goffin & Ghuysen, 1998). The serine residue of the motif SXXK is central to catalysis and is involved in enzyme

acylation by the C-terminal alanine. For transpeptidation, an amino group from the pentaglycine bridge serves as the proton acceptor of the reaction, resulting in the cross-linking of two stem peptides from PG strands. On the other hand, carboxypeptidation can further results in a permanently shortened stem peptide (4 amino acids) that is unable to be cross-linked. Finally, the proton is back-donated to the active site, breaking the acyl-enzyme bond and recharging the PBP for a new reaction (Ghuysen, 1991) (Figure 1.3.1).

Though PBPs are named based on their binding and catalytic hydrolyzing activity on β-lactam along with *in-vitro* transpeptidation/carboxypeptidation activity with synthetic dipeptides, independently of its function in the cell; no concrete evidence for direct PG biosynthesis participation has been reported for some PBPs (e.g. *Streptomyces* K15 and R61 transpeptidases). Moreover, precise cellular functions of *Streptomyces* K15 and R61 transpeptidases are still unknown (Macheboeuf et al 2006).

1.3.3 Penicillin-recognizing enzymes

PBPs are a large family of cell wall biosynthetic enzymes which are ubiquitous among all gram-positive and gram-negative bacteria with the exception of mycoplasmal species due to lacking of PG (Macheboeuf et al., 2006). PG is a major constituent of the bacterial cell wall where D-amino acids are found and these D-residues are synthesized by the help of a racemase that converts L-amino acids to D-amino acids. β-Lactam antibiotics interfere with PG biosynthesis by inhibiting the D-ala-D-ala transpeptidation reaction to form a penicilloyl- enzyme complex with a long half-life, this results in cell

lysis by preventing the synthesis of PG. The active site serine residue plays a key role for this catalysis.

However, aside from D,D-carboxypeptidases/transpeptidse, a large group of structurally similar protein family with an active-site serine at SXXK motif can recognize penicillin. These proteins are known as "penicillin-recognizing active-site serine enzymes (PRPs)" (Asano et al., 1996). These active-site serine proteins have been shown to catalyse a wide variety of substrates and reactions. They include β-lactamases which hydrolyze amide bond of β-lactam antibiotics, DD carboxypeptidase/transpeptidase which catalyze the removal of last D-Ala from the PG precursor), esterase which hydrolysis of esterase bond, D,L-endopeptidases which eleave DL bonds in stem peptides, D-aminopeptidases hydrolyze the removal of D-Ala from N-terminus of peptide (Asano et al., 1989; Bourne et al., 2001; Ghuysen, 1991; Komeda and Asano, 2000, Wagner et al., 2002). A brief description of above enzymes is provided below.

DD-Carboxypeptidase (EC 3.4.17.14) catalyzes a step in cell wall biosynthesis by hydrolyzing D-Ala- D-Ala bonds using a reactive site serine residue (Asano et al., 1989). Acting on pentapeptide muropeptides, it thus controls the extent of crosslinking between adjacent PG macromolecules. Most of the low molecular weight PBP's are referred to DD-carboxypeptidase. Representative substrate for these carboxypeptidase /transpeptidase (PBPs) is N_{α},N_{ε}-Diacetyl-Lys-D-Ala-D-Ala which is an analogue of short stem peptide with the sequence: L-alanyl-D-isoglutaminyl-L-Lysyl-D-Alanyl-D-alanine which is attached to each N-acetylmuramic acid in PG (Scheffers & Pinho, 2005) (Fig 1.3.1).

D-aminopeptidase (DAP) (EC 3.4.11.19) purified from *Ochrobactrum anthropi* which can hydrolyse peptides with a free N-terminal D-amino acid has shown various β-lactam inhibition and share ∼25% sequence identity with the R61 DD-carboxypeptidase and the class C β-lactamases. The sequence motif SXXK which is conserved among all penicillin-recognizing proteins is also essential for D-aminopeptidase activity (Asano et al., 2000). Delmarcelle et al. has illustrated a specificity inversion of D-aminopeptidase to D,D-carboxypeptidase via a loop deletion (the 476–486 γ-loop of domain C was replaced by a single glycyl residue) along with site-directed mutagenesis (Asn275Arg mutation), which is an unique example of drastic specificity change in the serine penicillin-recognizing proteins (Delmarcelle et al., 2005). DAP is very specific to peptides with free D-amino acid at NH_2 terminus D-Ala-L-Ala-LAla (Asano et al. 1992); However, D-Ala-paranitroanilide can be used as a substrate to assess the D-aminopeptidase activity. As yet, biological role of these enzymes are still not clear (Remaut et al., 2001).

β-Lactamases (EC 3.5.2.6) are members of penicillin-recognizing active-site serine enzymes. They hydrolyze all classes of β-lactam antibiotics (Asano et al., 2000). These enzyme catalyses the irreversible hydrolysis of the amide bond of the β-lactam ring, thus yielding biologically inactive products. All β-lactamases harbor the active-site SXXK motif and follow a catalytic pathway similar to DD-peptidases where active-site serine resides close to the amino terminus of the protein. However, the deacylation step of the penicilloyl-enzyme complex formed after a nucleophilic attack of the active-site Ser of β-lactamases to the carbonyl carbon of the amide linkage is much faster than DD-carboxypeptidase (Davies et al., 1994).

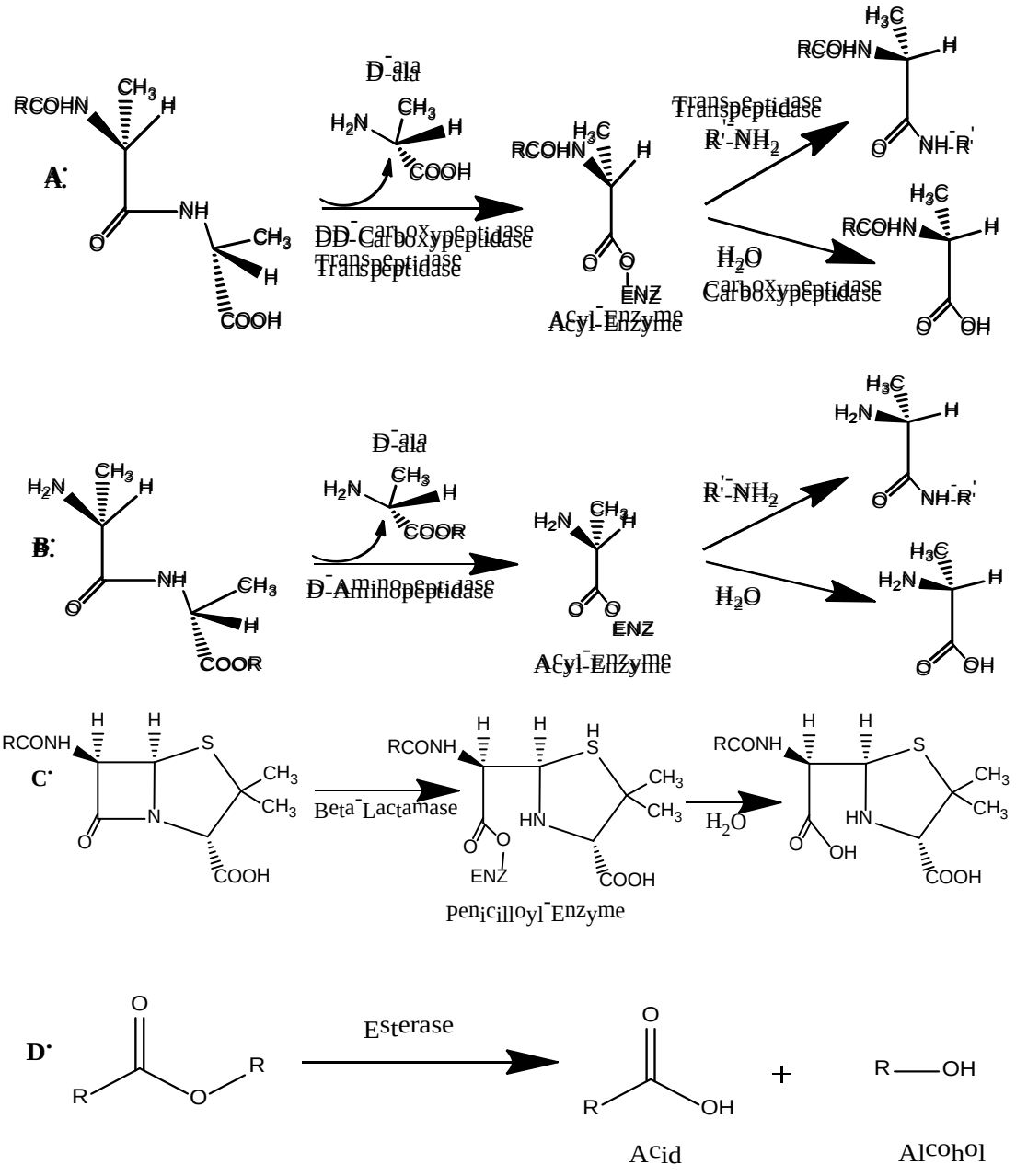

Figure 1.3.3 Reactions catalyzed by A. transpeptidase and DD-carboxypeptidase; B. D-aminopeptidase; C. β-lactamase and D. Esterase; Modified from Asano et al.1992.

Esterases (EC 3.1.1.) represent a diverse group of hydrolases ubiquitous in animals, plants, insects and microorganisms (Wagner et al., 2002). The esterases hydrolyse ester bonds and generate an acid and an alcohol as metabolites. The physiological role of a large number of esterases form a diverse sources is unknown. Esterases have a diversified preference for substrates and specificity; these include carboxylic, thio, phospho and other types of esters. Esterases are responsible for metabolism of several classes of exogenous and endogenous compounds (e.g hydrolysis of acetylcholine) (Isabela Reis Montella et al. 2012). Carboxylesterases catalyze the hydrolysis of short chain aliphatic and aromatic carboxylic ester compounds. Based on the catalytic activity on substrates of different acyl chain lengths, carboxylester hydrolases are classified as esterases or lipases. Esterases act preferentially on water-soluble short-chain fatty acids; on the other hand lipases display utmost activity towards water-insoluble long chain triglycerides (Tsujita et al., 1990). Based on the sequence homology, carboxylesterases are classified into eight families (families I–VIII) (Arpigny JL et al., 1999) . Superfamily VIII esterases have very high sequence similarity to class C β- lactamase and penicillin binding proteins (PBP). Family VIII esterases also represent a poorly characterized esterase family (Rashamuse et al., 2009). Family VIII carboxylesterases are structurally dissimilar from other carboxylesterases. Their modular structure is composed of a small helical domain and a α/β hydrolase domain that is similar of class C β-lactamases (Nacke et al., 2011). The nucleophilic serine residue in family VIII carboxylesterases occurs in the S-X-X-K motif, similar to class β-lactamases, rather

than the G-X-S-X-G motif. These enzymes have approximately 337 to 443 amino acids and a molecular mass of 36.9 to 48.6 kDa (Nacke et al., 2011).

Carboxylesterases, β-lactamases and PBPs share a two-step serine hydrolase mechanism that consists of two successive nucleophilic attacks by the serine residue and a water molecule. Due to the fact that family VIII of carboxylesterases have features similar to β-lactamases and PBPs, these enzymes might be expected to exhibit β-lactamase activity or DD-peptidase activity. Indeed few carboxylesterases have been reported to show β-lactamase activity (Wagner et al., 2002, Cha et al., 2013); however, as per my knowledge, no literature review in DD-carboxypeptidase activity has been reported.

According to Wagner et al. Esterases, D-alanyl-D-alanine-peptidases (DD-peptidases) and β-lactamases can be grouped into two distinct classes of hydrolases - one class comprised of esterases and the other one comprised of β-lactamases and DD-peptidases based on different folds and topologically unrelated catalytic residues. Due to similar chemical reactivity of esters and β-lactams towards hydrolysis scientists are interested to know the structural features that prevent esterases from displaying β-lactamase or PBP activity and vice versa (Wagner et al., 2002). Prediction studies indicate that proteins of the same family of penicillin-recognizing active-site serine enzymes may have evolved from a common ancestor (Ghuysen, 1991; Valegard et al., 2013). Based on their evolutionary distance, they may have acquired different amino acid sequences along with distinct functionalities and specificities while conserving the same polypeptide scaffolding (Ghuysen, 1991).

Table 1: catalytic residues (highlighted red) identified in the active sites of PBP/β-lactamase-fold enzymes. Classification based on Sauvage et al. (modified from Valegard et al. 2013)

Class/Type/ Subtype	Representative Protein	PDB code	Motif one (SXXK)	Motif two ([S/Y]DN)	Motif three KTG
β-Lactamase Class A	TEM-1	1M40	Ser^{70}/ Lys^{73}	Ser^{130}	Lys^{234}
β-Lactamase Class D	OXA10	1E3U	Ser^{67}/ Lys^{70}	Ser^{119}	Lys^{214}
β-Lactamase Class C	P99	1BLS	Ser^{64}/ Lys^{67}	Tyr^{150}	Lys^{315}
PBP Class C / Type-5	PBP5	1Z6F	Ser^{44}/Lys^{47}	Ser^{110}	Lys^{213}
PBP Class C / Type-4	R39 DD-peptidase	1W79	Ser^{49}/Lys^{52}	Ser^{298}	Lys^{410}
PBP Class B / Type-B1	PBP2a	1VQQ	Ser^{403}/Lys^{406}	Ser^{462}	Lys^{597}
PBP Class A / Type-A3	PBP1a	2C6W	Ser^{370}/Lys^{373}	Ser^{428}	Lys^{557}
PBP Class C /Type-7	K15 DD-peptidase	1SKF	Ser^{25}/Lys^{38}	Ser^{96}	Lys^{213}
PBP Class C / Type-AmpH	R61 DD-peptidase/ transpeptidase	3PTE	Ser^{62}/Lys^{65}	Tyr^{159}	His^{298}
D-Aminopeptidase	DAP	1EI5	Ser^{62}/Lys^{65}	Tyr^{153}	His^{287}
D-Amino acid amidase	DAA	2DRW	Ser^{60}/Lys^{63}	Tyr^{149}	His^{307}
Esterase family VIII	EstB	1CI8	Ser^{75}/ Lys^{78}	Tyr^{133}	Tyr^{181}
Nylon amidase	NylC	1WYC	Ser^{112}/Lys^{115}	Ser^{217}	Tyr^{215}
Esterase family X	ORF12	2XEP	Ser^{173}/ Lys^{176}	Ser^{234}	Lys^{375}
Unknown	**FmtA**		**Ser^{63}/ Lys^{65} & Ser^{127}/ Lys^{130}**	**Tyr^{211}**	

1.3.4 Teichoic acids

Teichoic acids are an important component of cell wall in Gram-positive bacteria. They play vital roles in bacterial survival under unfavorable conditions and in other basic cellular processes (Weidenmaier and Peschel, 2008). In *S. aureus*, these constitutively produced anionic glycopolymers can be classified into two types: wall teichoic acids, (WTA); which are usually covalently connected to PG and extend through and beyond cell wall and lipoteichoic acids (LTA); which are usually connected to cytoplasmic membrane and extend from cell surface into PG layers. Most of the Gram-positive bacteria produce both teichoic acids (Swoboda et al., 2010).

S. aureus WTAs consist of 1, 5-linked repetitive poly ribitol phosphate attached to N-Acetylmuramic acid (MurNAc) of PG via a disaccharide linkage unit to consisting of N-Acetylglucosamine (GlcNAc), MurNAc and two to three glycerol phosphate residues. WTA repeating polymer is composed of 11–40 poly ribitol phosphate (PolyRboP) repeating units in most *S. aureus* strains (Brown et al., 2008). *S. aureus* produces polyRboP WTAs whereas *B. subtilis* produces either polyRboP or polyGroP (poly glycerol phosphate) depending on strains (Brown et al., 2012). On the other hand, LTAs consist of 1,3-linked repetitive polyol (glycerol) phosphate repeating units attached to the cytoplasmic membrane via a glycol lipid anchor (diacylglycerol). LTA repeating backbone is composed of repeating units of polyGroP (Figure 1.3.4.2).

Teichoic acids structural diversity exhibit due to tailoring modifications on the repeating polymers. The hydroxyls groups from repetitive polyol (ribitol or glycerol) phosphate in teichoic acids can be modified. In WTA, cationic D-alanine esters

modifications are found at the second position of ribitol where as sugars, mono- or oligosaccharides, mainly glucose or GlcNAc are incorporated at the fourth position of ribitol (Brown et al., 2013). These modifications are commonly observed in all *S. aureus* strains. Recently, it has been reported that modification of WTA by GlcNAc could be fully α-glycosylated, fully β-glycosylated or mixture of both. However, anomeric configuration of the glycosidic linkage to the repeat unit of ribitol varies, sometimes in the same cell (Brown et al., 2013). Recently, it has been demonstrated that glycosylation of WTA may contribute to methicillin resitance (Brown et al., 2012). Although there is structural similarity between WTA and LTA, most of their biosynthesis follows different pathways and precursors (Xia and Peschel, 2008). Usually, modifications of LTA are observed at the second position of the glycerol, where hydroxyl group is substituted with D-alanyl ester or a-GlcNAc (Fischer, 1988).

Characterization of the WTA biosynthetic pathway was challenging due to the attachement of the precursor undecaprenyl phosphate (C55-P) lipid chains in the substrate which is difficult to handle. Biosynthesis of WTA starts in the cytoplasm catalyzed by *tag* family of enzymes in *S. aureus*. Genes involved in conserved steps like poly-Gro-P and poly-Rbo-P WTA biosynthetic pathways are referred as ‘tag’ while ‘tar’ is used only for those genes that are additionally required for incorporation of Ribitol phosphate units (Xia and Peschel,2008). WTA biosynthetic pathway can be divided into five steps and uses an undecaprenyl phosphate (C55-P) which is also known as bactoprenyl phosphate (Xia and Peschel, 2008). The first step of WTA biosynthesis is initiated by disaccharide linkage unit by the enzymes TagO and TagA which transferr GlcNAc-1-phosphate and a

ManNAc, respectively, from UDP-activated precursor to C55-P. Further, phosphoglycerol units are attached to the WTA precursors with the help of TagB and TarF. Incorporation of repeating units of poly ribitol phosphate into WTA is mediated by a group of polymerizing enzymes (Xia and Peschel, 2008). With the help of the transporter TagGH, it is flipped to the external surface of the cytoplasmic membrane (Brown et al., 2008; D'Ellia et al., 2009) (Figure 1.3.4.1).

Prior to the WTA export to the cell surface, WTA glycosylation occurs, where RboP WTA α-O-GlcNAc modifications are installed by TarM and β-O-GlcNAc modifications are installed by TarS. Another major modification of WTA, D-alanylation occurs in the cell surface. Four enzymes encoded by the *dltABCD* operon participate in the esterification of hydroxyl group of ribitol with D-Ala. D-Ala biosynthesis starts in the cytoplasm where DltA activates D-alanine as an AMP ester and then transfer the aminoacyl adenylate to DltC. It is believed that this is transported to the cell surface and attached to WTA with the help of DltB and DltD. Though it has been shown that both LTAs and WTAs require the same enzymes (DltABCD) for incorporation of D-alanine, the enzymology of these biosynthetic process remains to be elucidated (Xia et al., 2010; Brown et al., 2013; Reichmann et al., 2013).

Teichoic acids play an important role in cell wall based on their location, abundance and nature. However, the actual cellular functions are poorly understood. Generally, TAs are known to protect Gram-positive bacteria against harmful molecules and environmental stresses, control enzyme activities in the cell surface, control cation

concentrations in the cell envelope and bind to receptors (toll-like receptors 2 and 4) and assist bacteria to attach to the surfaces (Weidenmaier et al.,2003; D'Elia et al., 2009).

Due to teichoic acids anionic nature, they bind cations and thus play a role in cation homeostasis (Marquis et al. 1976). However, most TAs display zwitterionic properties because of the presence of negatively charged phosphate groups and tailoring D-alanine residues on the repeating units, which have a positively charged amino group (Weidenmaier and Peschel, 2008). The *dltA* gene deletion removes D-alanine esters from both LTAs and WTAs. Recent studies on *dltA* deletion mutant have shown that absence of D-alanyl esters increases the overall negative cell surface charge. Controlling cell surface charges by altering the amount of teichoic acid D-alanylation is a very important mechanism by which Gram-positive bacteria can protect themselves against host-defense mechanism (Kristian et al., 2005).

D-alanine deficient *S. aureus* has shown an increased susceptibility to endopeptides: lysostaphin and lysozyme, to phagocytes and to neutrophil killing (Collins et al., 2002). Furthermore, a reduction of D-alanylation in teichoic acid resulted in increased susceptibility to glycopeptide antibiotics and to certain cationic antimicrobial peptides (CAMPs) (Collins et al., 2002). The amount of D-alanyl esters and the presence of WTAs influence bacterial interactions with various surfaces (Neuhaus et al., 2003). WTA-lacking cells have shown reduced capability to form biofilms (Gross et al., 2001). Moreover, strains lacking D-alanine showed decreases biofilm formation and bacterial adhesion to plastic and glass surfaces. A decreased in D-alanine content in WTA could result in an increased of negative cell surface charge creating an increased repulsive force

between bacteria and a surface which could result in reduced adherence (Holland et al., 2011). Reduced cationic binding capacity of WTA could result due to the masking of negative charge of WTA by D-alanylation.

It has been hypothesized that ion binding to WTAs could help prevent fluctuations in osmotic pressure between the inside and the outside of the cell (Heptinstall et al., 1970). It has been demonstrated that the *dlt* operon could be a target for antivirulence agents as strains lacking teichoic acid or D-alanine esters are strongly attenuated in animal infection models (Weidenmaier et al., 2003). Given the importance of D-alanylation in teichoic acids they remain a potential target for prospective drug development.

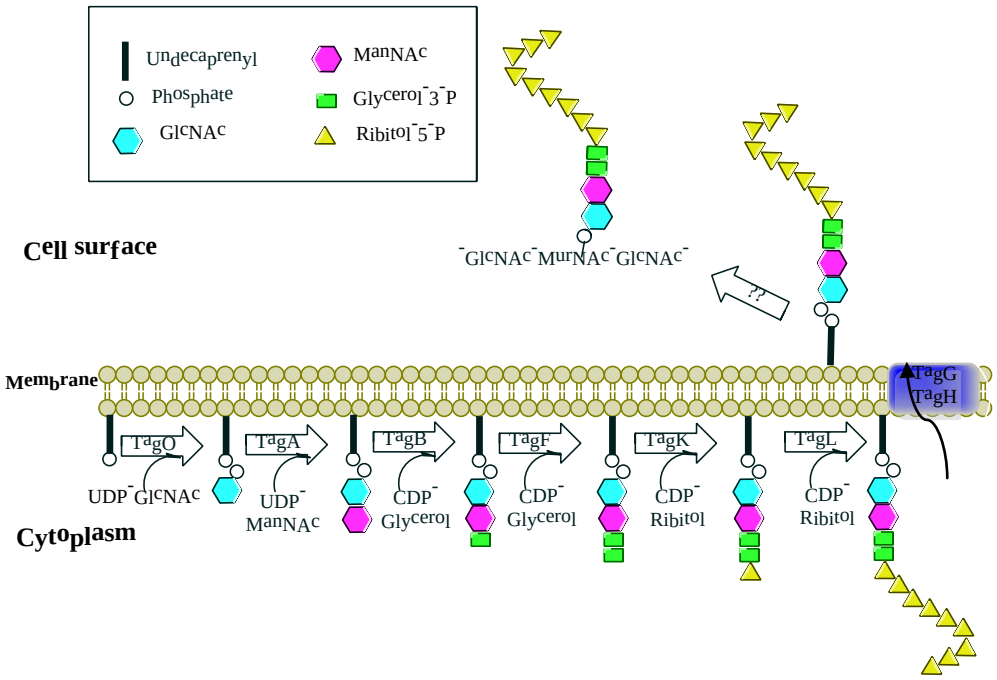

Figure 1.3.4.1 Path ways of *S. aureus* wall teichoic acid (WTA) biosynthesis
CDP, cytidyldiphosphate; Glc, glucose; GlcNAc, N- acetylglucosamine;; ManNAc, N-acetylmannosamine; MurNAc, N-acetylmuramicacid; UDP-Glc, uridine-5'-diphosphate-glucose; UDP-GlcNAc, uridine-5'-diphosphate-N-acetyl-glucosamine; UDP-ManNAc, uridine-5'diphosphate-N-acetyl-mannosamine.

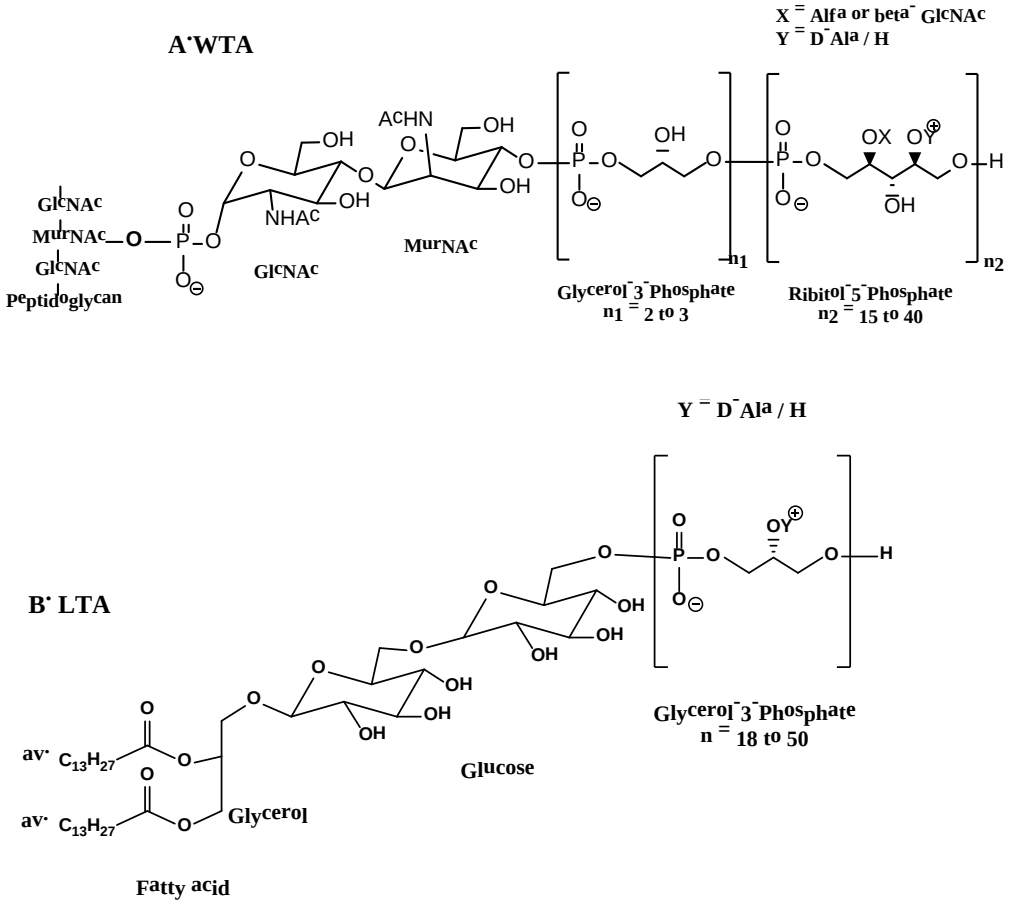

Figure 1.3.4.2 Chemical structure of *S. aureus* wall teichoic acid (WTA) and lipoteichoic acid (LTA)

A) Wall teichoic acid (WTA) linkage unit is composed of N-acetylglucosamine (GlcNAc) and N-acetlymuramic acid (MurNAc) followed by two to three $_{D}$-alanylated glycerol phosphate residues. The WTA backbone on average ranges from 15 - 40 units of ribitol phosphates (substituted with D-Ala and/or α- or β- GlcNAc). B) Lipoteichoic acid (LTA) linkage unit is composed of a diacylglycerolconnected to disaccharide. The LTA backbone on average is 18 to 50 units of glycerolphosphates (substituted mainly with $_{D}$-Ala).

1.4 Methicillin resistance *S. aureus* (MRSA)

S. aureus has four major penicillin-binding proteins (PBPs): PBP1, PBP2, PBP3 and PBP4 (Machebouf et al., 2006). Out of these, PBP1, PBP3 and PBP4 show

monofuctional transpeptidase activity, which can cross link the pentaglycine bridge to other stem peptide containing terminal D-ala-D-ala (Atilano et al., 2010). β-lactam antibiotic inhibits penicillin-binding proteins (PBPs), specifically transpeptidase activity that are involved in the synthesis of PG, an essential mesh-like polymer that surrounds the cell. This then leads to death of the bacterial cell due to osmotic instability or autolysis (Stapleton, 2002). After penicillin resistance by β-lactamase in *S. aureus* was observed, a new β-lactam antibiotic name Methicillin was introduced. However, *S. aureus* became resistant to methicillin and other β-lactam antibiotics through the expression of a foreign PBP, PBP2a that shows low affinity for methicillin.

Methicillin resistant *Staphylococcus aureus* (MRSA) harbour a large mobile genetic element called staphylococcal cassette chromosome, mec (SCCmec) which was acquired through the horizontal transfer. It carries the mecA gene that encodes for an alternative penicillin binding protein, PBP2a that has reduced affinity for β-lactams. This allows MRSA strains to grow in antibiotic concentrations when all native PBPs are inactivated (Ito et al., 1999). Expression of PBP2a is inducible through a signal transduction system encoded by genes *mecRI* and *mecI* on the SCCmec genetic element, and involves an integral membrane protein sensor (MecR1) and a transcriptional repressor (MecI). MecRI, a receptor protein contains an extracellular penicillin-binding domain that undergoes a conformational change inducing autocleavage of the intracellular protease domain upon the binding of β-lactams. Further, de-repression of *mecA* transcription as well as the *mecI-mecRI* operon starts by the cleavage of MecI by active MecRI (Mallorqui-Fernandez et al., 2004). Besides the regulation of *mecA* expression by

its cognate MecI and MecRI regulators, it can also be regulated structurally and functionally by β-lactamase regulators, BlaI and BlaRI (champbers, 1997) (Figure 1.4).

Apart from methichillin resistance, *S. aureus* has developed resistance to other class of semisynthetic β-lactamase-insensitive β-lactams: oxacillin and nafcillin by using the same mechanism of acquired *mecA* gene described above. The expressed PBP2a has a molecular mass of 78 kDa enzyme and consists of a transpeptidase domain and a non-penicillin binding domain (Goffin & Ghuysen, 1998). Compare to other native PBPs that synthesize highly cross-linked peptidoglycan, PBP2a, emerged to be a rather poor active enzyme *in-vitro* (de Jonge et al., 1993). It has been shown that the cooperative functioning of PBP2A and the penicillin-insensitive transglycosylase (TGase) domain of PBP2 is required for high-level of resistance (Pinho et al., 2001). Recent crystallographic studies have revealed that PBP2a has an allosteric binding domain which can control the substrate entry to active site by conformational changes (Otero et al., 2013).

To treat MRSA associated infections, Vancomycin, a glycopeptide was introduced in hospitals. Unfortunately, it didn't take long for *S. aureus* to exhibit resistance or reduced susceptibility to glycopeptides. Vancomycin resistant strains follow an alternative pathway for PG biosynthesis where these strains synthesize D-Ala-D-Lac instead of D-Ala-D-Ala in the stem peptides which are the binding site for vancomycin. In addition to alteration of stem peptides, other mechanisms of over production of PG precursors also contribute to antibiotic resistance. Based on the vancomycin susceptibilities, vancomycin-resistant *S. aureus* are categorized into three classes: vancomycin-intermediate *S. aureus* (VISA), heterogenous vancomycin-intermediate *S.*

aureus (hVISA), and high-level vancomycin-resistant *S. aureus* (VRSA) (Howden et al., 2010). VRSA shows fully vancomycin-resistance due to acquisition of the *vanA* gene from vancomycin-resistant enterococci. In case of VISA strains, abnormally thickened cell wall was observed. Unfortunately, it didn't take long to spread worldwide the resistance phenotype after the first report of VISA and hVISA from Japan (Hiramatsu et al., 1997).

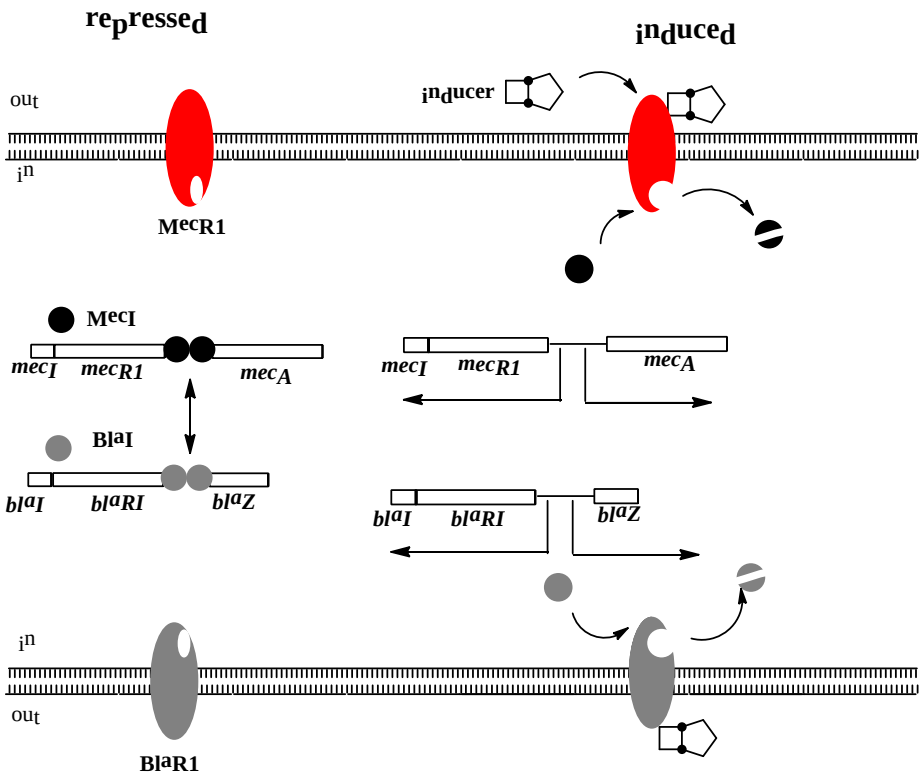

Figure 1.4 Induction of PBP2a and penicillinase
Two pathways regulate PBP2a (*mecA*) and penicillinase (*blaZ*) production. MecI represses transcription of *mecA* and *mecR1-mecI*. Upon binding of an inducer to the sensor domain of MecR1, the sensor transducer is autocatalytically cleaved, activating the intracellular zinc peptidase domain, which in turn cleaves the MecI repressor, thereby relieving *mecA* and *mecR1-mecI* repression. In analogy, *BlaI* represses *blaZ*, and induction occurs through BlaR1. While the repressors are interchangeable and can recognize both the mec and bla regulatory regions, cleavage of the repressors by BlaR1 or MecR1 is specific.

1.5 Factors affecting methicillin resistance levels

In MRSA, PBP2a plays a major role for resistance. Methicillin resistance in *S. aureus* is classified into two categories: homogeneous and heterogeneous. The level of resistance varies extremely among strains. Non-consistency in the phenotypic expression of antibiotic resistance is known as heteroresistance (Tomasz et al., 1991). The ability of heterogeneous *S. aureus* population to produce PBP2a is essential for their methicillin resistance but there is no correlation between cellular concentration of PBP2a and the observed difference in resistance levels (Berger-Bachi & Rohrer, 2002). Hence, involvement of additional factors from chromosomal genes is proposed for optimal methicillin resistance. In addition to *mecA*, several other accessory factors were identified for high level of methicillin resistance in *S aureus* which were named *fem* genes (for factor essential for methicillin resistance) or aux genes (for auxiliary genes) (Chambers, 1997). These series of fem and aux genes: *llm*, *fmt*, *pbpD*, *lytH*, *dlt operon*, *VraSR* and *sigB* were identified as factors which are independent of the *mec* locus and affect the methicillin resistance level. Most of these factors are considered to be associated with PG biosynthesis directly or indirectly (Schneider, 2004). However, precise functions of most of these genes are still not well understood.

1.5.1 FmtA (a factor which affects methicillin resistance and autolysis in presence of Triton X-100)

FmtA was first identified as a methicillin resistance factor by Komatsuzawa's research group in Japan in 1997. Inactivation of *fmtA* has been shown to affect the

sensitivity of MRSA strains result in decreased level of highly cross-linked PG in the presence of Triton X100 (Komatsuzawa et al., 1999). Transcriptomics studies of *fmtA* have reported to be involved in cell wall stress stimulon. Cell wall stress stimulon is a group of genes that is commonly induced when treated with cell wall active antibiotics, such as β–lactams. Transposon insertions (Tn551) in *fmtA* resulted in decreased biofilm formation and reduced attachment of wall teichoic acids to cell wall (Boles et al., 2010).

Dr.Golemi-Kotra's lab showed that FmtA is capable of biding with fluroscent labeled penicillin and can form stable acylenzyme species; however, the interaction is weak (Fan et al, 2007). Recently, this group reported that SarA, a global regulatory transcription factor is responsible for regulation of *fmtA*. SarA is also known to be involved in methicillin resistance and regulation of many virulence factors (Zhao et al., 2012). In another study by a former PhD graduate student, Dr.Aneela Qamar revealed that FmtA interacts with WTA and localizes in cell division septum (Qamar and Golemi-kotra, 2012). However, the biological functions of FmtA remain to be elucidated.

1.6 Aims of Research

S. aureus is one of the major reasons for mortality and morbidity due to hospital- and community-acquired infections. Due to extensive use of antibiotics, *S. aureus* has acquired resistance to β-lactams. Methicillin, a β-lactam antibiotic was introduced to overcome the penicillin-resistance of *S. aureus*, but subsequently resulted in resistant strains soon after its introduction into therapeutics. Moreover, Methicillin-resistant *S. aureus* (MRSA) strains have become resistant to almost all the clinically used antibiotics and this is a serious threat to all over the world. Besides *mecA* gene, which encodes PBP2a, many other genes are recognized for phenotypic expression of methicillin resistance. FmtA is one of the methicillin resistance factors, connected to biofilm formation and cell wall synthesis. To date, the biological function of FmtA is not well understood. It has been shown *in-vitro* that FmtA has very low carboxypeptidase activity and can interact with WTA. Understanding of FmtA's functional role and mechanism towards methicillin resistance would provide insights into development of novel antibacterial molecules. Hence the aim of my thesis research work was to characterize the functions of FmtA by investigating potential role of WTA as an activator ligand for low carboxypeptidase activity of FmtA, which is described in chapter two. Moreover, potential protein-protein interaction activity of FmtA and cell wall associated protein such as AtlA are addressed in chapter 3.

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CHAPTER TWO

INTERACTION OF FMTA$^{\Delta 27}$ WITH WTA

2.1 Introduction

The peptidoglycan (PG) is composed of repeating units of the glycan chains made up of the alternating amino sugars N-acetylglucosamine and N-acetylmuramic acid. Stem pentapeptides (L-Ala-D-Glu-L-Lys-D-Ala-D-Ala) are attached to the carboxyl group of each N-acetylmuramic acid, and interpeptide bridges (pentaglycines, made up of glycine residues) connect the lysine component of one stem peptide to the penultimate D-Ala of a neighboring stem peptide where the glycan strands are cross-linked with short peptide bridges (Vollmer et al. 2008).

Gram-positive bacteria synthesize peptidoglycan at the interface of the membrane and the inner layer of the cell wall, where new peptidoglycan strands are assembled and cross-linked by PBPs (Silhavy et al., 2010). PBPs can exhibit transglycosylase, transpeptidase or DD-Carboxypeptidases activity (Sauvage et al., 2008). Both transpeptidase and carboxypeptidase activities of PBPs occur at the D-Ala-D-Ala terminus of a murein precursor. Antibiotics having β-lactam ring inactivate transpeptidases by reacting with the active site nucleophile of transpeptidases. *S. aureus* has four native PBPs and based on their molecular weight they are named as PBP1, PBP2, PBP3 and PBP4, which differ in their cell wall biosynthetic functions and affinity to β-lactam antibiotics. In addition, a non native PBP2a is observed in methicillin resistance *S. aureus* (MRSA). PBP1 and PBP3 are mono-functional transpeptidase whose function is

primarily related to cell division (Wada et al., 1998). PBP2 is the only bifunctional PBP in *S. aureus* shows transpeptidase as well as trasglycosylase activity and important for viability in *S. aureus* (Pinho et al., 2001). PBP4 is responsible for higly cross linked peptidoglycans (Leski et al., 2005). PBP2a in methicillin resistance strain shows transpeptidase activity and low affinity towards β-lactams (Pratt, 2008; Sauvage et al., 2008). Beside PBP2a, other factors named as fem (factor essential for methicillin resistance) and aux (auxilary) can affect methicillin resistance level in *S. aureus* (Komatsuzawa et al., 1999).

Teichoic acids are long anionic polymer composed of ribitol or glycerol phosphates appear to extend to the layers of the peptidoglycan. Teichoic acid that are covalently attached to peptidoglycan are known as the Wall Teichoic Acids (WTA) where as teichoic acids that are anchored to the head groups of membrane lipids are referred as Lipoteichoic Acids (LTA) (Neuhaus et al., 2003).

It is believed that attachment of WTA to peptidoglycan occur during septum formation of the cell division cycle (Formstone et al., 2008; Atilano et al., 2010). In gram-positive bacteria, WTAs are highly abundant in the cell walls (Matias and Beveridge, 2007). The structures of WTA vary widely between bacterial to bacteria and often even between species. Teichoic acid biosynthesis in *S. aureus* is discussed in chapter one. In *S. aureus*, WTA D-alanyl residues are attached to second position of ribitol contribute positive charge to WTA (Swoboda et al., 2010). The amount of D-Ala ester in ribitol phosphate varies a lot and depends on environmental conditions such as pH, salt concentrations and temperature (Paschel et al., 1999). On the other hand, WTA sugar

substituents are generally enclosed at the fourth position of ribitol and do not appear to alter with changes in the cellular environment (Brown et al., 2012). Both WTA and LTA show zwitterionic properties because of the presence of negatively charged phosphate groups and additional positively charged amino groups of D-Ala residues on the repeating units (Neuhaus and Baddiley, 2003).

The FmtA protein was first identified in a methicillin resistant *S. aureus* (MRSA) by using a transposon mutagenesis (Tn551 insertion of COL strain) screening technique by Komatsuzawa et al. It has been identified as a part of the core cell wall stimulon and is known to be involved in methicillin resistance of *S. aureus* (Komatsuzawa, et al. 1997; Komatsuzawa, et al. 1999). Inactivation of *fmtA* in MRSA resulted in enhanced autolysis, increased sensitivity to β-lactams and decreased level of highly cross-linked peptidoglycans. Expression of *fmtA* was found to be upregulated in the presence of cell wall inhibitors (Komatsuzawa, et al. 1997; McAleese F, et al. 2006, Utaida et al., 2003). It has been speculated that FmtA might be involved in peptidoglycan biosynthesis under antibiotic-induced cell wall stress conditions (Xin et al., 2007). Analysis of the primary structure of FmtA reveals that of carries two of the three conserved motifs of PBPs, SXXK and S(Y)XN and absence of the third conserved motif KTG. Dr. Golemi-Kotr's group showed that FmtA binds to β-lactams and forms a covalent species. However, this interaction is vey weak (Xin et al., 2007).

Recently, Dr. Golemi-Kotra and co-worker demonstrated that FmtA has very low DD carboxypeptidase activity on Nα,Nε-diacetyl-L-Lys-D-Ala-D-Ala peptide, which is a surrogate of peptidic moiety of PG precursor. FmtA interacts with WTA and localizes in

the cell division septum, most likely by binding to the cross wall. Furthermore, FmtA can alter the autolysis and biofilm formation which requires the presence of WTA and depends on the concentration of FmtA (Qamar and Golemi-kotra, 2012).

Although, it was proposed that FmtA could be a novel penicillin binding protein, its precise function was not demonstrated. Several hypotheses were raised for FmtA's low DD carboxypeptidase activity. It was proposed that FmtA may require a ligand to become active in the periplasmic space of *S. aureus*. Here in, we probed whether WTA could serve as an activator of FmtA activity.

2.2 MATERIALS AND METHODS

2.2.1 Materials and chemical

Growth media were purchased from EMD Bioscience. Enzymes (trypsine, DNase, RNase, horseradish peroxidase, D-amino acid oxidase) and chemicals (Tris, Cytochrome C, LTA) were purchased from Sigma (Oakville, Canada) and Thermo-Fisher (whitby, Canada); unless otherwise stated. Amplex Red (AR) was purchased from Molecular Probes, Inc.

2.2.2 Expressions and purifications of FmtA $^{\Delta 27}$ wild type and mutant proteins

Expressions of FmtA$^{\Delta 27}$ wild type and mutant proteins were performed as described by Xin Fan and Dr. Aneela Qamar (Fan, 2006; Qamar, 2011). Briefly, 1 ml of overnight seed culture of *E. coli* BL21 (DE3) containing pET24a (+):: *fmtA*$^{\Delta 27}$ or the mutant variants were used to inoculate 800 mL of terrific Broth medium supplemented with kanamycin (30 µg/mL). A glycerol stock of BL21(DE3), containing pET24a (+)::

fmtA$^{\Delta 27}$ or the mutant variants, was used to start a seed culture which was grown overnight at 37°C in 5 mL Luria Bertani medium supplemented with kanamycin (30 µg/mL). A 1 mL overnight seed culture was used to inoculate 800 mL of Terrific Broth medium supplemented with kanamycin (30 µg/mL), 0.4 M D-sorbitol; and 2.5 mM β-betaine. The inoculated medium was grown at 37°C, 180 rpm, until OD_{600nm} reached 0.6 to 0.8, at which time protein expression was induced by addition of Isopropyl β-D-thiogalactopyranoside at a final concentration of 1 mM. Expression of protein was carried out for 16 hours at 25°C, 120 rpm and the cells were harvested at 7000 x g for 20 min at 4°C. Resulting pellets were frozen at -80°C if subsequent purification steps were not carried out on same day.

Purifications of FmtA$^{\Delta 27}$ wild type and mutant proteins were performed as described by Xin Fan and Dr. Aneela Qamar with few modifications (Fan, 2006; Qamar, 2011). All purification steps were carried out at 4°C. Harvested cells were resuspended in 50 mM sodium phosphate buffer, pH 7.2. Sonication (5 cycles of 1 min; 30% power, 60% pulse) was used to liberate the protein and centrifugation at 21000 x g for 60 min was carried out to remove cell debris. The resulting supernatant was applied to a Sp-Sepharose column (80 mL column volumes) pre-equilibrated with sodium phosphate buffer, pH 7.2. The target protein (FmtA$^{\Delta 27}$ or mutants) was eluted at 60-70% of a liniear gradient of 0 to 1 M sodium chloride in 50 mM sodium phosphate buffer, pH 7.2. The fractions containing the target protein were combined and concentrated to 4 mL and loaded to a gel filtration column (HiPrep 26/60 Sephacryl, 320 ml) washed and pre-equilibrated with sodium phosphate buffer, pH 7.2. Protein fractions containing pure protein were

concentrated using centrifugal filter Ultracel-3K (Amicon, Millipore). The purified protein concentration was determined BSA method.

2.2.3 Construction of *fmtA* conditional mutant and *fmtA* Deletion strains

To investigate the $FmtA^{\Delta 27}$ biological function, two mutant strains (*fmtA* conditional mutant and *fmtA* deletion strain) were constructed by Dr. Vidhu Verma.

2.2.4 Isolations of wall teichoic acid

Wall teichoic acid was isolated according to Former graduate student Michale Fridman from Dr.Golemi-Kotra's Lab (Fridman, 2013). Briefly, *S. aureus* (RN4220, FmtA Del, FmtA CM2, DltA) Cultures were incubated at 37°C in an orbital incubator (180 rpm) and harvested by centrifugation (5300 x g for 15 min, RT) once the OD_{660} had reached 0.6–0.7. Cells were suspended in 10 mL of 2 M NaCl and disrupted with sonicator((1 sec ON, 9 sec OFF, 2 min total, 35% amplitude) at RT. Cell wall fragments were recovered by centrifugation (14100 x g; 5 min at 4°C), the pellet suspended in phosphate-buffered saline (PBS) (prepared in the Lab) and recovered by centrifugation (14100 x g; 5 min at 4°C). Recovered wall materials were incubated with 20 mL of 4% w/v sodium dodecyl sulphate at 37°C for 4 h. Cell walls were recovered by centrifugation (14100 x g; 5 min at 25°C), washed three times by resuspending in water (10 mL each time) to remove non-covalently bound components by 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 $MgCl_2$ for 18 h with stirring. After recovery by centrifugation(14100 x g; 5 min at 4°C), wall material was washed with 1 M Tris–HCl

(pH 7.0) containing 1 M NaCl and then again with 1 M Tris–HCl (pH 7.0), recovered by centrifugation (14100 x g; 5 min at 4°C) and finally washed three times by resuspending in water (5 mL each time) (14100 x g; 5 min at 4°C) . WTA was released from peptidoglycan using 10% w/v aqueous trichloroacetic acid (TCA) at 4°C for 48 h. Peptidoglycan was removed by centrifugation (5300 x g; 45 min; 20°C) and WTA precipitated from the supernatant with 95% ice-cold ethanol and held 5 days at 4°C. After centrifugation (5000 x g; 30 min; RT), the pellet was washed two times with 95% 5 mL ethanol (5000 x g; 30 min; RT); after the final ethanol wash, the solvent was allowed to evaporate, purified WTA was lyophilized and stored at -20°C.

2.2.5 DD-Carboxypeptidase assay

This was carried out as described previously (Gutheil, Stefanova, and Nicholas et al., 2000) and modified by Dr. Aneela Qamar (Qamar, 2011). Briefly, Purified $FmtA^{\Delta 27}$ (10 µM) was incubated in 0.1 M Tris (pH 8.5) buffer with 6 mM Nα,Nε-Diacetyl-Lys-D-Ala-D-Ala for 60 min at 37°C in a total volume of 100 µL . In another reaction, different concentrations of WTA (10 µg/100 µL , 20 µg/100 µL, 30 µg/100 µL) and purified $FmtA^{\Delta 27}$ (10 µM) was incubated in 0.1 M Tris (pH 8.5) buffer (100 µL). At this time, 40 µM of Amplex Red (Molecular Probes, Inc) (in DMSO) and 2.5 µg/ml of flavin adenine dinucleotide (FAD), 0.75 units of horseradish peroxidase (HRP), and 0.05 units of D-amino acid oxidase (DAO) in a total volume of 200 µl were added to each sample (100 µL) and samples were incubated for another 30 min. D-Ala released from the tripeptide or WTA was determined by fluorescence spectrophotometer at excitation 563 nm and emissions 584 nm.

2.2.6 Nuclear Magnetic Resonance Spectroscopy (NMR) Experiments

WTA was isolated from various strains (RN4220, Δ*fmtA*, *fmtA* CM2) using trichloroacetic acid as described earlier. LTA (*S. aureus*) was purchased from Sigma. NMR experiments were performed with the help of Dr. Hunter (Manager NMR facilities, York University). The isolated WTAs were lyophilized and resuspended in 100% D_2O to a 5−10 mg/mL concentration and placed in a 3 mm NMR tube. LTAs were resuspended in 100% D_2O to a 5 mg/mL concentration and placed in a 3 mm NMR tube. One-dimensional ^1H NMR spectra were collected at 25 °C on a Bruker AV III 700 MHz spectrometer (operating frequencies of 700.28 MHz for 1H NMR and 176.096 MHz for 13C NMR). The spectrometer was controlled with TOSPIN version 3.2 software and equipped with a 5 mm 1H/13C/15N cryoprobe. For quantitative 1D 1H spectra, 1D 1H T1 analysis was completed using the inversion recovery experiment.

2.2.7 Enzymatic activity of FmtA $^{\Delta 27}$ by NMR

The isolated lyophilized WTA or LTA (Sigma) was dissolved in 100% D2O to a 0.25–10 mg/mL concentration and mixed with $FmtA^{\Delta 27}$ (10 µM, unless or otherwise mentioned) placed in a 3 mm NMR tube. LTA was resuspended in 100% D2O to a 5 mg/mL concentration and mixed with $FmtA^{\Delta 27}$ (10 µM) placed in a 3 mm NMR tube. Enzymatic reaction (formation of D-Ala from wall teichoic acid) was observed at different time intervals (25°C).

To check the activity on $FmtA^{\Delta 27}$ in presence of common esterase inhibitors, e.g. Sodium fluoride, Eserine, PMSF (1 mM) was added in the enzymatic reaction mixture. Briefly, $FmtA^{\Delta 27}$ at 10 µM was incubated with WTA at 5 mg/mL and the inhibitor (1 mM)

was added and the enzymatic reaction was checked over time at (25°C). In some cases, FmtA $^{\Delta 27}$ at 10 µM was pre-incubated with one of PMSF (1mM) for 24 hrs and the WTA at 5 mg/mL was added and the activity was monitored.

2.2.8 Investigation of the substrate specificity of FmtA$^{\Delta 27}$ by NMR

Esterase substrates, e.g p-nitro phenyl butyrate (p-NPB), p-nitro phenyl acetate (p-NPA), D-Ala methyl ester (1 mM) in 300 µL of sodium phosphate buffer, pH 7.0 was incubated with FmtA $^{\Delta 27}$ (10 µM) along with 300 µL of deuterium oxide. The reaction was monitored by NMR at different time intervals at 25°C for appearance of peaks around 8.15 ppm with disappearance of peaks around 8.35 ppm for p-NPA and p-NPB and appearance of peaks around 1.46 pp with disappearance of peaks at 1.53 ppm for D-Ala methyl ester.

2.2.9 Enzymatic activity of FmtA $^{\Delta 27}$ by continuous spectrophotometric assay

Esterase activity of FmtA was investigated by a continuous spectrophotometric assay using p-Nitrophenyl butyrate (p-NPB) or p-nitrophenyl acetate (p-NPA) as the substrate. DMSO was used to dissolve these substrates. The standard assay was measured in a final volume of 0.5 mL containing p-NPB or p-NPA (1mM), the enzyme FmtA$^{\Delta 27}$ WT or PBP4 or PBP2a (5 µM), and the assay buffer (50 mM sodium phosphate buffer, pH 7.0) at room temperature. The reaction was initiated by the addition of substrate. The hydrolysis of p-NPB or p-NPA was spectrophotometrically monitored for the formation of the p-nitrophenol at 405 nm ($\varepsilon = 0.2\ mM^{-1}\ cm^{-1}$).

2.2.10 Determination of whole-cell surface charge

To estimate the relative surface charge of cell envelope, cationic protein cytochrome C (Sigma) was used according to previously described method (Peschel et al., 1999). Briefly, cells were grown for overnight in 5 ml Tryptic Soy Broth (TSB) at 37°C 200 rpm. Overnight culture was diluted to 200 fold in 50 mL TSB (in a 250 mL flask) and further inoculated for 3 hours at 37°C 200 rpm. Cell densities were measured at Absorbance 650 nm, and an amount equivalent to 15 absorbance units (e.g., 15 ml of a culture with an A650 of 1.0) were pelleted by centrifugation (5000 x g for 5 min at 4°C) and washed twice by resuspending the pellet with 20 mM morpholinepropanesulfonic acid (MOPS), pH 7 (5000 x g for 5 min at 4°C). Washed cells were resuspend in 0.5 mg/ml cytochrome c and incubated for 10 min at room temperature. The mixture was centrifuged twice (5000 x g for 5 min at 4°C), and the amount of cytochrome C in the supernatant fraction was determined at A530 using standard Cyt C as a reference. Cyt C protein concentration in the supernatant was calculated as, % of applied protein = (A530 of sample / A530 of Std Cyt C) x 100.

2.2.11 Phosphate assay

The WTA phosphate content was determined by using QuantiChrom™ Phosphate Assay kit (BioAssay systems, USA). In a typical reaction, 50 µL of 30 µg /mL to 50 µg /mL WTA sample was transferred in a clean dry glass tube. WTA samples were dried at 150°C (Dr. Yousaf lab, LSB, 4th Floor) for 10 min. Samples were hydrolyzed for inorganic phosphate release by addition of 40 µL $HClO_4$ (70-72%) and incubated for 2 hr at 150°C. 500 µL distilled water was added into the incubated samples and analyzed by

QuantiChrom™ Phosphate Kit's instruction. Briefly, 50 µL blank (distilled water from kit), 50 µL standard (30 µM Pi) and 50 µL samples were transferred into clear flat bottom 96 well plate. 100 µL developing solution from the Kit was added into each sample, mixed gently and incubated for 30 min at room temperature. Change in color was determined by using the Synergy H4 Hybrid plate reader (BioTek, USA) at optical density at 620 nm. All the experiments were done in triplicates.

2.3 Results

2.3.1 Activity of FmtA$^{\Delta 27}$ WT against PG analogue (L-Lys-D-Ala-D-Ala) and WTA by DD-Carboxypeptidase assays

Previously, Dr.Golemi-Kotra's research group showed that FmtA$^{\Delta 27}$ has very weak D,D-carboxypeptidase activity. It was hypothesized that FmtA$^{\Delta 27}$ may require interaction with a ligand in the periplasmic space to become active (Qamar and Golemi-Kotra, 2012). In lieu of earlier findings that FmtA$^{\Delta 27}$ interacts with WTA, we raised the question whether this interaction has any effect on the activity of FmtA$^{\Delta 27}$ as a D,D-Carboxypeptidase. For this purpose we used the D,D-carboxypeptidase assay (based on Fluorescent coupled enzyme assays for D-Ala) to monitor the amount of D-Ala released from the tripeptide Nα,Nε-Diacetyl-Lys-D-Ala-D-Ala by FmtA$^{\Delta 27}$ in the presence and absence of WTA. Incubation of the tripeptide with FmtA $^{\Delta 27}$ resulted in very low amount of D-Ala release, which was comparable to the background levels. However, in the presence of WTA (0.1 µg/µL, 0.2 µg/µL, 0.3 µg/µL) the amount of D-Ala release increased 3 to 4 fold (Figure 2.3.1.1). We used PBP4 (1µM) and PBP2a (10 µM) as a

control, in these cases no significant free D-Ala formation was observed in the presence of WTA and substrate Nα,Nε-Diacetyl-Lys-D-Ala-D-Ala (6 mM) (Figure 2.3.1.3 and Figure 2.3.1.5). WTA contain D-Ala on its second position of ribitol phosphate polymer. To exclude the possible source of D-Ala from WTA, we performed another experiment using WTA isolated from *dltA* mutant *S. aureus* which lacks D-Ala in WTA. In this case no significant release of D-Ala was observed in the presence of WTA. In light of these results, we suspect that FmtA$^{\Delta 27}$ removes the D-Ala from WTA and that was the reason behind the observed significant increase in D-Ala in the reaction. To confirm this, we repeated the experiment without the tripeptide Nα,Nε-Diacetyl-Lys-D-Ala-D-Ala and observed the same trend for the increased signal in comparison to the experiment that was done in the presence of Nα,Nε-Diacetyl-Lys-D-Ala-D-Ala and WTA . Furthermore, we repeated this experiment with boiled FmtA $^{\Delta 27}$ protein and no significant amount of D-Ala was detected. These results strongly suggest that FmtA$^{\Delta 27}$ has a catalytic activity to release D-Ala from WTA, which is connected through ester bond with ribitol. Furthermore, FmtA $^{\Delta 27}$ S127A (mutation of serine at 127 poition of *fmtA*) showed less catalytic activity towards WTA which indicates the potential involvement of serine residue (Figure 2.3.1.6).

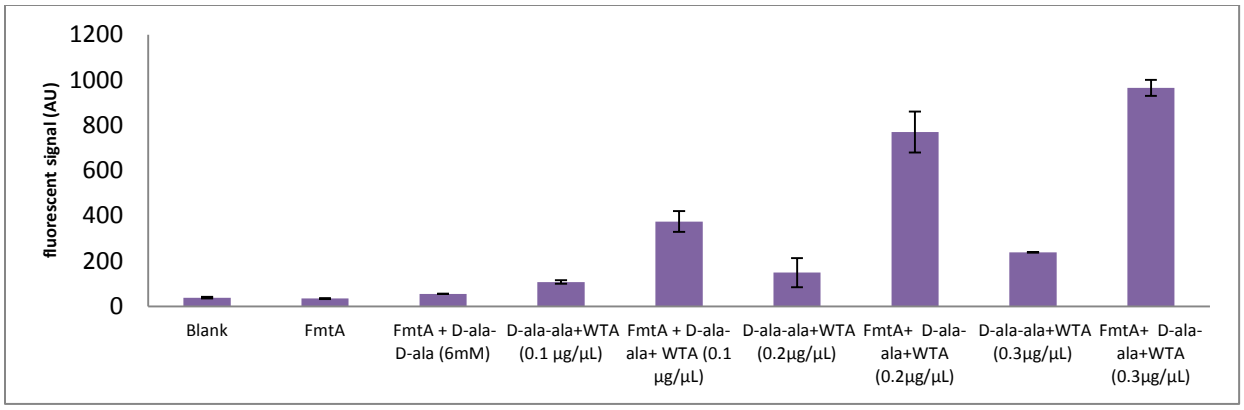

Figure 2.3.1.1 Activity of FmtA$^{\Delta 27}$ against PG analogue (L-Lys-D-Ala-D-Ala) by DD-Carboxypeptidase assay in presence of WTA as an activator

A reaction mixture containing FmtA $^{\Delta 27}$ (10 µM),Nα,Nε-Diacetyl-Lys-D-Ala-D-Ala (6 mM), and various concentrations of WTA (0.1 µg/µL, 0.2 µg/µL, 0.3 µg/µL) isolated from RN4220 was incubated at 37° C for 1 hr. Conversion of amplex red to resorufin which depend the presence of free D-Ala in the reaction mixture was detected by fluorescence signal at 584 nm. Reaction mixture without FmtA $^{\Delta 27}$ or substrate was used as a blank. Reactions with FmtA $^{\Delta 27}$ alone or WTA from RN4220 with Lys-D-Ala-D-Ala were used as controls.

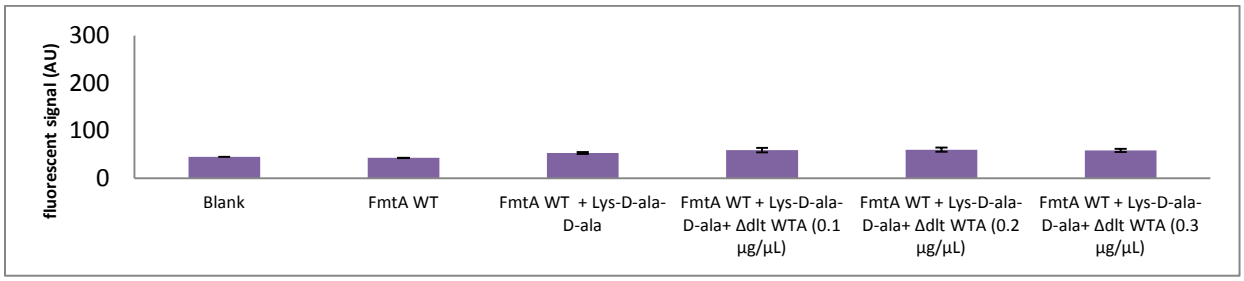

Figure 2.3.1.2 Investigation of the potential role of WTA as an activating ligand of FmtA $^{\Delta 27}$ activity (negative control)

A reaction mixture containing FmtA$^{\Delta 27}$ (10 µM), Nα,Nε-Diacetyl-Lys-D-Ala-D-Ala (6 mM), and various concentrations of WTA (0.1µg/µL, 0.2µg/µL, 0.3µg/µL) isolated from *ΔdltA* strain (no D-Ala in WTA) was incubated at 37° C for 1 hr. Conversion of amplex red to resorufin which depend on the presence of free D-Ala in the reaction mixture was detected by fluorescence signal 584 nm. Reaction mixture without FmtA$^{\Delta 27}$ or substrate was used as a blank. Reactions with FmtA $^{\Delta 27}$alone or WTA from *ΔdltA* strain were used as negative controls.

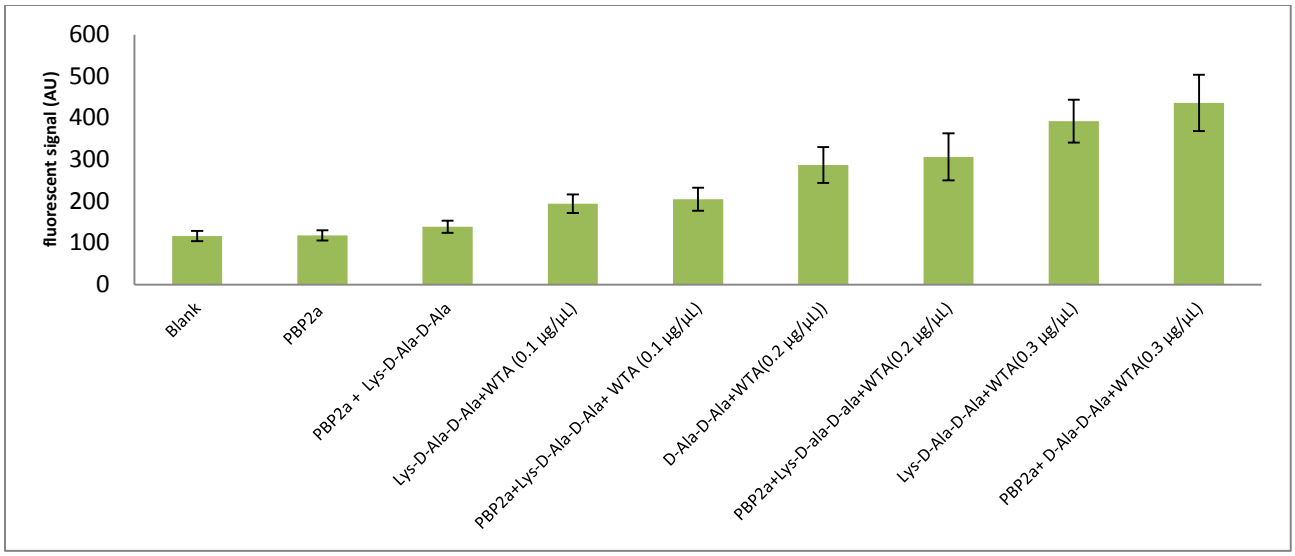

Figure 2.3.1.3 Investigation of the potential role of WTA as an activating ligand on PBP2a activity (Control for FmtA$^{\Delta 27}$)

A reaction mixture containing PBP2a (10 µM), Nα,Nε-Diacetyl-Lys-D-Ala-D-Ala (6 mM), and various concentrations of WTA (0.1µg/µL, 0.2µg/µL, 0.3µg/µL) isolated from RN4220 was incubated at 37° C for 1 hr. Conversion of amplex red to resorufin which depend the presence of free D-Ala in the reaction mixture was detected by fluorescence signal at 584 nm. Reaction mixture without PBP2a or substrate was used as a blank. Reactions with PBP2a alone or WTA from RN4220 with Lys-D-Ala-D-Ala were used as negative controls.

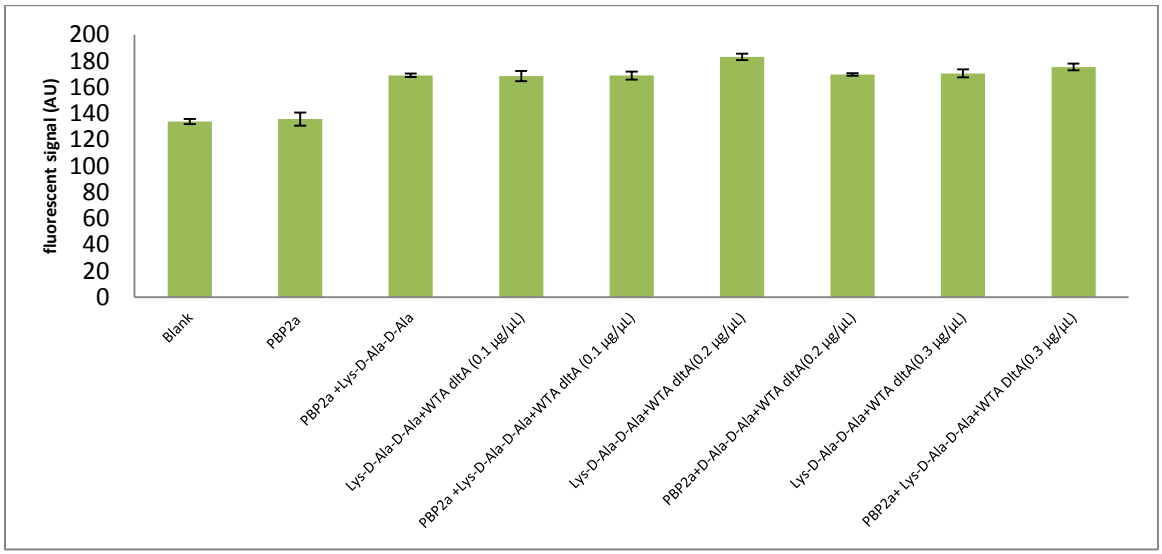

Figure 2.3.1.4 Investigation of the potential role of WTA as an activating ligand on PBP2a activity (negative control)

A reaction mixture containing PBP2a (10 µM), Nα,Nε-Diacetyl-Lys-D-Ala-D-Ala (6 mM), and various concentrations of WTA (0.1µg/µL, 0.2µg/µL, 0.3µg/µL) isolated from *ΔdltA* strain (no D-Ala in WTA) was incubated at 37° C for 1 hr. Conversion of amplex red to resorufin which depend on the presence of free D-Ala in the reaction mixture was detected by fluorescence signal 584 nm. Reaction mixture without PBP2a or substrate was used as a blank. Reactions with PBP2a alone or WTA from *ΔdltA* strain were used as negative controls.

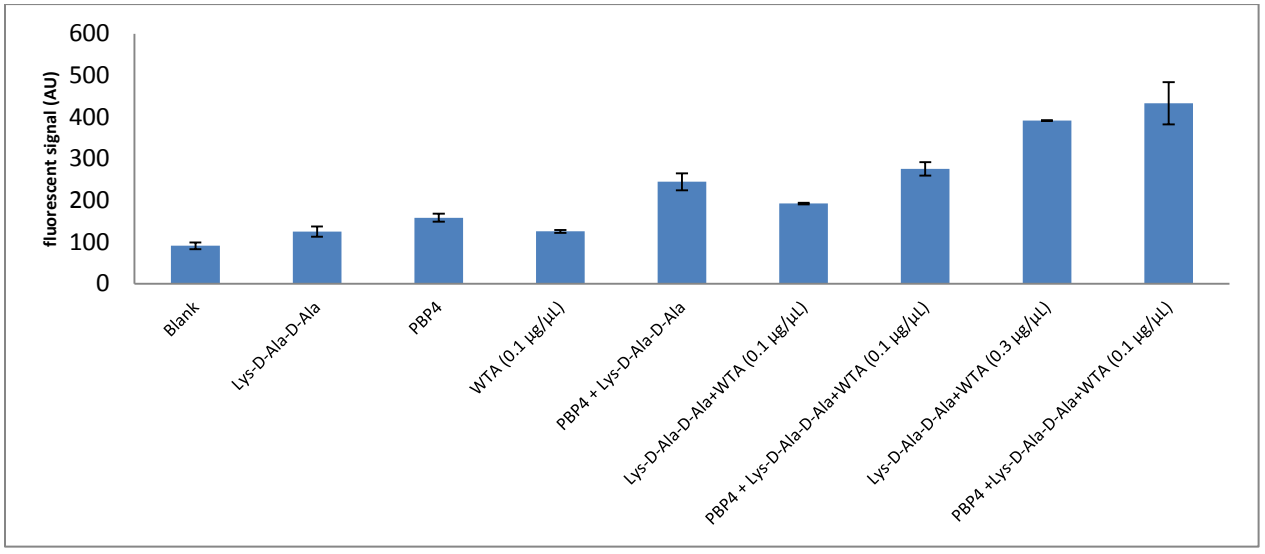

Figure 2.3.1.5 Investigation of the potential role of WTA as an activating ligand on PBP4 activity

A reaction mixture containing PBP4 (1 µM), Nα,Nε-Diacetyl-Lys-D-Ala-D-Ala (6 mM), and various concentrations of WTA (0.1µg/µL, 0.2µg/µL, 0.3µg/µL) isolated from RN4220 was incubated at 37° C for 1 hr. Conversion of amplex red to resorufin which depend the presence of free D-Ala in the reaction mixture was detected by fluorescence signal at 584 nm. Reaction mixture without PBP4 or substrate was used as a blank. Reactions with PBP4 alone or WTA from RN4220 with Lys-D-Ala-D-Ala were used as controls.

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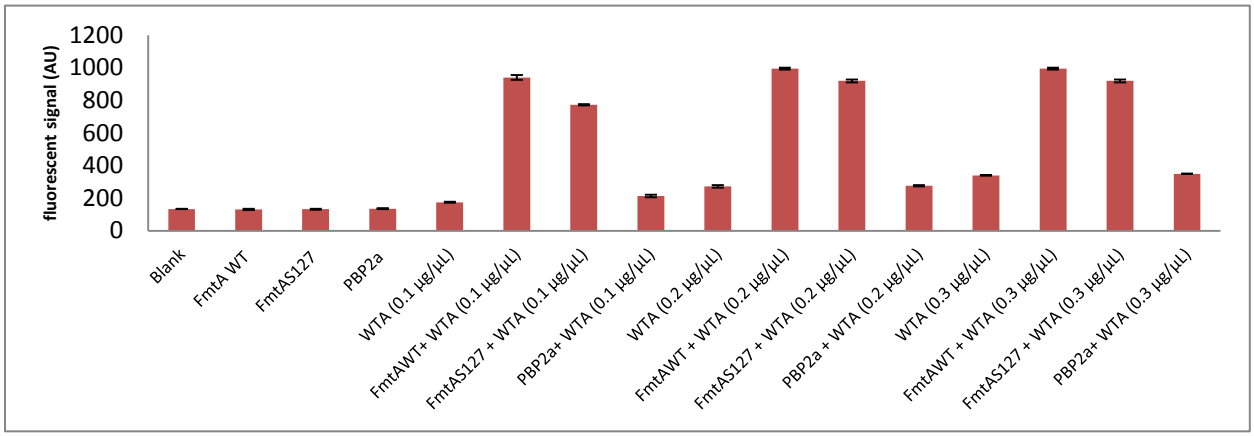


Figure 2.3.1.6 Investigation of the potential esterase activity of FmtA $^{\Delta 27}$ on WTA
A reaction mixture containing FmtA$^{\Delta 27}$ WT or FmtA$^{\Delta 27}$ S127 or PBP2a (10 µM) and various concentrations of WTA (0.1µg/µL, 0.2µg/µL, 0.3µg/µL) isolated from RN4220 was incubated at 37° C for 1 hr. Conversion of amplex red to resorufin which depend the presence of free D-Ala in the reaction mixture was detected by fluorescence signal at 584 nm. Reaction mixture without protein and substrate was used as a blank. Reaction with FmtA$^{\Delta 27}$, FmtA$^{\Delta 27}$S127, PBP2a alone or WTA from RN4220 was used as a control.

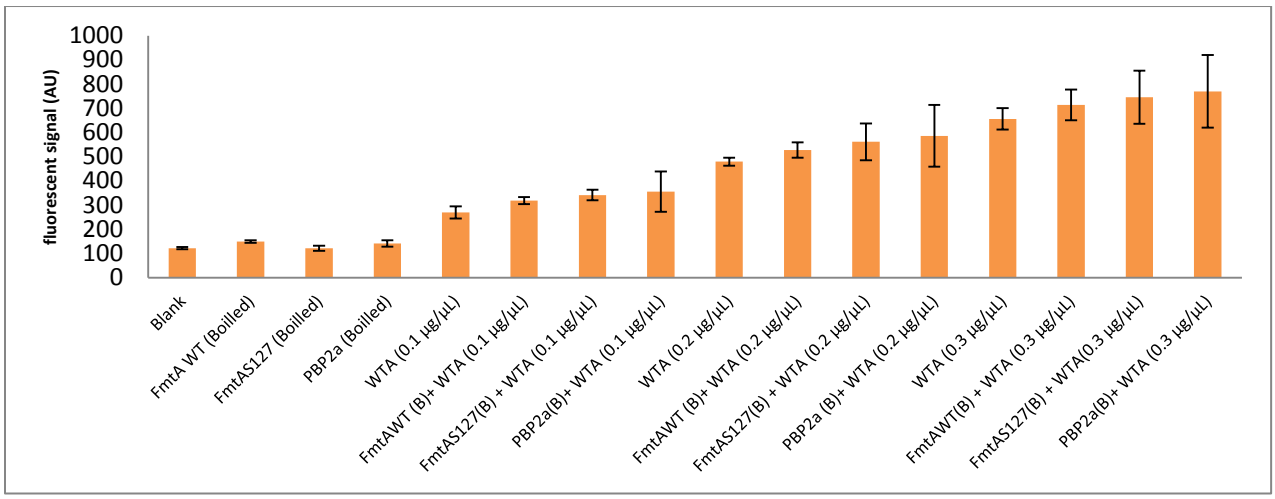


Figure 2.3.1.7 Investigation of the potential esterase activity of FmtA $^{\Delta 27}$ on WTA (control experiments)
A reaction mixture containing boiled FmtA$^{\Delta 27}$ WT or FmtA$^{\Delta 27}$ S127 or PBP2a (10 µM) and various concentrations of WTA (0.1µg/µL, 0.2µg/µL, 0.3µg/µL) isolated from RN4220 was incubated at 37° C for 1 hr. Conversion of amplex red to resorufin which depend the presence of free D-Ala in the reaction mixture was detected by fluorescence

signal at 584 nm. Reaction mixture without protein and substrate was used as a blank. Reaction with FmtA$^{\Delta 27}$, FmtA$^{\Delta 27}$S127, PBP2a alone or WTA from RN4220 was used as a control. (B): Boilled

2.3.2 Investigation of the esterase activity of FmtA$^{\Delta 27}$ on WTA by NMR

We observed that in the presence of WTA, the amount of free D-Ala increased in the reaction mixtures. To probe whether WTA solutions with FmtA $^{\Delta 27}$ contained free D-Ala, we have analyzed wall teichoic acid and furthermore monitored the changes in WTA in presence of FmtA $^{\Delta 27}$ by ^{1}H NMR. Results showed that indeed FmtA$^{\Delta 27}$ can remove D-Ala from wall teichoic acid. The cleavage of D-Ala ester from WTA was observed by the disappearance of the resonance at 1.65 ppm and the appearance of the free D-Ala resonance at 1.45 ppm. In another experiment, we included a control, which was only wall teichoic acid in deuterium oxide. In absence of FmtA$^{\Delta 27}$ there was no removal of D-Ala from WTA (Figure 2.3.2.1).

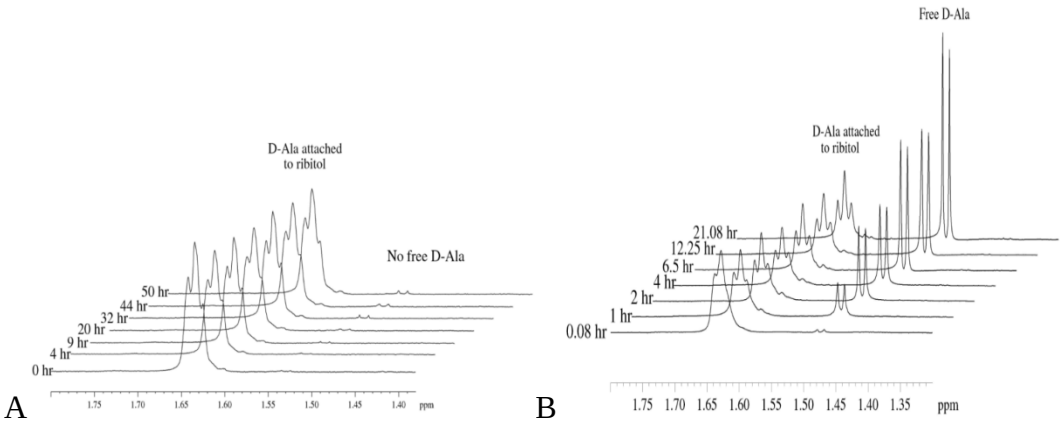


Figure 2.3.2.1 Removal of D-Ala of WTA by FmtA
^{1}H-NMR spectrum of WTAs (RN4220) in the absence or presence of FmtA$^{\Delta 27}$ (10 µM). A: WTA (5 mg/ml) in deuterium oxide (control); B: Reaction mixture containing FmtA$^{\Delta 27}$ (10 µM) and WTA (5 mg/ml) in deuterium oxide. Formation of free D-Ala was monitored as appearance of a resonance around 1.45 ppm.

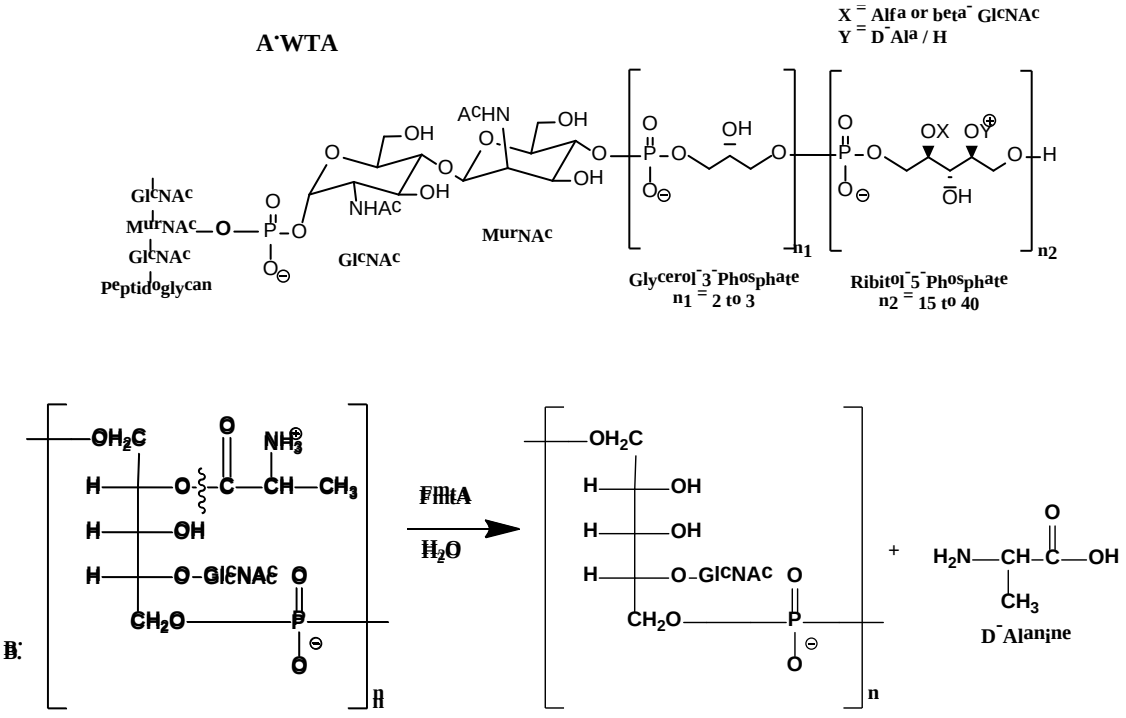


Figure 2.3.2.2 Catalytic function of FmtA$^{\Delta 27}$ on WTA.
A: Chemical structure of WTA B: In presence of water, FmtA removes D-Ala from WTA and produces free D-Ala.

2.3.3 Investigating the specificity of D-esterase activity of FmtA$^{\Delta 27}$ by NMR

Our NMR experiments confirmed that FmtA$^{\Delta 27}$ can remove D-Ala from WTA. Furthermore, I checked the effects of various enzyme and substrate concentrations on removal of D-Ala from WTA. The increase in FmtA$^{\Delta 27}$ concentration in the reaction mixture resulted in the increased in the D-Ala removal. The increase in substrate concentrations had a double effect and showed reaction rate chages as substrate concentaion chages. Interestingly, no reaction was observed in the presence of high substrate conecntration of WTA (10 mg/mL). This effect may be possible due to substrate inhibition.

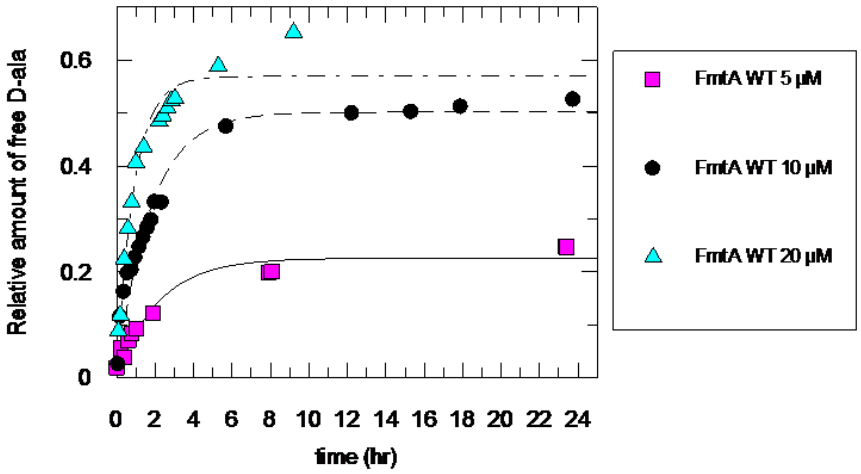


Figure 2.3.3.1 The effect of enzyme concentration in the rate of removals of D-Ala from WTA

FmtA $^{\Delta 27}$ WT enzymatic activity on WTA (RN4220) by ^{1}H NMR was monitored for reaction mixture containing different concentration of FmtA $^{\Delta 27}$ WT (5 µM, 10 µM, 20 µM) and WTA (5 mg/mL) isolated from RN4220. Release of free D-Ala was monitored as appearance of a resonance around 1.45 ppm over time.

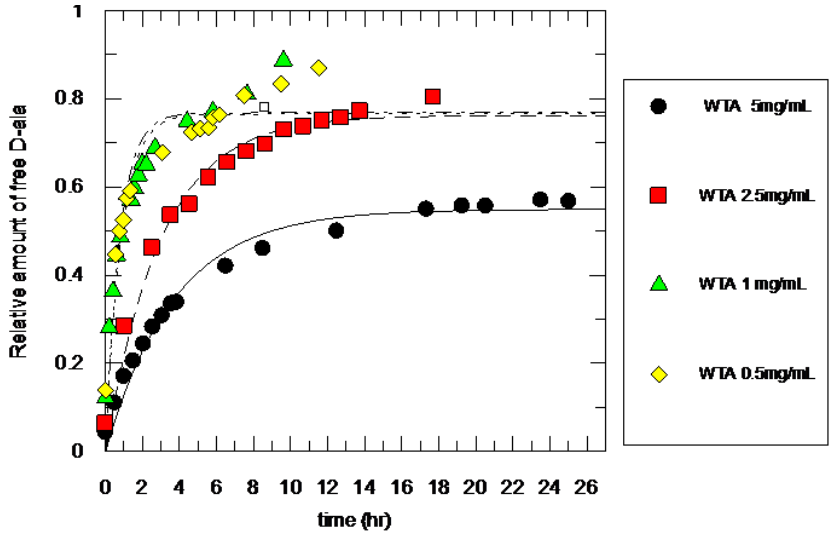
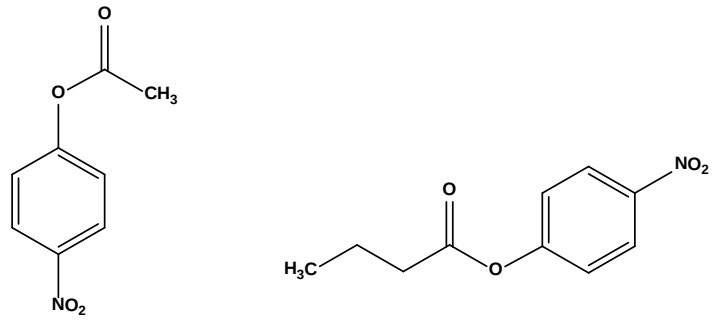


Figure 2.3.3.2 The effect of WTA concentrations in the rate of removals of D-Ala from WTA

FmtA$^{\Delta 27}$ WT enzymatic activity on WTA (RN4220) by ^{1}H NMR was monitored for reaction mixture containing FmtA$^{\Delta 27}$ WT (10 µM) and different concentration of WTA (0.5 mg/mL , 1 mg/mL, 2.5 mg/mL, 5mg/mL) isolated from RN4220. Release of free D-Ala was monitored as appearance of a resonance around 1.45 ppm over time.

2.3.4 Screening for esterase activity of FmtA$^{\Delta 27}$ by continuous spectrophotometric assay using small molecules

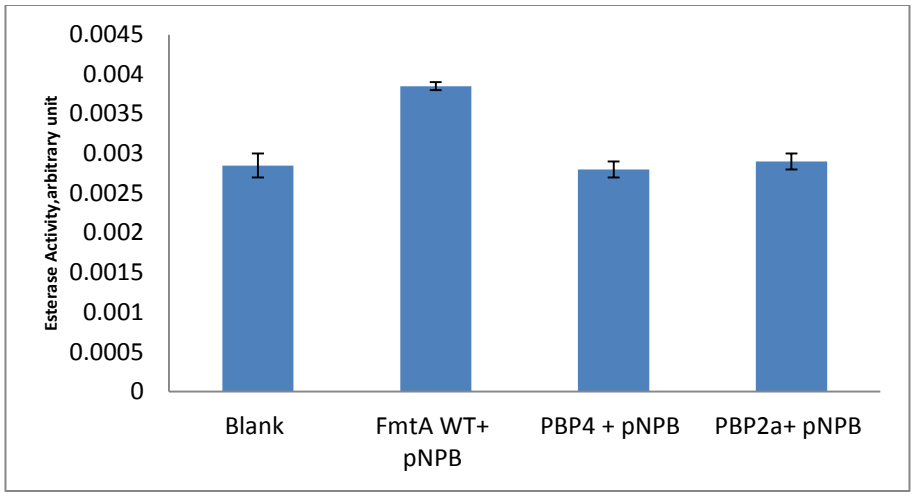
To check the substrate specificity for FmtA$^{\Delta 27}$, we tried several carboxylesterase substrates, e.g p-nitrophenyl butyrate (p-NPB) and p-nitrophenyl acetate (p-NPA). FmtA$^{\Delta 27}$ showed very poor esterase activity of p-nitrophenyl butyrate and p-nitrophenyl acetate by continuous spectrophotometric assay. Two control proteins (PBP4 and PBP2a) were included to compare esterase activity of FmtA$^{\Delta 27}$. The hydrolysis of pNPB or pNA was spectrophotometrically monitored for the formation of the p-nitrophenol at 405 nm. Due to high background hydrolysis of carboxylesterase substrates, we couldn't conclude any esterase activity of FmtA$^{\Delta 27}$ for pNPB or pNA. Therefore, we moved to NMR to investigate enzymatic esterase hydrolysis of pNPB or pNA by FmtA$^{\Delta 27}$.



p-Nitrophenyl acetate p-Nitrophenyl butyrate

Figure 2.3.4.1 Chemical structure of p-Nitrophenyl acetate and p-Nitrophenyl butyrate

A



B

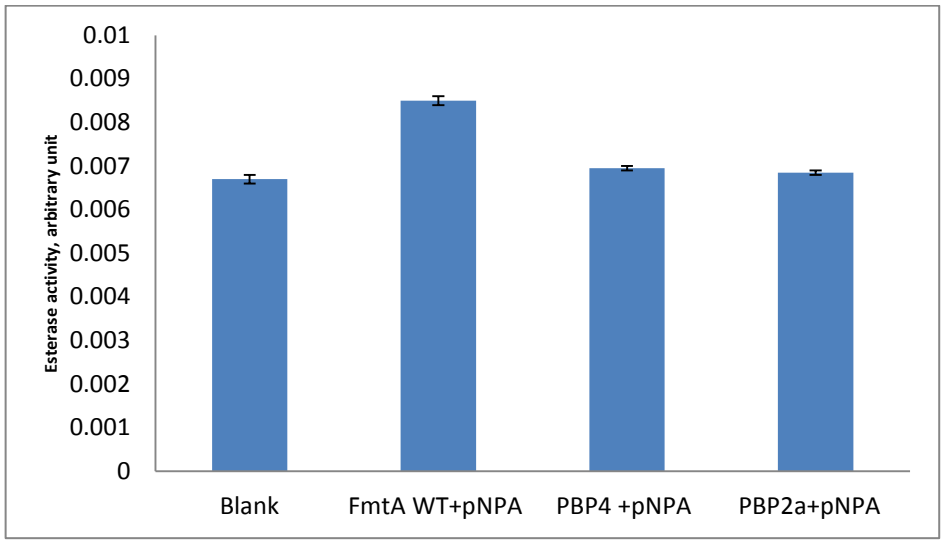


Figure 2.3.4.2 Esterase activity of FmtA$^{\Delta 27}$ against p-NPA and p-NPB by continuous spectrophotometric assay

p-NPB or p-NPA at 1mM was mixed with FmtA$^{\Delta 27}$, PBP4 or PBP2a at 5 µM in buffer (50 mM sodium phosphate buffer, pH 7.0) at room temperature. The hydrolysis of pNPB or pNA was spectrophotometrically monitored at 405 nm, which correspond to the formation of p-nitrophenol.

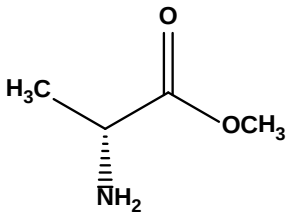
A: continuous spectrophotometric assay for p-NPB; B: continuous spectrophotometric assay for p-NPA

2.3.5 Assessment of esterase activity of FmtA$^{\Delta 27}$ by NMR

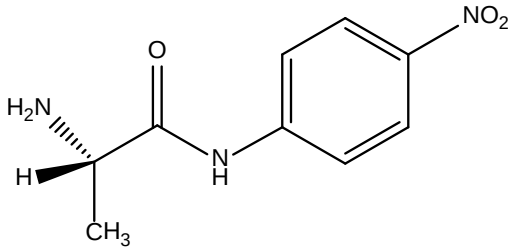
Due to high background hydrolysis of carboxylesterase substrates, we explored the catalytic activity of FmtA$^{\Delta 27}$ on pNPB and pNPA by NMR. Hydrolysis of these substrates was monitored as disappearance of a resonance around 8.35 ppm with appearance of new peak round 8.15 ppm which was observed due to the ortho protons on the benzene ring of the ester. Hydrolysis results from NMR revealed very poor activity of p-nitrophenyl butyrate and almost no activity on p-nitrophenyl acetate. Furthermore, we tried D-Alanine methyl ester and L-Alanine p-Nitroanilide for α-amino-acid esterase and alanine aminopeptidase activity, respectively. However, we couldn't see any catalytic activity against these substrates. Carboxylesterases catalyze the hydrolysis of short chain aliphatic and aromatic carboxylic ester compounds and all the carboxylesterase in family VIII are active towards nitrophenyl esters or triglycerides with short chain acyl moieties (Chahiniana et al., 2009). These results suggest that FmtA$^{\Delta 27}$ may be specific to D-Ala ester connected with ribitol polymer of WTA compare to synthetic substrate. Moreover, FmtA$^{\Delta 27}$ doesn't have any carboxylesterase or α-amino-acid esterase or aminopeptidase activity (Figure 2.3.5.2).

Most of the cephalosporins contain an ester bond (acetyl group) which might be susceptible to FmtA$^{\Delta 27}$ hydrolysis. Considering cephalosporin as a potential substrate for FmtA$^{\Delta 27}$, we explored esterolytic activity on cephalosporin derivatives by NMR. To study any potential esterase hydrolytic activity of cephalosporin, we used cephalothin (first generation cephalosporin) and cefoxitin (second generation cephalosporin) as a substrate

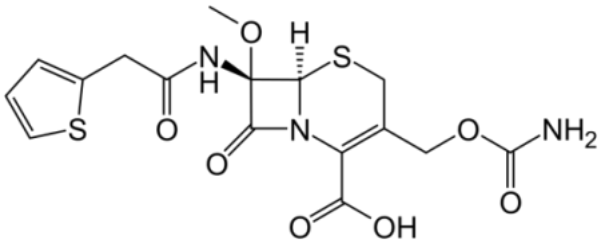
(Figure 2.3.5.1). However, we couldn't see any esterase activity on cefoxitin and cephalothin (Figure 2.3.6.3).



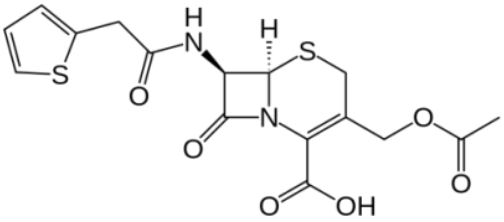
D-Alanine methyl ester



L-Alanine p-Nitroanilide



Cefoxitin



Cephalothin

Figure 2.3.5.1 Chemical structures of D-Alanine methyl ester, L-Alanine p-Nitroanilide, Ceforxitin and Cephalothin.

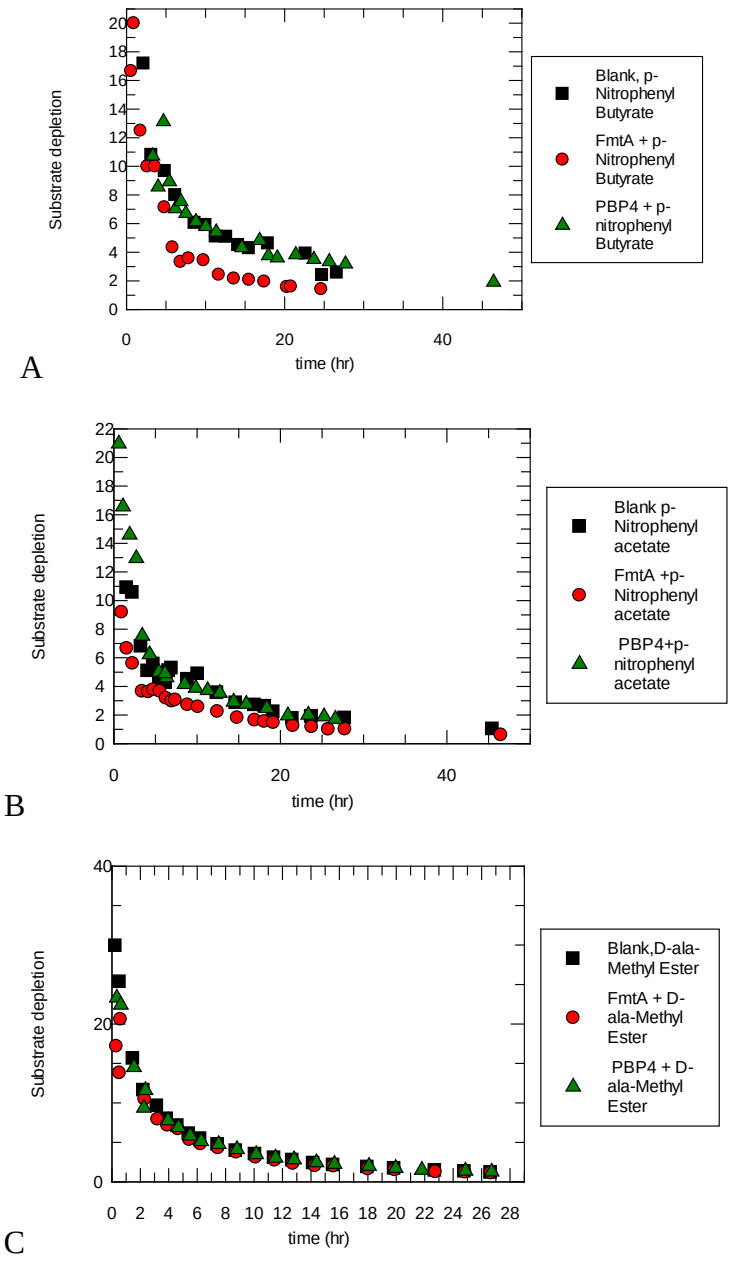


Figure 2.3.5.2 Assessment of esterase activity of FmtA$^{\Delta 27}$ by NMR
Substrate depletion around 8.35 ppm (ortho protons on the benzene ring) for reaction mixture containing FmtA$^{\Delta 27}$ (5 µM) or PBP4 (5 µM) with p-Nitrophenyl Butyrate (A) or p-Nitrophenyl acetate (B) or Substrate depletion around 1.53 ppm for D-alanine methyl ester (C) (1 mM) was monitored by NMR. A Reaction mixture containing no protein was included as a negative control.

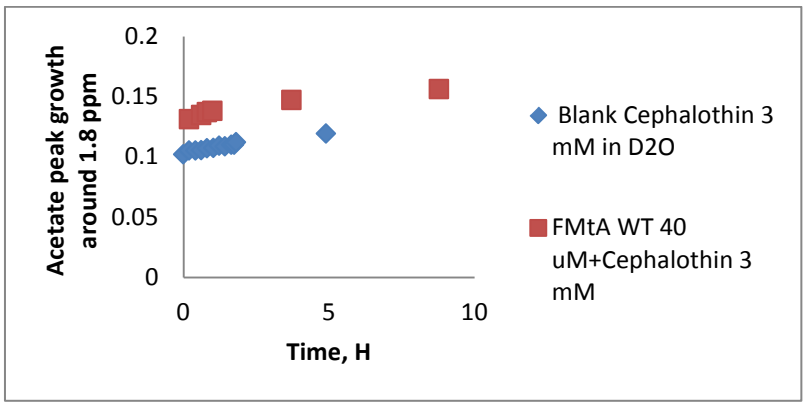


Figure 2.3.5.3 Screening for potential esterase activity of FmtA$^{\Delta 27}$ WT on cephalothin
FmtA $^{\Delta 27}$ WT enzymatic activity on cephalothin by ^{1}H NMR was monitored in a Reaction mixture containing FmtA $^{\Delta 27}$ WT (40 µM), cephalothin (3 mM). Any changes for acetate peak around 1.8 ppm were monitored.

2.3.6 Active site of FmtA$^{\Delta 27}$ WT for esterase activity (SXXK motif)

Esterases hydrolyze esters to produce acid and alcohol. Based on the literature, super family VIII esterases have very high sequence similarity to class C β- lactamase and penicillin binding proteins (Wagner et al., 2002). Family VIII esterases also represent a poorly characterized esterase family. The nucleophilic serine residue in family VIII carboxylesterases occurs in the S-X-X-K motif, like those in class β-lactamases, instead of in the G-X-S-X-G motif. These enzymes have approximately 337 to 443 amino acid sequence and a 36.9 to 48.6 kDa molecular mass (Nacke et al. 2011). FmtA$^{\Delta 27}$ has two S-X-X-K motifs, located at the position S63KLK and S127AQK. To explore the significance of these two motifs in activity, I monitored the activity of FmtA$^{\Delta 27}$ S63 and FmtA $^{\Delta 27}$ S127 mutants against WTA. A mutation of serine in the S^{63}KLK, with the other active site serine residue at 127 (S^{127}AQK) intact, was associated with a significantly

decrease in enzymatic activity (4 fold decrease in catalytic activity). Similar result was obtained with the mutation of serine in the S^{127}AQK motif. Mutation of lysine at the SXXK motif produced a 4 fold decrease in catalytic activity. These results indicate that serine at 63 and 127 positions and lysine at 130 position from SXXK motif are important for the catalytic activity of FmtA$^{\Delta 27}$.

Carboxylesterases and β-lactamases share a two-step hydrolase mechanism that consists of two successive nucleophilic attacks by a serine residue and a water molecule. Family VIII Carboxyesterase show a modular structure of a small helical domain and a α/β domain similar to that seen in class C β-lactamases (S.-S. Cha et al., 2013). The Crystal structure of EstB from *Burkolderia gladioli*, a representative of Family VIII carboxylesterases revealed that the mechanism of ester hydrolysis involves a Ser residue in the SXXK sequence as a catalytic nucleophile (Wagner et al., 2002).

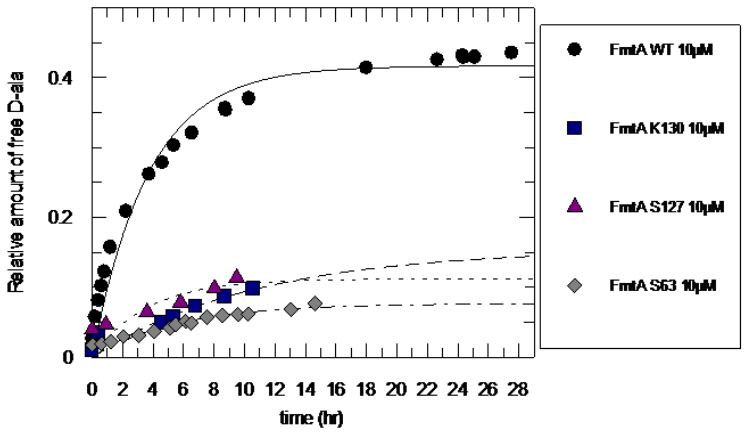


Figure 2.3.6 Active site (SXXK motif) of FmtA$^{\Delta 27}$ WT for esterase activity
FmtA $^{\Delta 27}$ WT enzymatic activity on WTA (RN4220) by ^{1}H NMR was monitored for reaction mixture containing FmtA $^{\Delta 27}$ WT or FmtA $^{\Delta 27}$ S63 (10 μM) or FmtA $^{\Delta 27}$ S127 or FmtA $^{\Delta 27}$ K130(10 μM) and WTA (5mg/ml) isolated from RN4220 . Formation of free D-Ala was monitored resonance around 1.45 ppm.

2.3.7 Esterase and protease inhibitors on the activity of FmtA$^{\Delta 27}$

Chemical compounds known to inhibit the activities of esterase and protease were used to investigate whether FmtA$^{\Delta 27}$ does rely on the Serine residue for its activity. Eserine (physostigmine) which is a reversible acetylcholinesterase inhibitor and sodium fluoride which is a known inhibitor for general esterase and serine/threonine phosphatases. These inhibitors showed partial inhibition (20 %) on the catalytic activity of FmtA$^{\Delta 27}$. Moreover, phenylmethylsulfonyl fluoride (PMSF) a protease inhibitor that reacts with serine residue could inhibit FmtA$^{\Delta 27}$ activity partially. These inhibitions were observed after 2.5 hours of the reaction.

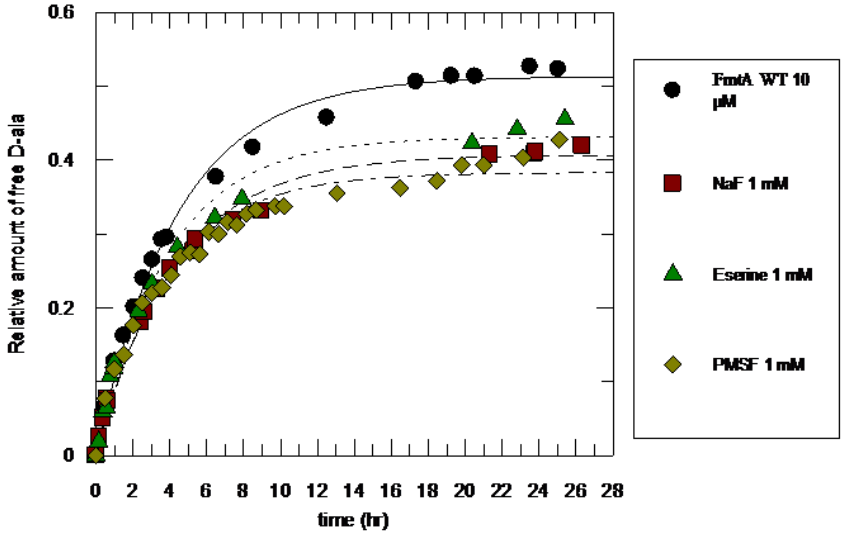


Figure 2.3.7 Inhibitors studies for esterase activity of FmtA$^{\Delta 27}$ WT
FmtA $^{\Delta 27}$ WT enzymatic activity on WTA (RN4220) by ^{1}H NMR was monitored in presence of inhibitors. Reaction mixture containing FmtA $^{\Delta 27}$ WT (10 μM), WTA (5mg/ml) isolated from RN4220 and inhibitors (1mM) was used for inhibitor studies. Formation of free D-Ala was monitored resonance around 1.45 ppm. A: Inhibitor studies for sodium fluoride and eserine; B: Inhibitor studies for PMSF.

2.3.8 Analysis of D-Ala modification of WTA isolated from *ΔfmtA* and *fmtA*-CM strain

I have shown *in-vitro* that FmtA $^{\Delta 27}$ can remove D-Ala from WTA. To investigate the *in-vivo* catalytic activity of FmtA$^{\Delta 27}$, I isolated WTA from *S. aureus* RN4220, *S. aureus fmtA*-CM in presence of 0.5 mM IPTG (a complete functional copy of *fmtA* gene under control of IPTG inducible promoter P*spac*) and *S. aureus fmtA* deletion mutant. To quantify the amount of D-Ala in WTA, GlcNAc peak was considered as 1 to integrate D-Ala peak. Results from ^{1}H NMR spectrum of purified WTAs showed that under *fmtA* CM in presence of IPTG (GlcNAc:D-ala, 1:0.76) had less D-Ala attached to ribitol of WTA compared to WT *S. aureus* RN4220 (GlcNAc:D-ala, 1:0.96).The *fmtA* deletion mutant (GlcNAc:D-ala, 1:1.17) showed slightly more D-Ala attached to ribitol compare to over-expressing *fmtA* conditional mutant with IPTG. *S. aureus fmtA*-CM has a complete functional copy of *fmtA* gene, and under the control of IPTG it induces the promoter P*spac*, to result in expression of fmtA at levels that are higher than in RN4220 (Qamar, 2011). This strain resulted reduction in the amount of D-Ala esterified to wall teichoic acids in the cell wall envelope of *S. aureus*. On the other hand, *S. aureus fmtA* deletion mutant has no *fmtA* gene, as a result more D-Ala was observed in the wall teichoic acids. Overall, these findings indicate that FmtA$^{\Delta 27}$ can remove D-Ala in vivo from WTA which is attached to ribitol by an esterase bond.

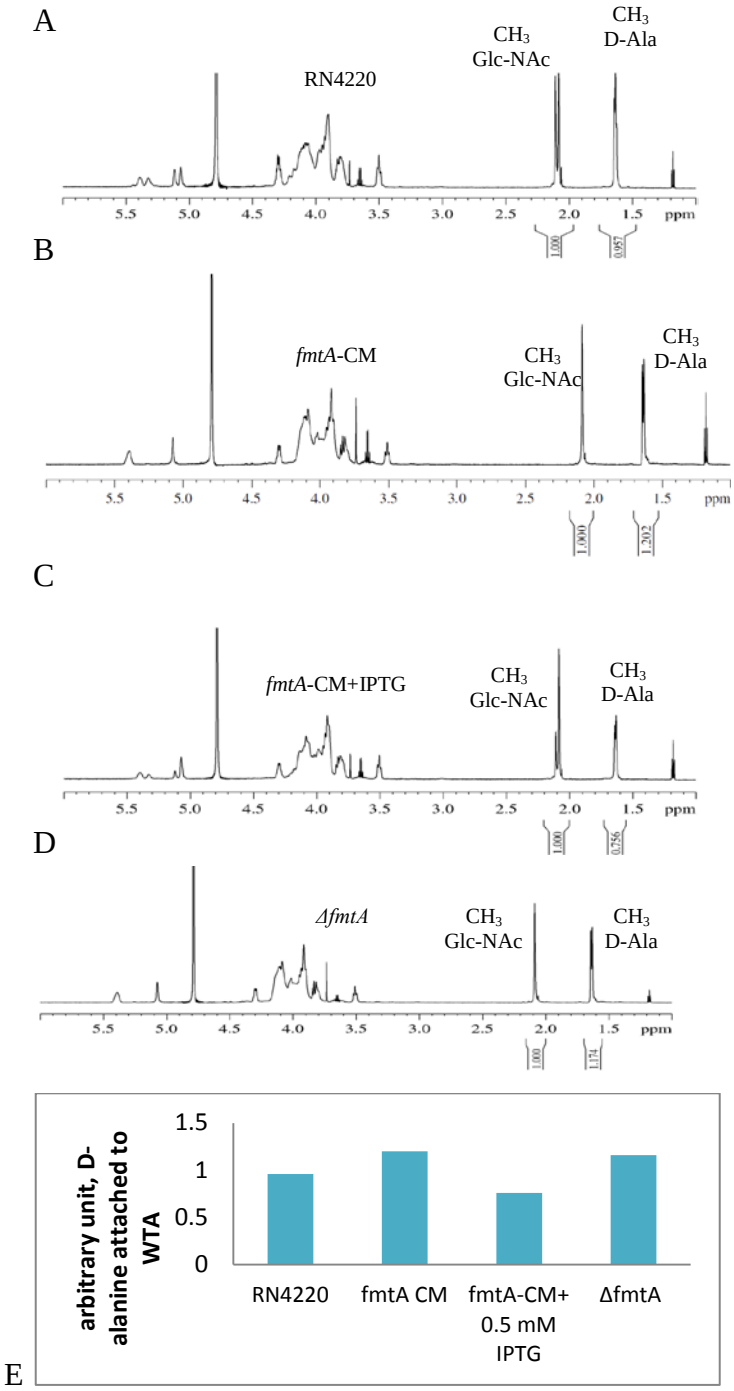


Figure 2.3.8 ^{1}H NMR spectrum of purified WTAs of different *S. aureus* strains (*S. aureus* RN4220, *S. aureus fmtA* conditional mutant and *S. aureus ΔfmtA* mutant)
A: WTA was isolated from WT *S. aureus* RN4220; B: WTA was isolated from over-expressing *fmtA*-CM without IPTG; C: WTA was isolated from over-expressing *fmtA*-CM in presence of 0.5 mM IPTG; D: WTA was isolated from *S. aureus fmtA* deletion mutant. E: comparison of D-Ala attached to ribitol phosphate of WTA from *S. aureus* RN4220, *S. aureus*-CM with or without IPTG and *S. aureus fmtA* deletion mutant

2.3.9 Inorganic phosphate assay for WTA

WTA of *S. aureus* is a zwitterionic cell wall glycopolymer composed of ~40 ribitol phosphate repeating units modified with *N*-acetylglucosamine and D-Ala. Released inorganic phosphate from WTA was measured with a Quanti ChromTM phosphate assay kit (DIPI-500) upon drying and then digesting WTA in 40 µl of 70% (w/v) $HClO_4$ at 165°C for 2 h. Inorganic phosphate analysis of fmtA-CM strain in presence of IPTG and *ΔfmtA* strain has very less effect in terms of phosphate content compare of wild type *S. aureus* RN4220. However, *fmtA*-CM strain gene without induction showed a little higher amount of phosphate content compare to wild type RN4220.

Table 2 Inorganic phosphate assay for WTA

Name of the Strain	Phosphate content in µM
S. aureus RN4220	1101.44± 0.005
S. aureus fmtA-CM	1280.93±0.003
S. aureus fmtA-CM+IPTG	946.42± 0.002
S. aureus Δ*fmtA*	974.16± 0.0003

2.3.10 Determination of whole-cell surface charge

Cytochrome c (Sigma) is a highly positively charged protein (pI 10.0; 12 kDa) and can be detected absorbance at wavelength 530 nm, binding of which is dependent on the net negative surface charge of *S. aureus* cells (Peschel et al., 1999). To determine whether the removal of D-Ala from WTA by FmtA$^{\Delta 27}$ caused an alteration in the overall charge of the cell envelope, the capacity of the wild-type and *fmtA* conditional mutant, over-expressing *fmtA* conditional mutant, *fmtA* Deletion mutant cells to positively charged cyt c protein were compared. *dltA* mutant was used as a control, where reduction in the amount of D-Ala esterified to teichoic acids in the cell envelope of *S . aureus* (brought about by disruption of the protein DltA) results in increased negative charge in the envelope and a concomitant increase in the electrostatic attraction of cationic protein cyt c towards the cell membrane (Peschel et al., 1999). Results revealed that *fmtA*-CM without IPTG had less positive charge in its cell envelope compare to wild type RN4220. When IPTG was used for *in-vivo* over expression of *fmtA*, opposite effect was observed i.e. overall cell surface charge was more positive compared to without induction of *fmtA* gene. The *fmtA* deletion mutant has no *fmtA* gene, hence theoretically, there should be more D-Ala in the WTA, as a result it should be more positively charge. Interestingly, whole cell surface charge of *fmtA* deletion mutant was a little more positively charged compared to *fmtA* conditional mutant but less than wild type RN4220. *S. aureus* cell surface is composed of peptidoglycan, teichoic acids (WTA and LTA) and surface proteins. Both WTA and LTA show D-Ala modifications in their polyol (ribitiol for WTA and glycerol for LTA) repeat units. We have shown that FmtA can remove D-Ala from WTA. We

don’t have enough knowledge about the regulation of *fmtA* gene to explain the opposite results of over expressing *fmtA* gene.

Table 3: Determination of whole-cell surface charge using cyt c

Name of the strain	% of applied protein
Cyt c	100±0
RN4220	64.93±4.51
***fmtA*-CM**	35.15±3.3
***fmtA*-CM+IPTG100**	62.49±3.68
***fmtA*-CM+IPTG 500**	55.66±5.95
ΔfmtA	35.61±3.53
ΔdltA	10.18±2.19

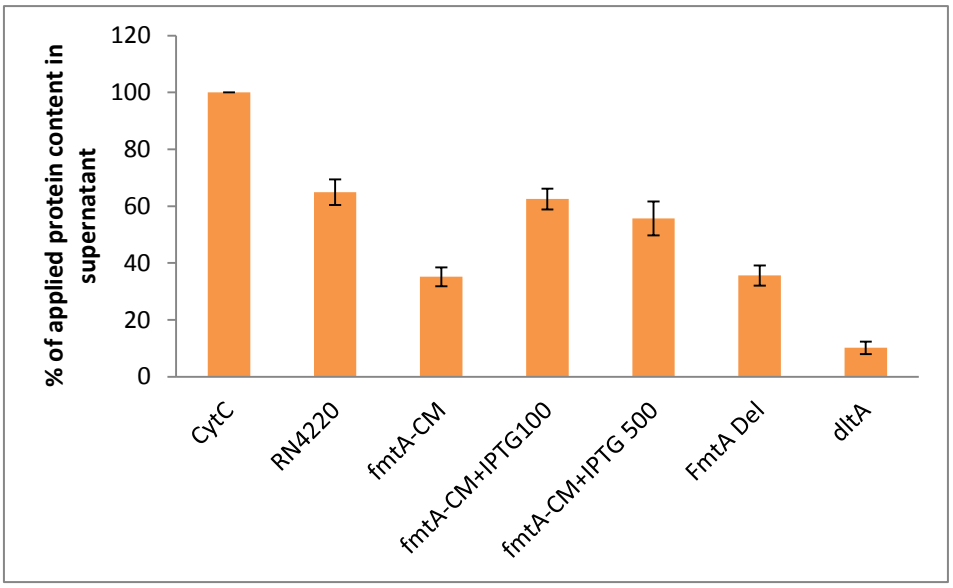


Figure 2.3.10 Determination of whole-cell surface charge using Cyt C
Cyt C is higly positively charged protein. Cell pellets from different strains (*S. aureus* RN4220, *S. aureus fmtA*-CM and *S. aureus ΔfmtA* mutant) was incubated with Cyt C and % of protein content in supernant indicates the amount of neragive charges in whole cell. *ΔdltA* mutant strain(WTA lacking D-Ala) was used as a control.

2.3.11 Investigation of Esterase activity of FmtA$^{\Delta 27}$ on LTA by NMR

Previously, I showed that FmtA $^{\Delta 27}$ can remove D-Ala from WTA. *S. aureus* cell wall has two types of teichoic acids: WTA and LTA. To investigate the effect of FmtA$^{\Delta 27}$ on LTA, I monitored the changes in LTA in presence of FmtA $^{\Delta 27}$ by ^{1}H NMR. Modification of LTA is observed at the second position of glycerol, where hydroxyl group is substituted with D-alanyl ester (Fischer, 19988). Results showed that FmtA$^{\Delta 27}$ can remove D-Ala from LTA. The cleavage of D-Ala ester from LTA was observed by the disappearance of the resonance at 1.65 ppm and the appearance of the free D-Ala resonance at 1.47 ppm. In another experiment, we included a control, which was only LTA in deuterium oxide. In absence of FmtA$^{\Delta 27}$ there was no removal of D-Ala from LTA (Figure 2.3.11.1).

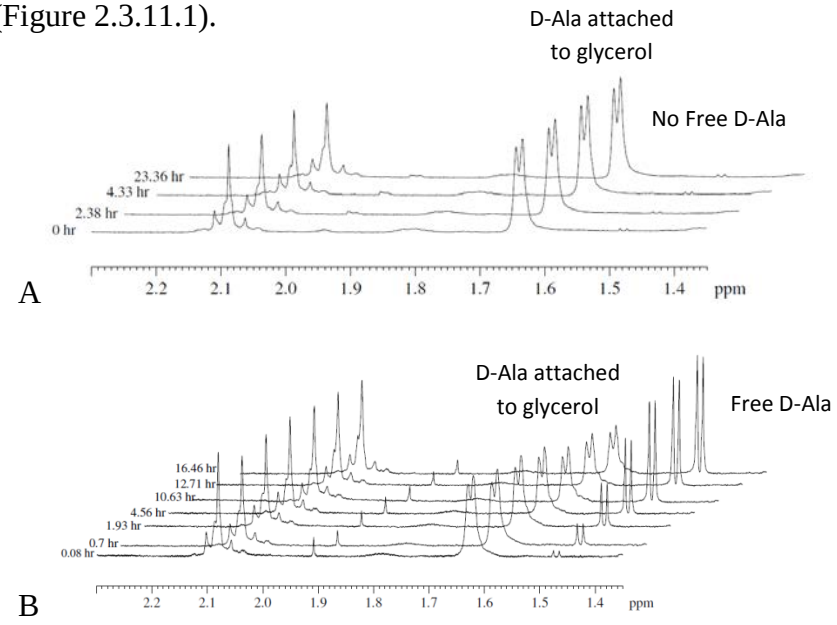


Figure 2.3.11.1 Removal of D-Ala of LTA by FmtA$^{\Delta 27}$
^{1}H-NMR spectrum of *S. aureus* LTA (Sigma) in the absence or presence of FmtA$^{\Delta 27}$ (10 µM). A: LTA (5 mg/mL) in deuterium oxide (control); B: Reaction mixture containing FmtA$^{\Delta 27}$ (10 µM) and LTA (5 mg/mL) in deuterium oxide. Formation of free D-Ala was monitored as appearance of a resonance around 1.47 ppm.

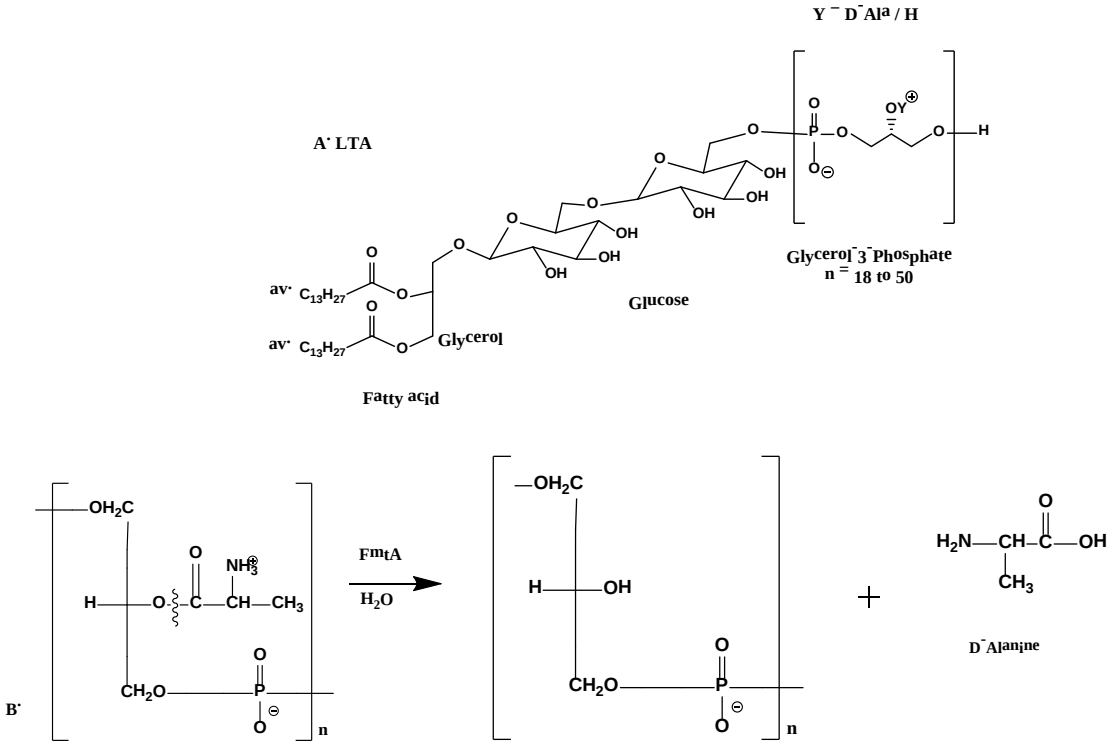


Figure 2.3.11.2 Catalytic function of FmtA$^{\Delta 27}$ on LTA.
A: LTA chemical structure; B: In presence of water, FmtA removes D-Ala from LTA and produces free D-Ala.

2.3.12 Esterase activity of PBP4 or PBP2a on WTA by NMR

Amino acid sequence homology comparison revealed that FmtA has sequence homology with penicillin binding protein (PBP) and β-lactamase (Fan et al., 2007). The sequence alignments revealed that FmtA has two of the three conserved motifs of serine active-site PBPs and β-lactamases: SXXK, Y(S)XN, and H(K/R)T(S)G, where X is any amino acid.

Previously it was shown that class B high molecular mass (HMM) PBP2a, a penicillin-binding protein that has transpeptidase activity interacts with WTA (Qamar, Golemi-kotra, 2012). Furthermore, from our experiments, it was clear that class B low

molecular mass (LMM) PBP4, that exhibit transpeptidase, carboxypeptidase , penicillinase activity can also bind with WTA . We have shown that FmtA can remove D-Ala from WTA. Furthermore, the active site serine from two SXXK motifs are indentified for this catalysis. In light of these facts, we were interested to see wheather PBP4 or PBP2a has similar esterase activiy on WTA.

Experiments of PBP4 or PBP2a with WTA by NMR revealed very low release of free D-Ala over time. We have examined the possibility of WTA hydrolysis in deuterium oxide and no hydrolysis of WTA was observed over time.

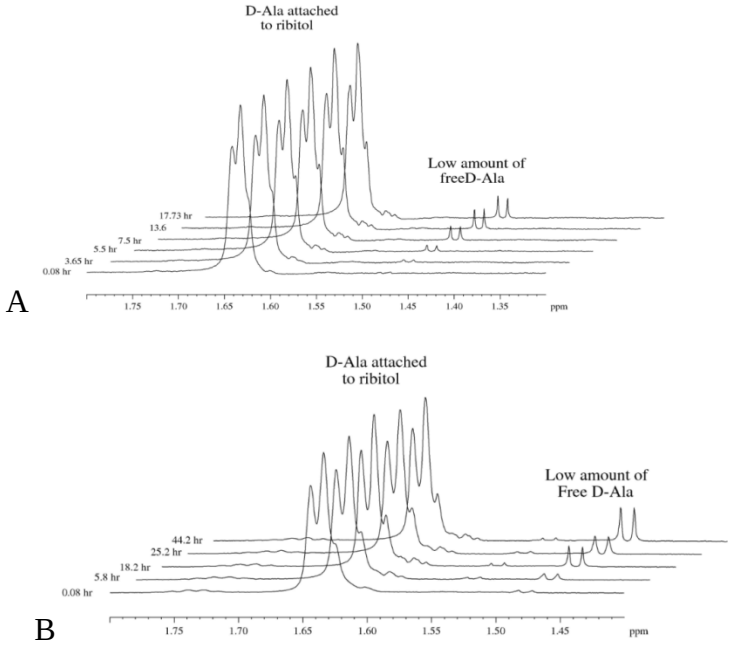


Figure 2.3.12 Esterase activity of PBP4 or PBP2a on WTA by NMR
Reaction mixture of Protein FmtA or PBP4 or PBP2a (10 µM) and WTA isolated from *S. aureus* RN4220 (5 mg/mL) was mixed in a total volume of 400µL deuterium oxide and the reaction was observed by ^{1}H NMR over time.
A: PBP4 with WTA
B: PBP2a with WTA

2.4 Discussion

The *fmtA* gene was first identified by Komatsuzawa et al. which was shown to effect autolysis in the presence of Triton X-100. Inactivation of *fmtA* in methicillin-resistant *S. aureus* (MRSA) strain is associated with reduced oxacillin resistance level and decreased level of highly cross linked peptidoglycan (Komatsuzawa et al., 1999). The primary structure of FmtA harbors two of the three conserved motifs of PBPs, β – lactamases, and acylate a serine residue of the SXXK motif but at very slow rate (Fan et al., 2007).

A previous graduate student Dr. Aneela Qamar in the lab of Dr. Golemi-Kotra showed that FmtA$^{\Delta 27}$ has very week D,D-carboxypeptidase activity (Qamar , 2011). It has been reported that some proteins related to peptidoglycan biosynthesis or remodeling require a native substrate or a particular ligand to become active. PBP1a, PBP1b of *E. coli* that are activated through interactions with membrane-bound proteins LpoA and LpoB (Qamar and Golemi-Kotra 2012). PBP5 of *E. coli* requires binding to the membrane to become active(Qamar and Golemi-Kotra 2012). It was hypothesized that FmtA may require interaction with a ligand in the periplasmic space to become active (Qamar and Golemi-Kotra, 2012). In lieu of our earlier finding by Dr. Aneela Qamar that FmtA $^{\Delta 27}$ interacts with WTA, we raised the question whether this interaction has any effect on the activity of FmtA as a DD-carboxypeptidase. To test this hypothesis, we used the DD-carboxypeptidase assay (also known as Fluorescent coupled enzyme assays for D-Ala) to monitor the amount of D-Ala released from the synthetic substrate Nα,Nε-Diacetyl-Lys-D-Ala-D-Ala by FmtA in the presence and absence of WTAs.

WTA of *Staphylococcus aureus* is a zwitterionic cell wall glycopolymer composed of ~40 ribitol phosphate repeating units modified with *N*-acetylglucosamine and D-Ala. In the presence of WTA (isolated from RN4220), the reaction mixture containing FmtA and the tripeptide Lys-D-Ala-D-Ala showed a significant increase in the amount of free D-Ala which was in agreement with our hypothesis. However, we were not sure about the source of D-Ala in the reaction mixture as WTA also have D-Ala in its second position of ribitol phosphate polymer. To exclude the possibility of D-Ala coming from WTA, I isolated WTA from *ΔdltA* mutant and used it for further experiments. The *dltABCD* operon is responsible for D-Ala incorporation into WTA and the first step of this process is activation of D-Ala as AMP ester in cytoplasm by DltA (Brown et al., 2013). Deletion of *dltA* gene in *S. aureus* lacks D-Ala in WTA (Peschel et al., 1999). Experiments containing FmtA and tripeptide Lys-D-Ala-D-Ala along with *ΔdltA* WTA (isolated from *ΔdltA* mutant) showed no significant release of D-Ala in the reaction mixture. In light of these results, we suspected that $FmtA^{\Delta 27}$ removes the D-Ala from WTA and that was the reason behind the observed significant increase in D-Ala in the reaction. To confirm this, we repeated the experiment containing $FmtA^{\Delta 27}$ and WTA (RN4220) but in the absence of tripeptide Lys-D-Ala-D-Ala; the results were the same as that obtained in the presence of tripeptide Lys-D-Ala-D-Ala. Furthermore, experiments of boiled $FmtA^{\Delta 27}$ and WTA (RN4220) showed no free D-Ala in the reaction mixture. These findings strongly indicated that free D-Ala from the reaction mixture containing $FmtA^{\Delta 27}$ and WTA was due to the catalytic activity of FmtA on D-Ala esterified wall teichoic acids.

Additionally, we have verified catalytic activity of FmtA on WTA by ^{1}H NMR where changes in WTA in the presence of FmtA showed cleavage of D-Ala ester from WTA by the disappearance of the resonance around 1.65 ppm and the appearance of the free D-Ala resonance around 1.45 ppm. Interestingly, Different enzyme concentrations (FmtA) and substrate concentrations (WTA) exhibited different catalytic effect of free D-Ala formation. Surprisingly, no enzymatic reaction was observed in the presence of high concentration of WTA (10 mg/ml). This behaviour could be due to substrate inhibition phenomenon. Many enzymes are inhibited by their own substrates and this inhibition often has important biological functions (Reed et al., 2010). These results strongly suggest that WTA is not an activator ligand for FmtA, but a substrate. Moreover, FmtA has an esterase catalytic activity to release D-Ala from WTA, which is connected through ester bond in the second position of ribitol in WTA.

A large family of penicillin-recognizing proteins with an active-site serine residue exhibit wide variety of activities but not limited to β-lactamases, D,D-endopeptidases, D,L-endopeptidases, D-amidases, D-esterases, and D-aminopeptidases (Delmarcelle et al., 2005). It has been reported that some esterases exhibit high sequence identity to class C β-lactamases and penicillin binding proteins. These are classified as Family VIII esterases (microbial carboxylesterases) based on a comparison of their primary structures (Arpigny and Jaeger, 1999). Family VIII esterases represent a poorly characterized esterase family. Enzymes from this family are distinct from those of other carboxylesterases. The nucleophilic serine residue in family VIII carboxylesterases occurs

in the S-X-X-K motif, like those in class β-lactamases, instead of in the G-X-S-X-G motif (Wagner et al., 2002).

The primary structure of FmtA harbors two of the three conserved motifs of PBPs and β–lactamase. The serine residue present in the sequence motif S^{127}-X-X-K (which is conserved among penicillin-binding proteins and β-lactamases) is the active-site nucleophile during the formation of acyl-enzyme species. S-X-X-K motif in FmtA is in duplicate, located at the positions S^{63}KLK and S^{127}AQK. Mutation studies of SXXK motif of $FmtA^{\Delta 27}$ revealed that both SXXK motifs are involved in the esterase catalytic activity of FmtA $^{\Delta 27}$. Substitution of serine in the S^{63}KLK or S^{127}AQK was associated with a significant decreased (75%) enzymatic activity. Substitution of lysine at 130 position ($SXXK^{130}$ motif) also produced a significant decrease in catalytic activity. These results indicate that serine (position at 63 and 127) and lysine (position at 130) are important for the catalytic activity of FmtA. Furthermore, both active sites of SXXK motif are involved in catalysis. However, no literature review was found on catalytic mechanisms of esterase that involve two active-site serine residues. Carboxylesterases and β-lactamases share a two-step hydrolase mechanism that consists of two successive nucleophilic attacks by the serine residue and a water molecule (Wagner et al., 2002). Family VIII Carboxyesterase has a modular structure of a small α-helical domain and an α/β domain similar to the class C β-lactamases (Cha et al., 2013). Crystal structure of EstB from *Burkolderia gladioli*, a representative of Family VIII carboxylesterases revealed that the mechanism of ester hydrolysis involves Ser residue of S-X-X-K sequence as a catalytic nucleophile (Wagner et al., 2002).

To check the substrate specificity for FmtA$^{\Delta 27}$, I tried a range of carboxylesterase substrates, e.g p-nitrophenyl butyrate (pNPB) and p-nitrophenyl acetate (pNPA). FmtA showed very poor esterase activity towards p-nitrophenyl butyrate and p-nitrophenyl acetate by continuous spectrophotometric assay**.** However, due to the high background hydrolysis of these carboxylesterase substrates, we explored the catalytic activity of FmtA toward p-NPB and p-NPA by NMR, and encountered the same problem. Hydrolyse results from NMR revealed very poor activity toward p-nitrophenyl butyrate and almost no activity toward p-nitrophenyl acetate. I used D-alanine methyl ester as a potential substrate, but no esterase activity was observed against it. The same observations were made for L-Ala p-Nitroanilide and these suggested that FmtA is not a prospective α-amino-acid esterase or aminopeptidase. Carboxylesterases catalyze the hydrolysis of short chain aliphatic and aromatic carboxylic ester compounds and all the carboxylesterase in family VIII are active towards nitrophenyl esters or triglycerides with short chain acyl moieties (Chahiniana et al., 2009). General esterase inhibitors (eserine, NaF) and phenylmethylsulfonyl fluoride (PMSF) could inhibit FmtA activity partially. These results clearly indicate that esterase catalytic of FmtA is very specific to D-Alanine ester connected with polymer of teichoic acid. Moreover, FmtA does not have any carboxylesterase or α-amino-acid esterase or aminopeptidase activity.

LTA consist of 1,3 linked repetitive glycerol phosphate repeating units attached to cytoplasmic membrane. Modification of LTA is observed at the second position of the glycerol, where hydroxylgroup is substituted with D-alanyl ester. I showed that FmtA can remove D-Ala from LTA. It is interesting that FmtA can show esterase activity toward

both teichoic acids from *S. aureus* but can't show any esterase activity toward synthetic esterase substrates.

I investigated the *in-vivo* esterase activity of FmtA towards WTA. Experiments with WTA isolated from *S. aureus* RN4220, *S. aureus fmtA* conditional mutant (a complete functional copy of *fmtA* gene under control of IPTG inducible promoter Pspac) and a *S. aureus fmtA* deletion mutant showed that over-expression of *fmtA* was associated with reduced amount of D-Ala modification of WTA whereas loss of *fmtA* gene in *S. aureus* resulted in higher content of D-Ala on WTA compare to wild type strain (*S. aureus* RN4220).

To determine whether removal of D-Ala from WTA by FmtA caused an alteration in the overall charge of the cell envelope, I used cytochrome C which due its positive charge binds to the negative charge derivatives of the cell wall. The binding capacities of the wild-type and mutant cells (*fmtA* conditional mutant and *fmtA* deletion mutant) to positively charged cyt C protein were compared. Cytochrome C (Sigma) is a highly positively charged protein (pI 10.0; 12 kDa) and can be detected at wavelength 530 nm, binding of which is dependent on the net negative surface charge of *S. aureus* cells (Peschel et al., 1999). The results of these experiments revealed that *fmtA* conditional mutant had less positive charge in its cell envelope compared to wild type RN4220. When IPTG was used for *in-vivo* over expression of *fmtA* (*fmtA* conditional mutant), the opposite effect was observed, *i.e.* the overall cell surface charge was more positive than that obtained without the induction of *fmtA* gene in the conditional mutant. The *fmtA* deletion mutant lacks a functional *fmtA* gene, hence theoretically, there should be more

D-Ala in the WTA, as a result, cell surface should be more positively charged. Interestingly, whole cell surface charge of *fmtA* deletion mutant was a little more positively charged compared to *fmtA* conditional mutant but less positively charged than wild type RN4220. *S. auresus* cell envelope is composed of peptidoglycans, teichoic acids (wall teichoic acids and lipoteichoic acid) and some surface proteins. Our results indicate that *fmtA* many not be solely responsible for controlling the overall cell envelope charge and moreover, that over-expression of *fmtA* gene could trigger a feedback signal. It has been reported that *fmtA* gene is a member of the core cell wall stimulon (McAleese et al., 2006). However, regulation of *fmtA* gene is not well understood.

We have shown that FmtA and PBP4 interact with WTA. Interaction studies between PBP2a and WTA using native-H^+/PAGE was shown by Aneela Qamar (Qamar, 2013). Additionally, I have shown that FmtA can display esterase enzymatic activity to remove D-Ala from D-alanyl ester attached to ribitiol. These findings led us to investigate any enzymatic changes of WTA by PBP4 or PBP2a. Our NMR data showed that indeed PBP4 and PBP2a can remove D-Ala from WTA. However, these esterase activity was very low (13 fold less) compare to FmtA.

Family VIII carboxylesterases revealed the mechanism of ester hydrolysis involve Ser residue of S-X-X-K sequence as a catalytic nucleophile (Wagner et al. 2002). Site directed mutagenesis for FmtA showed involvement of SXXK motif for esterase catalytic activity on WTA. Active serine and lysine from SXXK motif of PBP4 and PBP2a might be responsible for PBPs esterase activity on WTA. All PBPs and β-lactmases contain three conserved motif and they are SXXK, SXN and KTG (where X indicates any amino

acid. Predicted secondary structure of FmtA showed high level of similarity with D-ala-D-Ala carboxypeptidase and β-lactamase activity. FmtA harbors 2 out of three conserved motifs of PBPs (SXXK and SXN).

PBP4 is known to have DD-carboxypeptidase, where as PBP2a shows only transpeptidase activity. Both transpeptidase and carboxypeptidase activities of PBPs occur at the D-Ala-D-Ala terminus of PG. Apart from carboxypeptidase activity of PBP2a and PBP4, we have shown their cross functional esterase activity. Several reports have been noted for cross functional esterase activity for PBPs. PBP 2x in *S. pneumoniae* exhibits esterase and thiolesterase activities *in-vitro*, where C_6H_5-CO-NH-CHR^1-CO-X-CHR^2-COO^- (for esterase, X=O and for thiolesterase, X=S) was used as a substrate (Jamin et al., 1993). PBPs from *Streptomyces* sp. strain R61 and *Actinomadura* sp. strain R39 have reported for D, D-peptidase, esterase, and thiolesterase activities (Frere et al., 1975, Frere 2004). This is the first time to report that PBPs from *S. aureus* exhibit low esterase activity on WTA. However, the catalytic mechanism for PBPs as an esterase is not known at this point. More investigations are required for the esterase activity of PBPs.

In conclusion, WTA is not an activator ligand for FmtA and that FmtA shows esterase catalytic activity towards D-Alanyl ester of WTA and LTA. Both serine and lysine from $S^{127}XXK^{130}$ motif are involved in esterase catalysis. Mutation studies suggest that both the SXXK motifs in FmtA, located at position S63 and S127 are involved in catalysis and the enzymatic activity depends both on substrate and enzyme concentration. Furthermore, high WTA concentration can inhibit enzymatic reaction of FmtA. To date, biological functions of most esterases are unknown. To our knowledge, this is the first

study to report an esterase from *S. aureus* that has sequence similarity to PBPs and β-lactamase and very selective to its biological substrate WTA and LTA compare to synthetic esterase substrate.

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CHAPTER THREE

INTERACTIONS OF FMTA WITH *S. AUREUS* SURFACE PROTEINS INVOLVED IN AUTOLYSIN ATLA

3.1 Introductions

Most bacterial cytoplasm is surrounded by an external cell wall to prevent cell lysis as a result of turgor pressure generated by high internal osmolality. One of the cell wall components is known as murein or peptidoglycan (PG), a long polymer with stiff glycan strands cross linked by short peptides. The network of glycan strands forms cytoplasmic cytoskeletal elements that furnish an external physical barrier, which gives the cell shape and functions as a permeability barrier to macromolecules. In the final stage of PG synthesis cross-linked PG strands are generated by the help PBPs, which have transglycosylase and/or peptidase activities (Beise et al., 1987).

In order for the cell to grow and divide effectively, it is necessary to hydrolyze the covalent bonds of the peptidoglycan. Autolysins are enzymes that can hydrolyze the peptidoglycan, by cleaving specific bonds found in PG (Typas et al., 2011). Based on specificities, autolysins can be categorized into three major classes: N-acetylmuramoyl-L-alanine amidases, glycosaminidases, and endopeptidases. The N-acetylmuramoyl-L-alanine amidases specifically cleave the bond between the carboxylate group of muramic acid and the N-terminal-L-alanine (Sugai et al., 1990) and N-acetylglycosaminidases hydrolyze the β-1,4 glycosidic bonds on the reducing end of N-acetylglucosamine

(Figure 4.1.1). The stem peptide chains in PG are specifically hydrolyzed by endopeptidases of which only a few have been characterized. These autolytic enzymes are strongly regulated and ubiquitous among bacteria. Unregulated peptidoglycan degradation results in damage to the integrity and protective properties of the peptidoglycan structure (Typas et al., 2012). However, the regulation of autolytic systems remains incompletely defined, and appears to be different in various species of bacteria. Though unregulated autolysin could be suicidal, they are involved in vital aspects of bacterial physiology: assembly and disassembly processes of the PG layer such as PG growth, PG turnover and recycling, cell separation in cell division, remodeling the PG sacculus to determine cell shape, assembly of cell surface organelles, autolysis, resuscitation of dormant cells, biofilm formation, bacterial cell lysis in interbacterial species competition and lysis induced by the β-lactam antibiotics (Vollmer et al., 2008; Typas et al., 2011).

Staphylococcus aureus contains several peptidoglycan hydrolases. Based on their cleaveage site they are classified as N-acetylglucosaminidases, N-acetylmuramoyl-l-alanineamidases, endopeptidases and transglycosidases (Strominger and Ghuysen, 1967). Zymograms of crude cell extracts of different *S. aureus* strains showed five major bacteriolytic bands (Sugai et al., 1990). Some of the enzymes remain uncharacterized. However, little is known about the regulation of staphylococcal peptidoglycan hydrolase system. The most prominent autolysin of *Staphylococcus aureus* is Atl, a bifunctional three-domain enzyme of aproximately 137 kDa, which undergoes proteolytic cleavage to yield two catalytically active proteins: N-terminal region independently exhibits N-

acetylmuramoyl-L-alanine amidase (AM) and The C-terminal region of the protein has endo-β-N-acetylglucosaminidase domains (GL) (Heilmannet al., 1997; Sugai et al., 1995). Atl-AM is a 63.3 kDa protein that cleaves the amide bond between the N-acetyl muramic acid in the murein backbone and L-alanine in the stem peptide (Biswas et al., 2006). AM contains an enzymatic domain and two repeat domains that are involved in localization and substrate recognition (Biswas et al., 2006, T Baba et al.,1998). These three modules are separated by two linkers, L1 and L2. Two repeat domains, known as R1 and R2 are located at C-terminal. Studies of sequence alignments demonstrated that repeats R1 and R2 can each be further divided into a-type and b-type subunit (Marino et al., 2002). Recently M. Schlag et al has studied the role of repeat units by expressing R1 and R2 endogenously and externally. They tried to understand the mechanism of Atl-repeat unit attachment to the equatorial surface ring in staphylococci. It has been shown that the binding of the amidase repeats (R1, 2) is based on an exclusion strategy mediated by WTA.

Atl-GL is a 53.6 kDa enzyme that hydrolyzes the bond between N-acetyl-β-D-glucosamine and N-acetyl muramic acid and contains an enzymatic domain and a single repeat domain (Oshida et al., 1995). The repeat domain of GL is known as R3 and located at its N-terminus (Heilmann et al., 1997). Its role in partitioning of daughter cells in *S. aureus* is less clear. The R3 domain showed good lytic activity in zymograms, where *Micrococcus luteus* was used as a substrate (Heilmann et al., 1997).

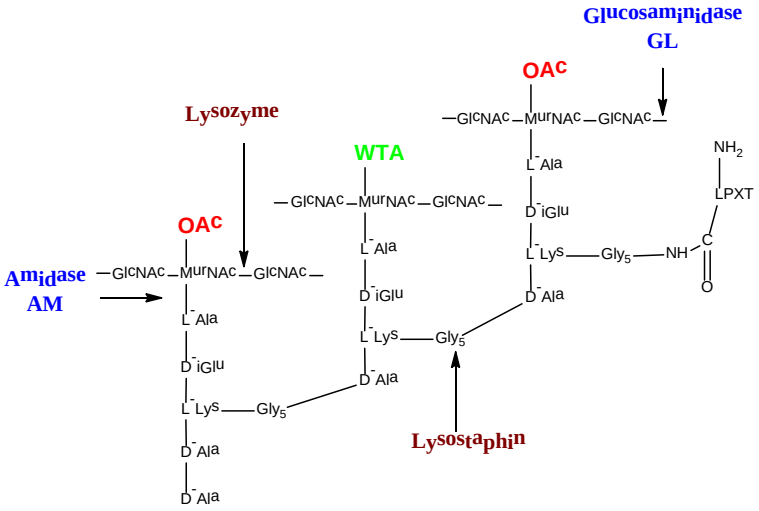


Figure 3.1.1 Cleavage site of the Atl amidase (AM) and glucosaminidase (GL). Shown is a section of *the S. aureus* peptidoglycan (PGN) structure composed of the glycan strand with alternating N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid(MurNAc), the peptide subunit and the penta-glycine interpeptide bridge. The cleavagesites of the amidase (AM, N-acetylmuramoyl-l-alanine amidase) and the glucosaminidase (GM, endo-β-N-acetylglucosaminidase) are indicated by arrows. For comparison the cleavage site of lysozyme (N-acetylmuramidase) and lysostaphin PGN-endopeptidases are indicated. Also shown are the three typical PGN modifications in *S. aureus*: OAc (O-acetylation) and WTA (wall teichoic acid) both are tethered at the C6 position of MurNAc, and the covalently cell wall bound proteins at the N-terminus of the pentaglycine peptide, with the conserved LPXT-motive.

Regulation of the autolytic enzymes in *S. aureus* is controlled at various transcriptional and posttranslational levels such as enzyme activity, processing and targeting. There are several two component regulatory systems which affects peptidoglycan hydrolase activity and autolysis. These included: lytSR, Sar, Arg, lrgAB, and cidABC regulatory network that modulate murein hydrolase expression and/or activity. Moreover, WalK/R and GraRS regulons are also involved in autolysis control. It has been shown that these types of regulations evolved as a way for the bacteria to maximize their ability to evade host immune responses, while simultaneously reducing

their susceptibility to β -lactam antibiotics. These two component system are out of this chapter's scope, hence not discussed here.

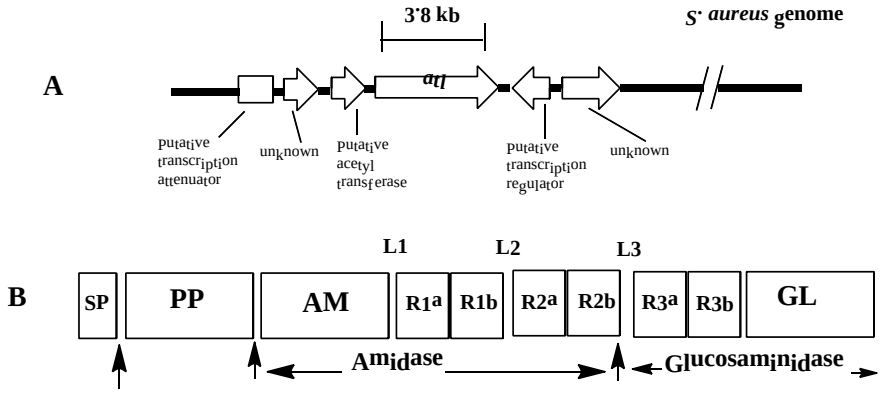


Figure 3.1.2 Genomic location of atl (A) and domain arrangement of the bifunctional precursor protein (B).
In most sequenced staphylococcal genomes *atl* is flanked by the same orthologous genes. In all staphylococcal species analyzed so far the Atl homolog is organized as a bifunctional protein with an amidase (AM) and a glucosaminidase (GM) domain. In *S. aureus* Atl is post-translocationally processed (indicated by arrows) between the propeptide (PP) and amidase (AM) domain and between therepeat domains R2b and R3a to free AM-R1-2 and R3-GM. SP signalpeptide, PP propeptide, AM catalytic domain of amidase, GL catalytic domain of glucosaminidase, R1a, R1b, R2a, R2b, R3a, R3b repeat domains.

TAs are usually constitutively produced and either connected to PG (WTA) or to the cytoplasmic membrane (LTA). *S. aureus* WTA is covalently linked to the 6-OH of N-acetyl muramic acid (MurNAc) via a disaccharide composed of N- acetyl glucosamine (GlcNAc)-1-P and N-acetyl mannosamine (ManNAc) (Brown et al., 2008). Besides, teichoic acid and PG, cell wall has surface proteins on their outer membrane. WTAs help in anchoring cell surface proteins. It has been shown that Atl and PBP4 localized to the cell septum in presence of WTA (Atilano et al., 2010; Schlag et al 2010). This binding is

modulated most likely by the charge of WTA. Due to negatively charged phosphate groups and free positively charged amino groups D-Ala residues on the ribitol phosphate, teichoic acid exhibit zwitterionic properties (Weidenmaier and Peschel et al, 2008; Kohler et al., 2009). Reduced D-Ala in bacteria showed more negatively charged cell surface and this allowed increased binding of cationic proteins (Wecke et al., 1997). In absence of D-Ala and or less D-alanine, the cell surface charges become more negative due to less positive charge in WTA and that allows for increased binding of cationic proteins like Atl. The negatively charged bacterial surface is important for the activity of cationic autolysins and could direct an increased autolytic rate (Walter et al., 2007). Amidases are normally localized to the septum region, but in the absence of wall teichoic acids the amidases are delocalized and are distributed along the cell surface. It is hypothesized this mislocalization results in the increased autolysin activity seen in WTA deletion mutants (Schlag et al., 2010).

WTAs are proposed to be temporal and spatial regulators of peptidoglycan cross-linking that operate by mediating localization of proteins associated with PG biosynthesis and remodeling, such as PBP4 and AtlA (Schlag et al., 2010). Furthermore, FmtA localizes at the cell division septum, most likely by binding to the cross wall. More specifically, FmtA localizes at the division pole and moves further forward to the center of the bacterium as the cross-wall is synthesized. A similar localization pattern was also observed for PBP4 of *S. aureus* (Atilano et al., 2010.) In the absence of WTA, FmtA is delocalized throughout the cell. However, no delocalization was observed in the presence of cell wall inhibitors, which excluded the possibility of FmtA recruitment to the division

septum by PG. Like FmtA, amidase delocalize and distribute along the cell surface in the absence of WTA. However, there are reports that demonstrated the absence of mature WTA in the cross-wall region (Schlag et al., 2010). Studies have shown that WTAs control the autolytic activity of AtlA most likely by modulating WTA negative charge. FmtA is known to have effect on autolysis in presence of triton X-100. Like FmtA, AtlA also interacts with WTA and localized in cell division septum. We have shown that FmtA interacts with WTA and removes D-Ala from ribitol (chapter 2). Binding of FmtA to WTAs may activate AtlA by modulating the negative charge of WTAs in cell division septum; however, there is a possibility of interactions between FmtA and AtlA.

The interactions between FmtA and WTA suggest that WTA might recruit FmtA to cell wall. Same types of observation were noted for PBP4 and PBP2a. However, there is a possibility that FmtA, PBP4 and /or PBP2a may work cooperatively (Qamar and Golemi-Kotra, 2012). To address these questions, we tried to explore the WTA interaction with FmtA, PBP4 and Atl. We designed pull down experiments to study potential protein-protein interactions of FmtA with PBP4 and/or PBP2a. Furthermore, we have cloned and purify Atl-AM and Atl-GL. we have shown the biochemical evidence of interactions between Atl (AM and GL) and WTA. Finally, protein-protein interactions between Atl and FmtA were studied by gel filtration chromatography, ITC and native PAGE based on their mobility.

3.2 Materials and Methods

3.2.1 Materials and chemicals

Growth media were purchased from EMD Bioscience. Enzymes and chemicals were purchased from Sigma (Oakville, Canada) and Thermo-Fisher (whitby, Canada) and New England BioLabs (*Eco RI*, *NotI*); unless otherwise stated. All primers were purchased form Sigma (Oakville, Canada). The DNA polymerases PfuTurbo® was purchased from Agilent Technologies.

3.2.2 Cloning of atlAM gene

Cloning of Atl AM was done by Dr. Atul Kumar Singh. First the atl_{AM} gene was amplified from genomic DNA of *S. auresu* strain MU50 by polymerase chain reaction (PCR). The Primers were: Dir 5' – ACG **CAT ATG** GCT TCA GCA CAA CCA AG-3' and Rev 5' – ACG **AAG CTT** TTA TTA TTT TAC AGC TGT TTT TGG TTG-3' (*Nde I* and *Hind III* restriction sites are bolded and underlined). Finally the atl_{AM} gene was cloned into commercial pET24a (+) vector. The resulting clones were sequenced for correct insert at the center for applied genomics, the hospital for sick children (Toronto, Canada). Finally the correct insert was transformed into *E. coli* BL21 (DE3) cells for protein expression.

3.2.3 Expression and purifications of Atl-AM

A single colony of BL21(DE3), containing the required expression construct in pET24 a(+):: atl_{AM} vector was used to start a seed culture which was grown overnight at

37°C in 5 mL Luria Bertani (LB) medium supplemented with kanamycin (30 µg/mL). A 200 fold of overnight seed culture was used to inoculate 800 mL of Terrific Broth medium supplemented with kanamycin (30 µg/mL) and 0.1 M D-sorbitol. The inoculated medium was grown at 37°C, 180 rpm, until OD_{600nm} reached 0.6-0.8, at which protein expression was induced by addition of Isopropyl β-D-thiogalactopyranoside at a final concentration of 0.1 mM. Expression of protein was carried out for 16 hours at 16°C, 160 rpm and the cells were harvested at 7,000 x g for 20 min at 4°C. Resulting pellets were frozen at -80°C if subsequent purification steps were not carried out on same day.

All purification steps were carried out at 4°C. Harvested cells were resuspended in 50 mM sodium phosphate buffer, pH 7.6. Sonication (5 cycles of 1 min; 30% power, 60% pulse) was used to liberate the protein and centrifugation at 21000 x g for 60 min was carried out to remove cell debris. The resulting supernatant was applied to a S- Support column (Strong Cation exchange) (90-100 mL column volumes) pre-equilibrated with sodium phosphate buffer, pH 7.6. The target protein (AtlAM) was eluted at 40-50% of a liniear gradient of 0 to 1 M sodium chloride in 50 mM sodium phosphate buffer, pH 7.6. Protein fractions containing pure protein were concentrated using centrifugal filter Ultracel-3K (Amicon , Millipore). The purified protein concentration was determined BSA method.

3.2.4 Theoretical PI, MW calculation and Structural prediction of Atla-AM

Theoretical PI, MW calculation and Structural prediction of Atla-AM was calculated using the software Ex-pasy. For theoretical PI and MW computation, Amino

acid sequence was submitted to at http://web.expasy.org/compute_pi/. For Structure prediction, Amino Acid sequence was submitted to http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index and http://zhanglab.ccmb.med.umich.edu/I-TASSER/

3.2.5 Expression and purifications of Atl-Gl

Atl Gl was cloned by Michael Fridman. A single colony of BL21(DE3), containing the required expression construct in pET24 a(+):: atl_{GL} vector was used to start a seed culture which was grown overnight at 37°C in 5 mL Luria Bertani (LB) medium supplemented with kanamycin (30 µg/mL). A 200 fold of overnight seed culture was used to inoculate 800 mL of Terrific Broth medium supplemented with kanamycin (30 µg/mL) and . The inoculated medium was grown at 37°C, 180 rpm, until OD_{600nm} reached 0.6-0.8, at which protein expression was induced by addition of IPTG at a final concentration of 0.5 mM. Expression of protein was carried out for 16 hours at 16°C, 160 rpm and the cells were harvested at 7,000 x g for 20 min at 4°C. Resulting pellets were frozen at -80°C if subsequent purification steps were not carried out on same day.

All purification steps were carried out at 4°C. Harvested cells were resuspended in 50 mM sodium phosphate buffer, pH 7.6. Sonication (5 cycles of 1 min; 30% power, 60% pulse) was used to liberate the protein and centrifugation at 21000 x g for 60 min was carried out to remove cell debris. The resulting supernatant was applied to S-Support column (Strong Cation exchange column) (90-100 mL column volumes) pre-equilibrated with sodium phosphate buffer, pH 7.6. The target protein (Atl-AM) was eluted at 40-50%

of a liniear gradient of 0 to 1 M sodium chloride in 50 mM sodium phosphate buffer, pH 7.6.Protein fractions containing pure protein were concentrated using centrifugal filter Ultracel-3K (Amicon , Millipore). The purified protein concentration was determined BSA method.

3.2.6 Circular Dichroism (CD) spectroscopic studies

Near UV (200 nm- 260 nm) CD spectra of Atl AM and GL was recorded on a Jasco J-810 instrument using a cuvette with 1.0 mm light path length at 22 °C. A blank spectrum was recorded for buffer and subtracted from proteins spectrum.

3.2.7 Enzymatic activity of Atl-AM and Atl-GL by zymography

A single colony of *S. aureus* RN4220 was used for a seed culture which was grown overnight at 37°C in 5 mL Tryptic Soy Broth (TSB) medium. A 200 fold of overnight seed culture was used to inoculate 500 mL of TSB medium for overnight. Further, Cells were harvested by centrifugation at 7000 x g by using 10.5 JLA rotar and transferred in to 50 mL tube. Cell pellet were diluted with 2-3 mL TSB media and boiled for 15 min to inactivate any pathogenic factors. Second harvest was followed by centrifugation at 16000 x g for 15 min and supernatant was removed. Finally, cell pellet were lyophilized at Dr. Krylov's lab facilities.

Zymogram gel was prepared by using 0.6% w/v of lyophilized RN4220 in 12.5% SDS-PAGE gel preparation. 0.25 µg lysostaphin was used as a positive control. SDS dye was used for protein sample loading. For enzymatic activity on the zymogram, gel was washed 3 to 4 times with water to remove any SDS in the gel (1-2 hrs). Furthermore, the

gel was incubated in 1% triton X-100-25 mM Tris-HCL (pH-8.0) for overnight at 37°C. Zone of clear area showed the activity of Hydrolysis of murein on gel. Image was taken against light. Gel was stained with 1% w/v methylene blue in 0.01% w/v KOH (around 1 hr) followed by destaining with water.

3.2.8 Investigation of Atl-AM or Atl-GL and WTA interaction by acidic nPAGE/H^+

Interaction of WTA with Atl AM or Atl GL was investigated with native polyacrylamide gel electrophoresis (nPAGE/H^+). A typical reaction mixture (15 µL) consisted of Atl AM or Atl GL (10 µM) and 5, 10, 15, 20 µg of WTA in 50 mM sodium phosphate (pH 7.0) buffers were mixed and incubated for 1 hr at room temperature. 5X loading dye (0.2 M acetate KOH pH-6.8, glycerol 50%, Methyl green) was added to the mixture prior loading onto acidic native gel. The composition of the separating gel was 10 % Acrylamide-Bis acrylamide in 0.2 M acetate KOH pH-6.8, glycerol 50%, 1.5 M acetate KOH pH-4.3. The PAGE gels were stained with Coomassie blue. WTA used for these experiments were extracted previously by TCA method from *S aureus* RN4220 or *dltA* mutant (No D-alanine in WTA).

3.2.9 Investigation of FmtA$^{\Delta 27}$ and Atl-AM or Atl-GL interaction with by acidic nPAGE/H^+

Interaction of FmtA $^{\Delta 27}$ with Atl-AM or Atl-GL was investigated with native polyacrylamide gel electrophoresis (nPAGE/H^+). A typical reaction mixture (15 µL) of FmtA$^{\Delta 27}$ (0 to 40 µM) with Atl-AM (0 to 28 µM) or Atl-GL (0 to 30 µM) in 50 mM

sodium phosphate (pH 7.0) buffers were mixed and incubated for 1 hr at room temperature. 5X loading dye (0.2 M acetate KOH pH-6.8, glycerol 50%, Methyl green) was added to the mixture prior loading onto acidic native gel. The composition of the separating gel was 10 % Acrylamide-Bis acrylamide in 0.2 M acetate KOH pH 6.8, glycerol 50%, and 1.5 M acetate KOH pH 4.3 The PAGE gels were stained with Coomassie blue.

3.2.10 Investigation of FmtA$^{\Delta 27}$ and Atl AM interaction with by Isothermal Titration Calorimetry (ITC)

ITC experiments were performed using a ITC_{200} instrument (MicroCal., Inc., USA). Purified proteins were dialyzed with 50 mM sodium phosphate buffer, pH 7.2. Titrations were performed by injecting of FmtA wild type (46 µM) into the sample cell containing Atl AM (4 µM) at 25°C, DP 10 and 300 rpm. The binding stoichiometry and binding constants were determined by fitting the data to a one-site binding model. The ITC data were fit using Origin 7.0 (MicroCal., Inc., USA).

3.2.11 Investigation of FmtA$^{\Delta 27}$ and Atl GL interaction with by size exclusion chromatography

FmtA $^{\Delta 27}$ WT 42 µM or FmtA $^{\Delta 27}$ S127 40 µM was mixed with Atl-GL 10µM in a final volume of 40 µL in 50 mM Sodium phosphate buffer (pH 7.0) and incubated for 1 hr at room temperature. The resulting mixture was analyzed by high-performance liquid chromatography (HPLC). FmtA $^{\Delta 27}$ WT 42 µM or FmtA$^{\Delta 27}$ S127 40 µM or Atl-GL 10µM in a final volume of 40 µL was used for reference analysis. HPLC analysis of the protein

mixtures was conducted on an Size exclusion column G2000SW$_{XL}$ for HPLC (7.8 x 300 mm, 5 µm, TOSHOH bioscience LLC) using 50 mM Tris, 50 mM potassium Chloride, 5 mM Magnesium Chloride (pH 7.4). The program was run for 20 min at a flow rate of 1 mL/min.

3.3 Results

3.3.1 Expressions and Purifications of Atl-AM

The pET24a (+):: *atl* $_{AM}$ construct, containing R1 and R2 unit was cloned by Dr. Atul Kumar Sing. Furthermore, construct was transformed into *E. coli* BL21 (DE3) contains an IPTG inducible T7 promoter for cytoplasmic expression. The protein was best expressed in cytoplasm of *E. coli* BL21 (DE3) cells by using optimized expression conditions (0.1 mM IPTG in presence of 0.1M D-sorbitol at 16°C for 16 hrs). Following induction with IPTG sufficient amount of soluble Atl AM was obtained, as seen in the figure 3.3.1 for subsequent purification steps. Theoretical pI of Atl AM was calculated to be 9.62 with a molecular weight of 63070.80 Da. The purification strategies involve passing the supernatant through S-support column (strong cation exchange, due to SO_3^- group). The desired protein was eluted between 45-60 % linear gradient of 1 M NaCl. The resulting fairly pure fractions were concentrated and final protein homogeneity was assessed by 12.5% SDS-PAGE.

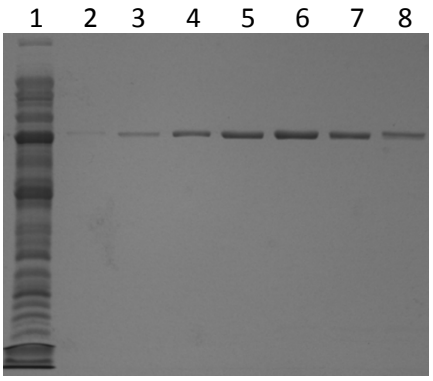


Figure 3.3.1 Purifications of Atl AM
12.5 % SDS-PAGE was loaded with fractions from S support column. Lane 1: supernatant from *E. coli* BL21 (DE3) cells expressing target protein. Lane 2 to lane 8 fractions eluted by 45-60 % linear gradient of 1 M NaCl

3.3.2 Expressions and Purifications of Atl GL

The pET24a (+):: atl_{GL} construct, containing R3 unit was cloned by Michael Fridman. Furthermore, construct was transformed into *E. coli* BL21 (DE3) contains an IPTG inducible T7 promoter for cytoplasmic expression. The protein was best expressed in cytoplasm of *E. coli* BL21 (DE3) cells by using 0.5 mM IPTG at 16°C for 16 hrs. Theoretical pI of Atl GL was calculated to be 9.64 with a molecular mass of 53567.53Da. The purification strategies involve passing the supernatant through S-support column (strong cation exchange, due to SO_3^- group). The desired protein was eluted between 45-60 % linear gradient of 1 M NaCl. The resulting fairly pure fractions were concentrated and final protein homogeneity was assessed by 12.5% SDS-PAGE.

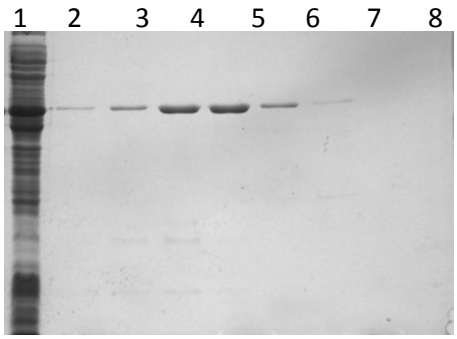


Figure 3.3.2 Purifications of Atl GL
12.5 % SDS-PAGE was loaded with fractions from S support column. Lane 1: supernatant from *E. coli* BL21 (DE3) cells expressing target protein. Lane 2 to lane 8 fractions eluted by 45-60 % linear gradient of 1 M NaCl

3.3.3 Zymographic detection of the autolytic activity of Atl AM and Atl GL

A suitable substrate (heat killed *S. aureus* RN4220) was embedded in the resolving gel during preparation of the 12.5% SDS acrylamide gel. Following electrophoresis, the SDS was removed from the gel (or zymogram) by incubation in water. Finally, the gel was incubated in a buffer containing Triton X-100 for an optimized length of time at 37°C. The zymogram is subsequently stained (alchene blue), and areas of digestion appear as clear bands against a dark background where the substrate has been degraded by the enzyme. Atl AM and Atl GL has a molecular weight 63 kDa and 53.5 Kda, respectively. Atl AM cleaves the amide bond between the N-acetyl muramic acid in the murein backbone and L-alanine in the stem peptide and Atl GL shows endo-β-N-acetylglucosaminidase activity that hydrolyzes bond between N-acetyl-β-D-glucosamine and N-acetyl muramic acid (Biswas, R et al., 2006, Bose, J L., et al 2012). Lysostaphin was used as a positive control. Clear bands in zymogram analysis showed Atl AM and Atl GL were active and had an enzymatic activity on substrate *S. aureus* RN4220

peptidoglycan cells. However, AM is more active on substrate *S. aureus* RN4220 compare to GL.

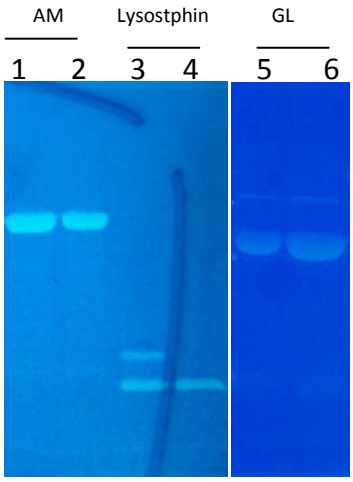


Figure 3.3.3 Zymographic detection of the autolytic activity of Atl AM.
Atl AM Protein Purified from *E. coli* BL21 cells grown with IPTG were used for enzymatic activity. The protein extract was separated by 12.5% SDS-gel electrophoresis. The SDS gel was impregnated with 0.8% heat killed *S. aureus* RN4220 cells. Autolytic activity was detected by renaturing the Atl AM by incubation of the gel in 50 mM Tri-HCl buffer, pH 8, containing 0.1% Triton X-100. Lane 1and 2: Atl AM ; lane 3: Lysostaphin . Lanes 4: Lysostaphin boiled, Lane 5 and 6: Atl GL

3.3.4 Circular Dichroism (CD) spectroscopic studies for Atl AM

Circular Dichroism is defined as the unequal absorption of left-handed and right-handed circularly polarized light. When asymmetric molecules interact with light, they may absorb right and left handed circularly polarized light to different extents (hence the term circular dichroism). CD is an excellent method of determining the secondary structure of proteins. When the chromophores of the amides of the polypeptide backbone of proteins are aligned in arrays, their optical transitions are shifted or split into multiple transitions due to “exciton” interactions. The result is that different structural elements have characteristic CD spectra. For example, α-helical proteins have negative bands at

222 nm and 208 nm and a positive band at 193 nm. Proteins with well-defined antiparallel β-pleated sheets (β-helices) have negative bands at 218 nm and positive bands at 195 nm, while disordered proteins have very low ellipticity above 210 nm and negative bands near 195 nm.CD analysis of Atl AM showed that Atl AM has both α and β sheet.

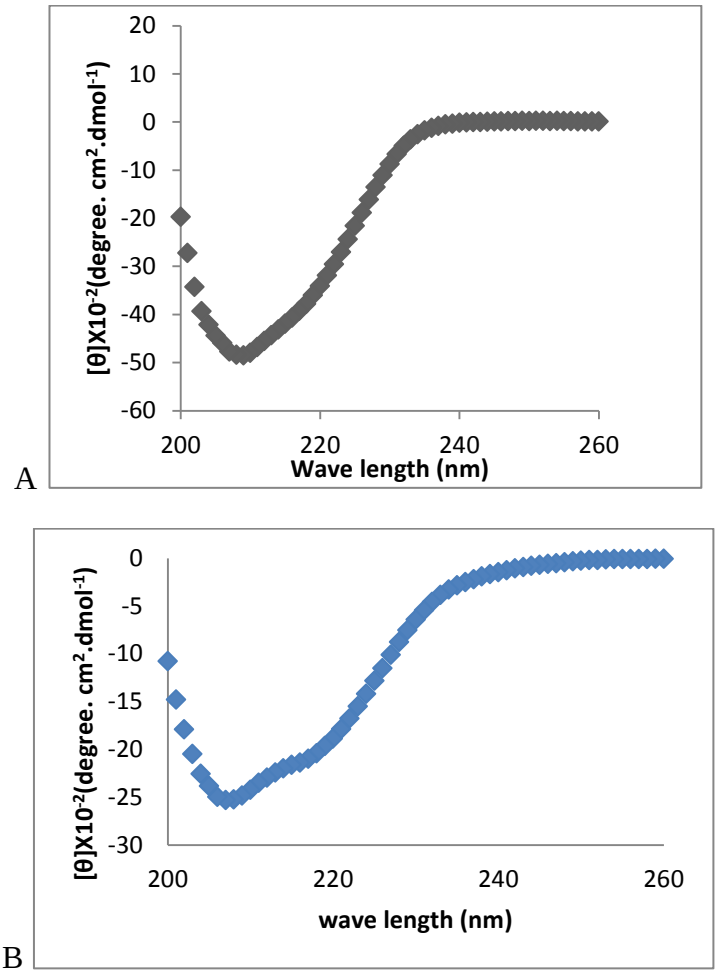


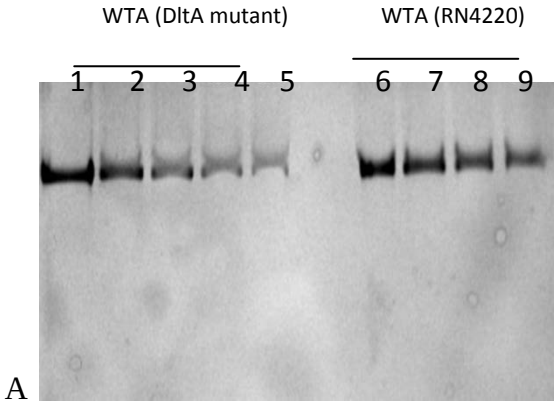
Figure 3.3.4 Circular Dichroism (CD) spectroscopic studies for Atl
A: Atl AM 10 µM in 50 mM sodium phosphate buffer (pH 7.6) at 22°C. Data points were corrected for buffer signal.
B: Atl GL 10 µM in 50 mM sodium phosphate buffer (pH 7.6) at 22°C. Data points were corrected for buffer signal.

3.3.5 Investigation of Atl and WTA interaction by acidic nPAGE/H^+

This interaction was investigated through native acidic polyacrylamide gel electrophoresis (native-H^+/PAGE). "Native" or "non-denaturing" gel electrophoresis is run in the absence of SDS. While in SDS-PAGE the electrophoretic mobility of proteins depends primarily on their molecular mass, in native PAGE the mobility depends on both the protein's charge and its hydrodynamic size. The electric charge driving the electrophoresis is governed by the intrinsic charge on the protein at the pH of the running buffer. This charge will, of course, depend on the amino acid composition of the protein. Since the protein retains its folded conformation, its hydrodynamic size and mobility on the gel will also vary with the nature of this conformation (higher mobility for more compact conformations, lower for larger structures like oligomers). If native PAGE is carried out near neutral pH to avoid acid or alkaline denaturation, then it can be used to study conformation, self-association or aggregation, and the binding of other proteins or compounds (protein-protein or protein-ligand) due to their mobility.

The WTA-binding properties of Atl were studied using WTAs isolated from *S. aureus* RN4220 and *dltA* mutant (where no D-Ala is present in the WTA). Incubation of Atl with WTAs resulted in a lack of electrophoretic resolution of the protein by the use of a native acidic gel. These reported results were reproduced by Shamina Prova. When protein and WTA interact together the protein is prevented from entering the gel. In these experiments protein concentration was kept constant and varied the WTA amount in the assay. These experiments show both AM and GL interacts with WTA but the amount of WTA needed to prevent GL from entering the gel is less than AM. Furthermore, *dltA*

mutant WTA showed higher affinity for AM and GL compare to *S. aureus* RN4220 WTA. WTA extracted from *dltA* mutant has no D-Ala in second position of ribitol. As a result, WTA from *dltA* mutant has more negative charge, which may attract cationic autolysins AM and GL.



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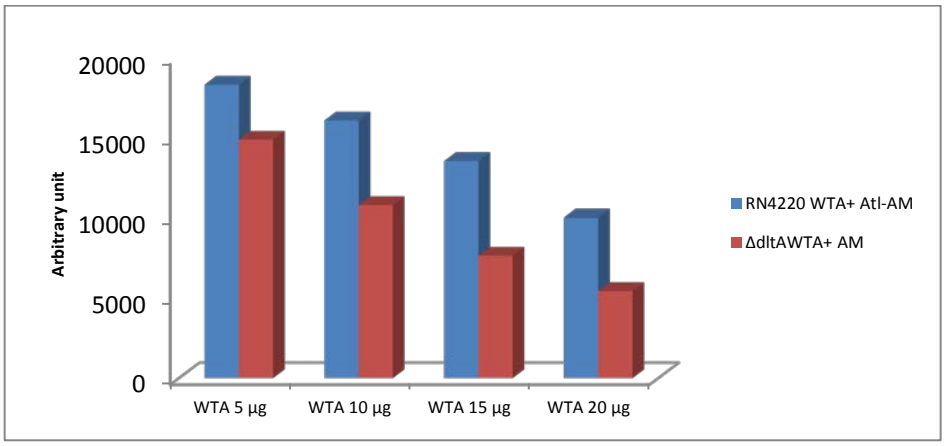
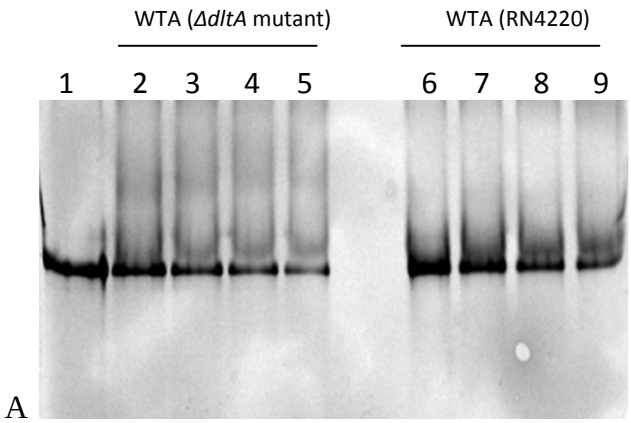


B

Figure 3.3.5.1 Investigation of Atl AM and WTA interaction by acidic nPAGE/H$^+$
A: Lane 1: Atl AM 10 µM, Lane 2: Atl AM 10 µM with 5 µg WTA (*ΔdltA* mutant), Lane 3: Atl AM 10 µM with 10 µg WTA (*ΔdltA* mutant), Lane 4: Atl AM 10 µM with 15 µg WTA (*dltA* mutant), Lane 5: Atl AM 10 µM with 20 µg WTA (DltA mutant), Lane 6: Atl AM 10 µM with 5 µg WTA (RN4220), Lane 7: Atl AM 10 µM with 10 µg WTA (RN4220), Lane 8: Atl AM 10 µM with 15 µg WTA (RN4220), Lane 9: Atl AM 10 µM with 20 µg WTA (RN4220)
B: Atl AM interaction comparison with RN4220 WTA and *ΔdltA*WTA



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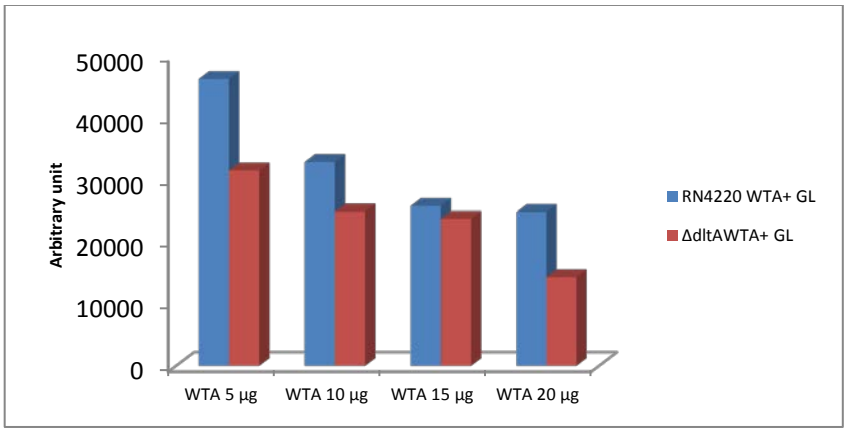

B

Figure3.3.5.2 Investigation of Atl GL and WTA interaction by acidic nPAGE/H$^+$
Lane 1: Atl GL 10 µM, Lane 2: Atl GL 10 µM with 5 µg WTA (*ΔdltA* mutant), Lane 3: Atl GL 10 µM with 10 µg WTA (*ΔdltA* mutant), Lane 4: Atl GL 10 µM with 15 µg WTA (*ΔdltA* mutant), Lane 5: Atl GL 10 µM with 20 µg WTA (*ΔdltA* mutant), Lane 6: Atl GL 10 µM with 5 µg WTA (RN4220), Lane 7: Atl GL 10 µM with 10 µg WTA (RN4220), Lane 8: Atl GL 10 µM with 15 µg WTA (RN4220), Lane 9: Atl GL 10 µM with 20 µg WTA (RN4220)
B: Atl GL interaction comparison with RN4220 WTA and *ΔdltA*WTA

3.3.6 Investigation of potential FmtA$^{\Delta 27}$ and Atl interaction by acidic nPAGE/H$^+$

Native PAGE can be used to study conformation, self-association or aggregation, and the binding of other proteins or compounds (protein-protein or protein-ligand) due to

their mobility. Acidic nPAGE/H^+ was used to investigate potential $FmtA^{\Delta 27}$ and Atl interactions. Different concentration of $Fmta^{\Delta 27}$ WT and Atl AM or Atl GL mixture was analyzed by 10% Acidic Native-PAGE. No changes in the mobility of $Fmta^{\Delta 27}$ WT and Atl AM or Atl GL from the mixed protein were observed compared to reference $Fmta^{\Delta 27}$ WT or AtlA-AM in the same gel. These results indicate no potential interactions between $Fmta^{\Delta 27}$ WT and Atl.

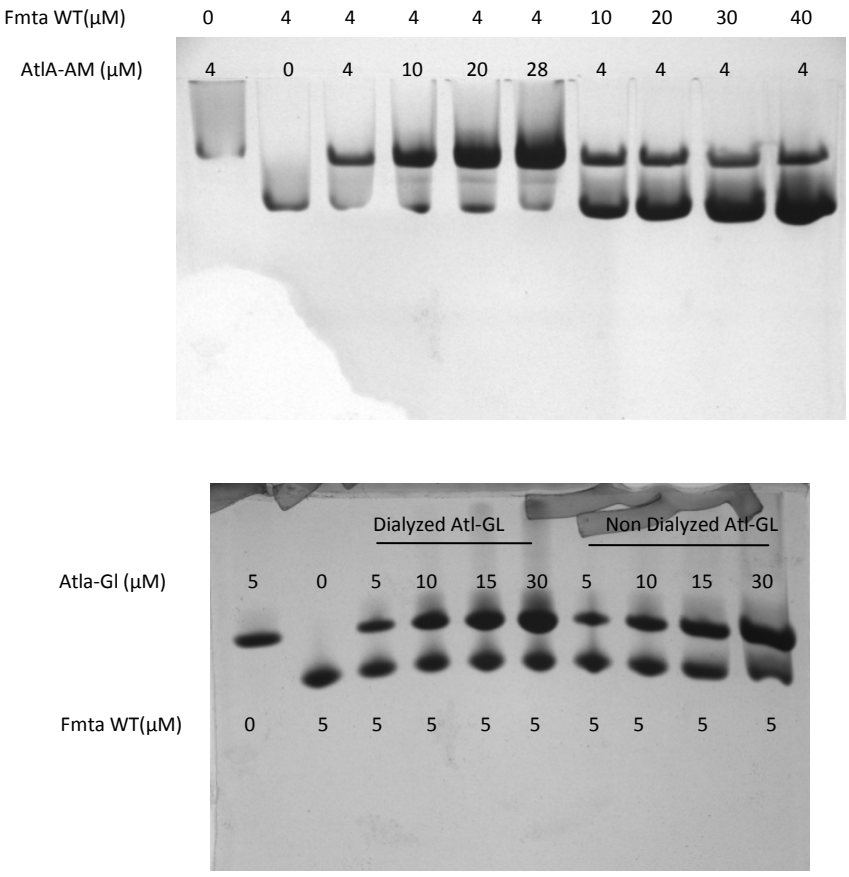

Figure 3.3.6 Investigation of $FmtA^{\Delta 27}$ and Atl AM/ Atl GL interaction with by acidic nPAGE/H^+

Different concentration of $Fmta^{\Delta 27}$ WT and AtlA-AM /Atl GL was mixed and incubated at RT for 1 hr in a total volume of 15 µL. Native loading dye was added with protein and

analyzed by 10% Acidic Native–PAGE. Protein bands were visualized by coomassie blue staining. Only Fmta $^{\Delta 27}$ WT or AtlA-AM/ Atl GL was used as a control.

3.3.7 Investigation of FmtA$^{\Delta 27}$ and Atl interaction with by gel filtration (HPLC)

Gel filtration (or size-exclusion) chromatography is a method for separating macromolecules on the basis of differences in Stokes' radii (Ackers et al, 1975). The chromatographic resin consists of porous particles made up of cross-linked polymers. Separation of proteins is achieved by partitioning between the mobile phase, the solvent that is exterior to the chromatographic particles, and the stationary phase, consisting of the solution within the porous particles themselves. The volume at which a protein elutes from the column is a direct reflection of the amount of time it spends within the porous particle. Thus, larger proteins, which are less likely to penetrate the particle, elute earlier, and smaller proteins, which effectively spend more time in the stationary phase, elute later. Gel filtration chromatography has been used extensively for protein purification and for estimation of protein molecular weight. Because the interaction between two or more proteins results in the formation of a complex having a larger Stokes' radius than its constituents, gel filtration chromatography can also be applied to the detection and characterization of protein–protein interactions.

To study protein-protein interactions between FmtA and GL, reference FmtA and GL was loaded in the gel filtration column besides the mixure of FmtA and GL. Atl GL showed one single peak around 7.5 to 9 min when eluted with 50 mM Tris, 50mM potassium Chloride, 5 mM Magnesium Chloride (pH 7.4) on Size exclusion column G2000SW$_{XL}$. On the other hand, FmtA showed two peaks, one around 9 min and other

one around 10 min; most likely due to their dimer nature. HPLC analysis of Atl GL and FmtA mixture on Size exclusion column revealed same retention time compared to reference Atl GL or FmtA WT protein elution. These results suggest no potential interactions between FmtA and Atl GL.

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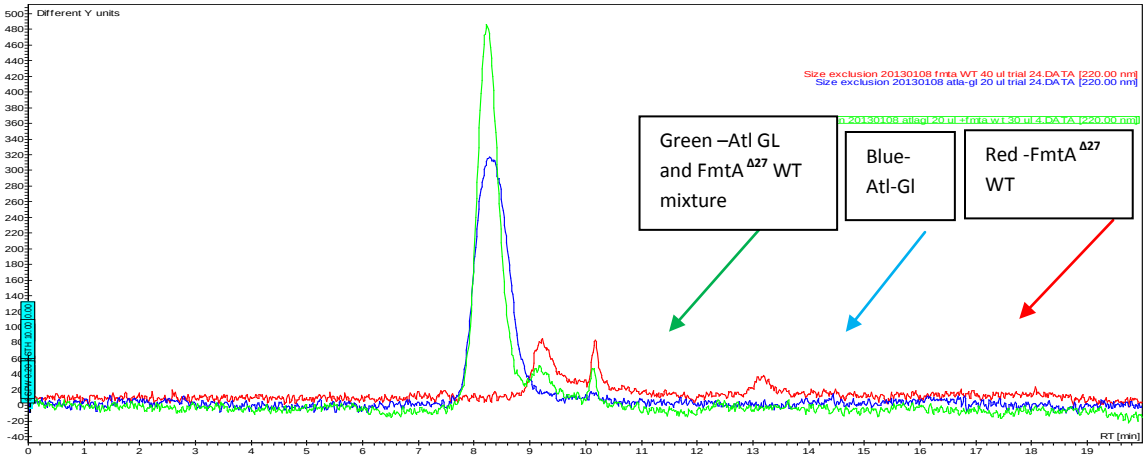

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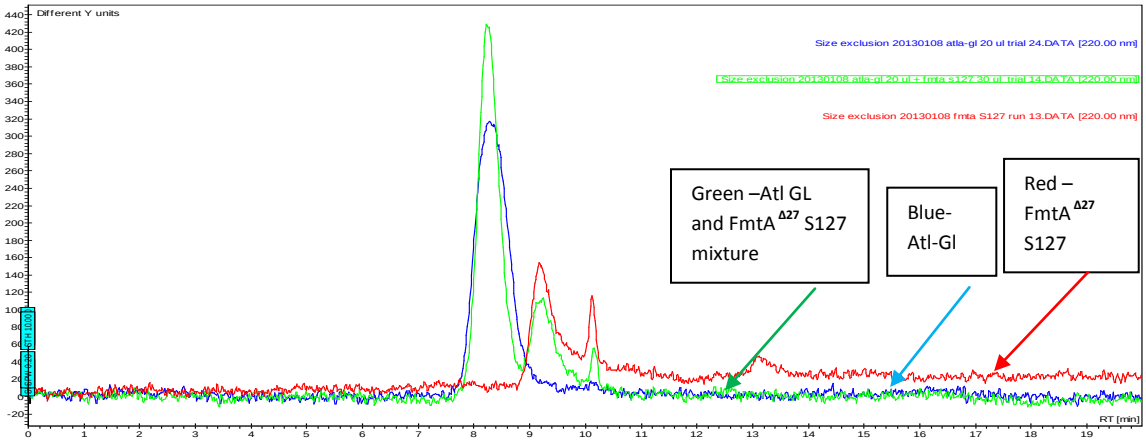
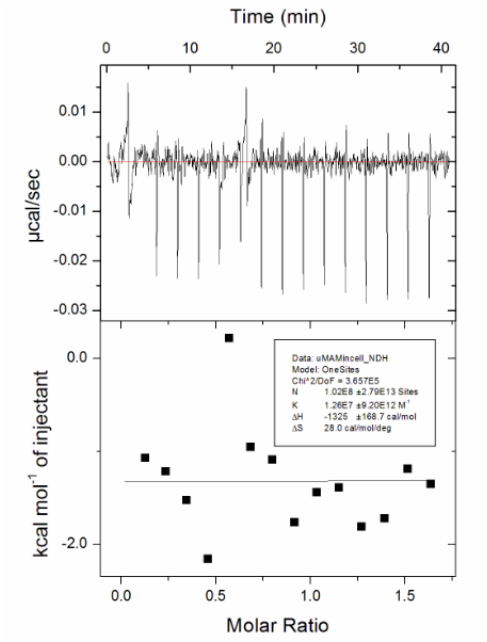


Figure 3.3.7 Investigation of FmtA$^{\Delta 27}$ and Atl interaction with by gel filtration (HPLC)

Atl GL and FmtA were injected separately as a reference for Atl GL and FmtA mixture analysis. Red color line represents FmtA WT, Blue color line represents Atl GL and green color line represents FmtA WT or FmtA S127 and Atl GL mixture. A: FmtA WT and Atl GL mixture analysis B: FmtA S127 and Atl GL analysis.

3.3.8 Investigation of FmtA$^{\Delta 27}$ and Atl interaction with by Isothermal Titration Calorimetry (ITC)

Isothermal Titration Calorimeters (ITC) measure the heat change that occurs when two substances interact. Heat is liberated or absorbed as a result of the redistribution of noncovalent bonds, for example, when the interacting molecules go from the free to the bound state. An ITC mixes the binding partners and monitors these heat changes by measuring the power required to maintain zero temperature difference between the reference and sample cells. When two proteins bind, there are changes in the thermodynamic potentials (ΔG, ΔH, ΔS), which can be measured directly by highly sensitive calorimetry. Investigation of protein-protein interactions between FmtA$^{\Delta 27}$ and AM by ITC showed no significant heat change. As a result, the data were not fitting well according to binding curve. These results suggest no potential interactions were FmtA$^{\Delta 27}$ and Atl AM and GL.



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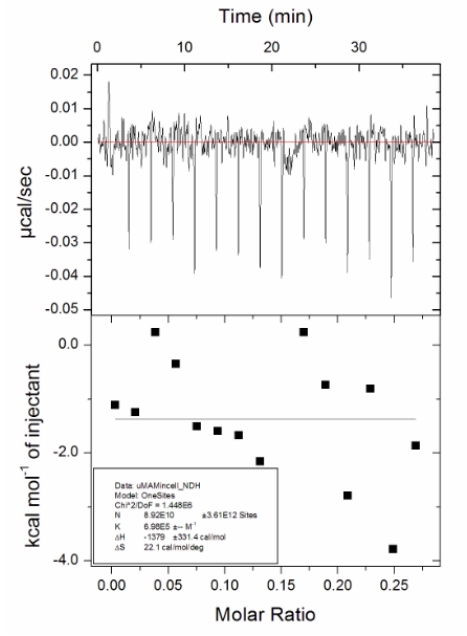

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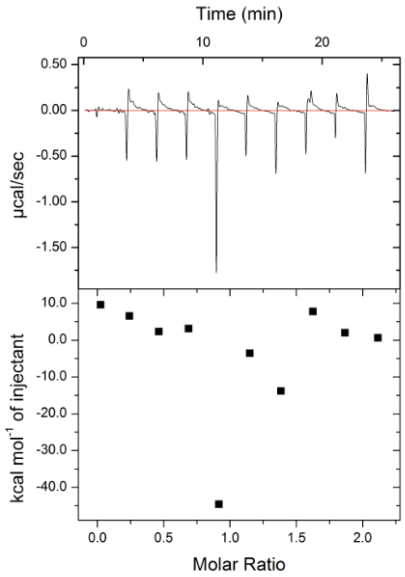

C

Figure 4.3.8 ITC analysis of the interaction between FmtA$^{\Delta 27}$ and Atl AM.
(A) Titration by injecting of FmtA wild type (46 µM) into the sample cell containing Atl AM (4 µM) (B) Titration by injecting of FmtA wild type (46 µM) into the sample cell containing Atl AM (28 µM) (C) Titration by injecting of FmtA wild type (63 µM) into the sample cell containing Atl GL (5 µM)

3.4 Discussion

The major enzyme that involved in cell lysis in *S. aureus* is autolysin (Atl). Atl is processed to generate two extracellular lytic enzymes, amidase, and an endo-β-N-acetylglucosaminidase which are noncovalently attached to the staphylococcal cell surface. We have cloned Atl AM with two repeat units (R1,2) and Atl GL with R3 unit from *S. aureus* Mu50. These proteins were expressed and purified to homogeneity. Atl AM (PI 9.62) and Atl GL (PI 9.64) are cationic proteins. These proteins were purified using one step purification strategy with S-support column, which is a strong cationic exchange column due to SO_3^- group. Furthermore, zymogram was used to check the enzymatic activity of these proteins, where substrate (heat killed *S. aureus* RN4220) was embedded in the resolving gel during preparation of the SDS-PAGE gel and triton X-100 was used for autolysis. Clear bands in zymogram showed Atl AM and Atl GL were active and had an enzymatic activity on substrate *S. aureus* RN4220 peptidoglycan cells. However, AM was enzymatically more active than GL on substrate *S. aureus* RN4220 peptidoglycan cells which is in agreement with reported literature (Bose et al., 2012). Currently it is unknown why *S. aureus* cell walls have a higher sensitivity to AM when compared to GL.

WTA is one of the key components of cell wall. WTA has many different functions: target and control of peptidoglycan biosynthetic proteins, resistance to harmful molecules such as antimicrobial fatty acids, antimicrobial peptides, and lysozyme. It has been shown that WTA plays a crucial role in targeting the Atl to the septum region. Recent studies have shown that presence of WTA reduces the affinity of Atl and of other

autolysins for *S. aureus* peptidoglycan. Atl binds preferentially to the cell division site where WTA appears to be less abundant or not yet fully matured (M Schlag et al., 2010). Concanavalin A (ConA) is a carbohydrate binding protein binds preferentially to teichoic acids. Binding studies with ConA-FITC conjugate revealed that the polymerization of WTA gradually increases with the distance from the cross-wall. This may explain the binding of AM and GL to cross wall for cell separation from daughter cell instead of binding to old cell wall (Götz et al., 2014).

It is very important to understand how WTA controls autolysin activity. Teichoic acids exhibit zwitterionic properties Due to negatively charged phosphate groups and positively charged amino groups in D-Ala residues. Negatively charged bacterial surface is important for the activity of cationic autolysins. So far there is no biochemical evidence of direct interaction between autolysin Atl and WTA. We have investigated the biochemical interactions between Atl and WTA by acidic nPAGE/H^+ and this is the first time to report about Atl and WTA direct biochemical interactions. WTA used for these experiments were extracted from well known laboratory strain *S. aureus* RN4220 and *ΔdltA* mutant (no D-Ala in WTA). Results from Atl and WTA experiments showed both AM and GL interacts with WTA, however, the amount of WTA required to prevent GL from entering the gel is less than AM. Moreover, WTA from *ΔdltA* mutant has showed higher affinity for AM and GL compare to wild type *S. aureus* RN4220 WTA. WTA extracted from *ΔdltA* mutant has no D-Ala in second position of ribitol. As a result, WTA from *ΔdltA* mutant has more negative charge, which may attract cationic autolysins AM and GL.

FmtA interacts with WTA and removes D-Ala from ribitol (Chapter 2). Therefore, FmtA might affect the activity of AtlA by modulating the negative charge of WTAs. FmtA localizes at the cell division septum, most likely by binding to the cross wall. AtlA also interacts with WTA and localized in cell division septum. To explore the possibility of interactions between FmtA and AtlA, we have examined protein-protein interactions of Atl and FmtA by using acidic native page, gel filtration (HPLC) and isothermal titration calorimetry methods. However, no potential protein-protein interactions of FmtA and Atl were observed from our experiments.

In conclusion, FmtA and Atl localize in cell division septum and in absence of WTA they distributed along the cell surface. We have shown that in absence of D-Ala in WTA, Atl interacts more to WTA most likely due to more negative charge in WTA. This may suggest that FmtA removes D-Ala from WTA and makes zwitterionic WTA (Chapter two) more negatively charge to facilitate the localization of Atl at cell division septum. It would be interesting to investigate the localization of Atl in *ΔfmtA* Strain. Our results also suggest no potential protein-protein interactions between FmtA and Atl AM or GL.

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CHAPTER FOUR

SUMMARY AND FUTURE DIRECTIONS

5.1 Summary and Future directions

Staphylococcus aureus is a virulent pathogen that is currently the most common cause of infections in hospitalized patients. Over time *S. aureus* has evolved to acquire resistance against various antibiotics. Methicillin resistant *S. aureus* (MRSA) shows resistance to not only methicillin but also almost all β-actams. It has developed two primary strategies to combat against β-lactam antibiotics: expression of β-lactamase and production of additional penicillin-binding protein 2a (PBP2a). The expression level of β-lactamase and PBP2a do not always coincide with the methicillin resistance level, which confers other factors are involved in methicillin resistance in MRSA (Chambers 1997; Rohrer and Berger-Bachi 2003).

FmtA is one of the methicillin resistance factors that is shown to be a part of the cell wall stimulon, a group of genes that is commonly induced upon treatment with cell wall-active antibiotics. Inactivation of *fmtA* gene exhibited reduced peptidoglycan cross-linking and methicillin resistance. To date, the biological function of FmtA is not well understood. It has been hypothesized that FmtA may participate in peptidoglycan biosynthesis under antibiotic-induced cell wall stress conditions (Qamar and Golemi-kotra, 2012).

Studies have shown that FmtA has low affinity for binding with β-lactams and a very weak D,D-carboxypeptidase activity. In lieu of this finding that FmtA interacts with WTA, we raised the question whether this interaction has any effect on the activity of FmtA as a D,D carboxypeptidase. Investigation of the function of FmtA revealed that WTA is not an activator for FmtA and that FmtA shows esterase catalytic activity towards D-alanyl ester of WTA and LTA. Active site motif of this catalytic activity was found to be SXXK motif by mutagenesis studies, which is similar to Family VIII carboxylestease. Both Serine and lysine from $S^{127}XXK^{130}$ motif are involved in esterase catalysis. Furthermore, our mutation studies suggest that both serine from SXXK motifs (S63 and S127) in FmtA are involved in catalysis.

During detail investigation of the esterase enzymatic activity of FmtA, we have observed that FmtA is very substrate specific (WTA or LTA) compare to synthetic esterase substrate and its enzymatic activity depends both on substrate and enzyme concentrations. Interestingly, high substrate concentration can inhibit enzymatic reaction of FmtA. Many enzymes are inhibited by their own substrates and this inhibition often has important biological functions. For example, Substrate inhibition of acetylcholinesterase enhances the neural signal and allows rapid signal termination (Shafferman et al., 1992).

WTAs are known to protect gram positive bacteria against harmful molecules and environmental stresses, control enzyme activities in the cell surface, control cation concentrations in the cell envelope and bind to receptors and surfaces (Weidenmaier et al.,2003; D'Elia et al., 2009). Most of these activities are controlled by the alteration of

charges in WTA. Incorporation of D-Ala in WTA is known to be an important mechanism by which net charge of the WTA become more positive due to free positively charged amino group. Studies have shown that lack of D-Ala decoration to WTA leads to increased susceptibility to cationic antimicrobial peptides. We have shown that FmtA can remove D-alanine from WTA and this could be an important mechanism of *S. aureus* to control cation homeostasis, D-alanine recycling and metabolism.

Besides WTA, tailoring modifications of D-alanyl esters are also observed in lipoteichoic acid (LTA) at the second position of glycerol. Although WTA and LTA follow different biosynthetic pathways and precursors, both teichoic acids use same biosynthetic machinery (*dltABCD* operon) for incorporation of D-Ala in their repetitive polyol (ribitol or glycerol) unit. It has been proposed that D-Ala is transferred to LTA and then to WTA (Reichmann et al., 2013). Recently, a new member of *dlt* operon named *dltX* was indentified which is a small protein with expected size of 5.9 kDa with unknown function (Reichmann et al., 2013). It is interesting how *dlt* operon incorporate D-Ala in WTA as well as LTA and FmtA removes it.

So far, biological functions of most of the esterases are unknown. A putative esterase gene, *lr1516* belonging to the COG β-lactamase family of penicillin-binding proteins from *L. reuterish* showed up-regulation while studying genome wide expression under acidic conditions (Wall et al., 2007). Precise function of this putative esterase is not known. It was postulated to be involved in changing the cell wall and thus increasing the tolerance of the cells towards acid (Wall et al., 2007). FmtA is considered to be a part of cell wall stimulon (McAleese et al., 2006); but its regulation is still unknown.

Amidases (AM) are normally localized to the septum region, but in the absence of wall teichoic acids they are delocalized and are distributed along the cell surface. Teichoic acids exhibit zwitterionic properties due to negatively charged phosphate groups and positively charged amino groups in D-Ala residues. Strains lacking D-Ala in teichoic acids showed more negative charge in their cell surface and control the activity of cationic autolysins. However, there is no biochemical evidence of direct interaction between autolysin Atl and WTA. We have shown that both amidase (AM) and glucosaminidase (GL) from *S. aureus* interact with WTA, however, the amount of WTA required to prevent GL from entering the gel is less than AM.

FmtA interacts with WTA and removes D-alanine from ribitol. Therefore, FmtA might affect the activity of Atl by modulating the negative charge of WTAs. FmtA localizes at the cell division septum, most likely by binding to the cross wall. AtlA also interacts with WTA and localized in cell division septum. To explore the possibility of interactions between FmtA and AtlA, we have examined protein-protein interactions of Atl and FmtA by using acidic native page, gel filtration (HPLC) and isothermal titration calorimetry methods. However, no potential protein-protein interactions of FmtA and Atl were observed from our experiments.

In conclusion, WTA is not an activator for FmtA $^{\Delta 27}$ and FmtA $^{\Delta 27}$ shows esterase catalytic activity towards D-alanyl ester of WTA and LTA. Active site motif of this catalytic activity was found to consist of SXXK motif by mutagenesis studies, which is similar to Family VIII carboxylesterase. Both serine and lysine from $S^{127}XXK^{130}$ motif are involved in esterase catalysis. Mutation studies suggest that seine located at position

S63 and S127 from SXXK motifs are involved in catalysis. Furthermore, esterase catalytic activity of FmtA $^{\Delta 27}$ is very substrate specific (compared to synthetic substrate) and the enzymatic activity depends both on substrate and enzyme concentrations. High substrate concentration can inhibit enzymatic reaction of FmtA. We have also demonstrated the biochemical evidence of direct interaction of AM and GL with WTA. Furthermore, no potential protein-protein interactions between Atl and FmtA were observed from our experiments. It would be interesting to synthesize D-alanyl glycerol and ribitol and study the enzyme kinetics of FmtA as an esterase. To date, biological functions of most esterases are unknown. To our knowledge, this is the first study that reports an esterase from *S.aureus* that has sequence similarity to PBPs and β-lactmase and can remove D-Ala from its substrate WTA and LTA.

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Appendix A

Atl AM expression optimization

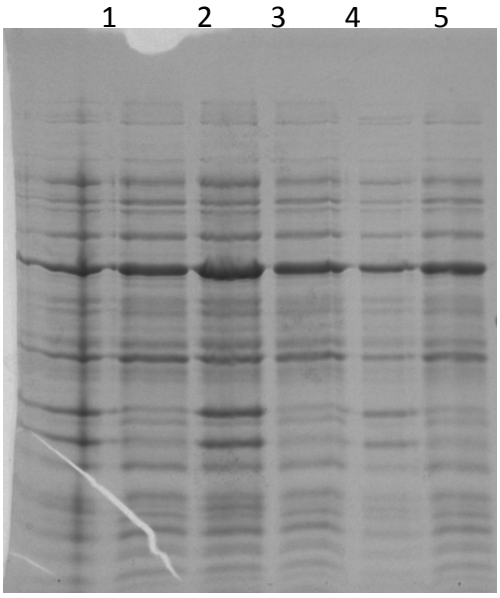

Figure: Atl AM expression optimization

Lane 1- pellet, 0.1 mM IPTG induced with 0.1M D-sorbitol and 2.5 mM β-Betaine at 16°C

Lane2- supernatant, 0.1 mM IPTG induced with 0.1M D-sorbitol and 2.5 mM β-Betaine at 16°C

Lane3- pellet, 0.1 mM IPTG induced with 0.1M D-sorbitol at 16°C

Lane4- supernatant, 0.1 mM IPTG induced with 0.1M D-sorbitol at 16°C

Lane5- pellet, 0.1 mM IPTG induced at 10°C

Lane6- supernatant, 0.1 mM IPTG induced at 10°C

Appendix B

Calculation for Activity of FmtA against PG analogue (L-Lys-D-Ala-D-Ala) by DD-Carboxypeptidase assay in presence of WTA as an activator (Figure 2.3.1.1)

				Fluorescence (AU) at 584 nm		
	Avg	**SD**	**Std Error**	**Trial 1**	**Trail2**	**Trial3**
Blank	38.43567	6.913199	3.991337	39.103	44.991	31.213
FmtA	34.613	4.387285	2.533	32.08	39.679	32.08
FmtA + D-Ala-D-Ala (6mM)	55.45833	2.146706	1.239401	53.329	57.622	55.424
D-Ala-D-Ala+WTA (0.1 µg/µL)	107.3987	13.81426	7.975666	91.917	111.812	118.467
FmtA + D-Ala-D-Ala+ WTA (0.1 µg/µL)	374.834	79.32415	45.79782	297.446	371.094	455.962
D-Ala-D-Ala+WTA (0.2µg/µL)	149.1129	111.03	64.10317	20.9457	210.453	215.94
FmtA+ D-Ala-D-Ala+WTA (0.2µg/µL)	769.3447	156.7096	90.47636	633.129	734.293	940.612
D-Ala-D-Ala+WTA (0.3µg/µL)	238.3373	2.32564	1.342709	238.707	240.456	235.849
FmtA+ D-Ala-D-Ala+WTA (0.3µg/µL)	964.8443	60.81348	35.11068	894.623	1000.008	999.902

Appendix C

Calculation for investigation of potential role of WTA as an activating ligand on FmtA $^{\Delta 27}$ activity (negative control) (Figure 2.3.1.2)

				Fluorescence (AU) at 584 nm	
	average	**Ave Dev**	**error**	**trial 1**	**trail 2**
Blank	44.439	0	0	44.439	44.439
FmtA WT	42.445	0.47	0.33234	41.975	42.915
FmtA WT + Lys-D-Ala-D-Ala	53.014	2.73	1.930402	50.284	55.744
FmtA WT + Lys-D-Ala-D-Ala+ *Δdlt* WTA (0.1 µg/µL)	58.951	6.665	4.712867	52.286	65.616
FmtA WT + Lys-D-Ala-D-Ala+ *Δdlt* WTA (0.2 µg/µL)	59.644	6.172	4.364263	53.472	65.816
FmtA WT + Lys-D-Ala-D-Ala+ *Δdlt* WTA (0.3 µg/µL)	58.2425	4.7845	3.383152	53.458	63.027

Appendix D

Calculation for investigation of potential role of WTA as an activating ligand on PBP2a activity (control for FmtA) (Figure 2.3.1.3)

				Fluorescence (AU) at 584 nm		
	AVG	**STD DEV**	**std error**	**Trial 1**	**Trial 2**	**Trial 3**
Blank	116.558	20.93426	12.0864	131.84	92.697	125.137
PBP2a	118.3303	21.33699	12.31891	132.506	93.791	128.694
PBP2a + Lys-D-Ala-D-Ala	138.8377	25.24123	14.57303	160.551	111.143	144.819
Lys-D-Ala-D-Ala + WTA (0.1 µg/µL)	194.1003	39.07136	22.55786	236.37	159.308	186.623
PBP2a+Lys-D-Ala-D-Ala+ WTA (0.1 µg/µL)	205.105	47.86551	27.63517	258.44	165.882	190.993
D-Ala-D-Ala + WTA (0.2 µg/µL)	287.384	74.53754	43.03427	373.106	237.841	251.205
PBP2a+Lys-D-Ala-D-Ala+WTA (0.2 µg/µL)	306.6293	97.80576	56.46818	419.248	257.651	242.989
Lys-D-Ala-D-Ala + WTA (0.3 µg/µL)	392.0643	89.25465	51.5312	494.413	330.404	351.376
PBP2a+ D-Ala-D-Ala + WTA(0.3 µg/µL)	436.0113	117.1785	67.65305	571.309	367.054	369.671

Appendix E

Calculation for investigation of potential role of WTA as an activating ligand on PBP2a activity (negative control) (Figure 2.3.1.4)

				Fluorescence (AU) at 584 nm	
	Average	**Std Dev**	**Std error**	**trail1**	**trial2**
Blank	133.79	2.757716	1.95	131.84	135.74
PBP2a	135.757	7.061168	4.993	130.764	140.75
PBP2a +Lys-D-Ala-D-Ala	168.996	1.907774	1.349	167.647	170.345
Lys-D-Ala-D-Ala+WTA Δ *dltA* (0.1 µg/µL)	168.4945	5.593922	3.9555	164.539	172.45
PBP2a +Lys-D-Ala-D-Ala+WTA Δ *dltA* (0.1 µg/µL)	168.8455	4.376284	3.0945	165.751	171.94
Lys-D-Ala-D-Ala+WTA Δ *dltA*(0.2 µg/µL)	183.029	3.423811	2.421	180.608	185.45
PBP2a+D-Ala-D-Ala+WTA Δ *dltA*(0.2 µg/µL)	169.579	1.359059	0.961	168.618	170.54
Lys-D-Ala-D-Ala+WTA Δ *dlt*A(0.3 µg/µL)	170.4555	4.277289	3.0245	167.431	173.48
PBP2a+ Lys-D-Ala-D-Ala+WTA Δ *dltA*(0.3 µg/µL)	175.3575	3.708775	2.6225	172.735	177.98

Appendix F

Calculation for Investigation of potential role of WTA as an activating ligand on PBP4 activity (Figure 2.3.1.5)

				Fluorescence (AU) at 584 nm	
	average	**std dev**	**std eror**	**trial 1**	**trial 2**
Blank	90.8655	10.90995	7.7145	83.151	98.58
Lys-D-Ala-D-Ala	124.9705	17.4224	12.3195	112.651	137.29
PBP4	158.363	13.55807	9.587	148.776	167.95
WTA (0.1 µg/µL)	125.4115	4.564374	3.2275	128.639	122.184
PBP4 + Lys-D-Ala-D-Ala	244.621	28.63782	20.25	264.871	224.371
Lys-D-Ala-D-Ala+WTA (0.1 µg/µL)	192.275	2.58094	1.825	194.1	190.45
PBP4 + Lys-D-Ala-D-Ala+ WTA (0.1 µg/µL)	275.1965	22.76672	16.0985	291.295	259.098
Lys-D-Ala-D-Ala + WTA (0.3 µg/µL)	391.3215	0.959544	0.6785	392	390.643
PBP4 +Lys-D-Ala-D-Ala + WTA (0.1 µg/µL)	432.855	71.38809	50.479	483.334	382.376

Appendix G

Calculation for investigation of potential esterase activity of FmtA $^{\Delta 27}$ on WTA (Figure 2.3.1.6)

				Fluorescence (AU) at 584 nm	
	Ave	**Std Dev**	**Std Error**	**trial 1**	**trial 2**
Blank	133.74	2.262742	1.6	132.14	135.34
FmtA WT	131.115	4.645692	3.285	127.83	134.4
FmtAS127	132.448	4.217185	2.982	129.466	135.43
PBP2a	135.2345	4.476693	3.1655	132.069	138.4
WTA (0.1 µg/µL)	174.83	5.048742	3.57	171.26	178.4
FmtAWT+ WTA (0.1 µg/µL)	940.6135	21.51514	15.2135	955.827	925.4
FmtAS127 + WTA (0.1 µg/µL)	773.1405	6.221833	4.3995	768.741	777.54
PBP2a+ WTA (0.1 µg/µL)	213.244	10.23325	7.236	206.008	220.48
WTA (0.2 µg/µL)	272.2555	10.59882	7.4945	264.761	279.75
FmtAWT + WTA (0.2 µg/µL)	994.725	7.459977	5.275	1000	989.45
FmtAS127 + WTA (0.2 µg/µL)	919.913	12.91601	9.133	929.046	910.78
PBP2a + WTA (0.2 µg/µL)	276.077	5.794033	4.097	280.174	271.98
WTA (0.3 µg/µL)	339.9575	0.880348	0.6225	339.335	340.58
FmtAWT + WTA (0.3 µg/µL)	993.995	8.492352	6.005	1000	987.99
FmtAS127 + WTA (0.3 µg/µL)	919.748	13.14936	9.298	929.046	910.45
PBP2a+ WTA (0.3 µg/µL)	349.423	1.636245	1.157	348.266	350.58

Appendix H

Calculation for investigation of potential esterase activity of FmtA $^{\Delta 27}$ on WTA (control experiments) (Figure 2.3.1.7)

				Fluorescence (AU) at 584 nm	
	avenge	**std dev**	**Std error**	**trial1**	**trial 2**
Blank	122.279	6.676502	4.721	127	117.558
FmtA WT (Boilled)	149.2805	7.11703	5.0325	154.313	144.248
FmtAS127 (Boilled)	122.028	15.39654	10.887	132.915	111.141
PBP2a (Boilled)	141.2935	18.48024	13.0675	154.361	128.226
WTA (0.1 µg/µL)	270.045	35.92385	25.402	295.447	244.643
FmtAWT (B)+ WTA (0.1 µg/µL)	318.291	20.26851	14.332	332.623	303.959
FmtAS127(B) + WTA (0.1 µg/µL)	341.472	31.22018	22.076	363.548	319.396
PBP2a(B)+ WTA (0.1 µg/µL)	355.82	117.0941	82.798	438.618	273.022
WTA (0.2 µg/µL)	479.8135	23.34513	16.5075	496.321	463.306
FmtAWT (B)+ WTA (0.2 µg/µL)	527.1345	44.87795	31.7335	558.868	495.401
FmtAS127(B) + WTA (0.2 µg/µL)	561.5895	107.6704	76.1345	637.724	485.455
PBP2a (B)+ WTA (0.2 µg/µL)	586.195	180.0379	127.306	713.501	458.889
WTA (0.3 µg/µL)	656.168	62.94947	44.512	700.68	611.656
FmtAWT(B) + WTA (0.3 µg/µL)	713.334	89.68518	63.417	776.751	649.917
FmtAS127(B) + WTA(0.3 µg/µL)	745.345	155.1661	109.719	855.064	635.626
PBP2a(B)+ WTA (0.3 µg/µL)	769.899	212.3442	150.15	920.049	619.749

Appendix I

The effect of enzyme concentration in the rate of removals of D-Ala from WTA by NMR (Figure 2.3.3.1)

FmtA WT 10 µM +WTA RN4220 5mg/mL (integration: CH3 of Glc-NAc,1) (Free D-Ala peak around 1.45 ppm)

trial 1		
time (hr)	**D-Ala-Ribitol**	**Free D-Ala**
0.08	0.9501	0.044
0.31	0.8898	0.1375
0.45	0.8546	0.1698
0.81	0.8261	0.2126
1.01	0.8014	0.2317
1.21	0.7848	0.2643
1.43	0.7568	0.2821
1.63	0.7336	0.2714
1.83	0.7204	0.2998
2.25	0.6983	0.3358
2.65	0.6793	0.3372
3.26	0.6551	0.3638
19.85	0.5631	0.5043
22.1	0.5488	0.5067

trial 2		
time (hr)	**D-Ala-Ribitol**	**Free D-Ala**
0.08	0.9405	0.0251
0.18	0.9128	0.1148
0.38	0.8732	0.1618
0.58	0.8363	0.197
0.78	0.7939	0.2031
0.98	0.7731	0.2262
1.2	0.7497	0.2465
1.4	0.7342	0.2644
1.6	0.7287	0.2824
1.8	0.7116	0.2973
2	0.7137	0.3315
2.35	0.6931	0.3308
15.33	0.5734	0.5021
17.9	0.564	0.5116
23.76	0.5539	0.5252

trial 3		
time (hr)	**D-Ala-Ribitol**	**Free D-Ala**
0.08	0.9504	0.0349
0.2	0.9121	0.1033
0.4	0.8806	0.1447
0.6	0.8505	0.1668
0.81	0.8237	0.172
1.01	0.8127	0.219
1.21	0.7809	0.2128
1.41	0.7598	0.2319
1.61	0.7471	0.2503
2.23	0.7097	0.2909
2.43	0.7028	0.3155
2.85	0.696	0.3602
3.66	0.6726	0.3784
4.06	0.6608	0.3825
4.48	0.6545	0.4017
4.9	0.6448	0.4024
5.3	0.6379	0.4063
5.71	0.6397	0.414
12.25	0.5845	0.444
21.08	0.555	0.4865
21.08	0.555	0.4865

FmtA WT 20 µM +WTA RN4220 5mg/mL		
time (hr)	**D-Ala Ribitiol**	**Free D-ala**
0.08	0.8953	0.0879
0.18	0.83	0.1182
0.38	0.7728	0.2241
0.58	0.7178	0.2818
0.78	0.6762	0.3313
1	0.6644	0.4066
1.4	0.6183	0.4359
2.21	0.5903	0.4847
2.41	0.5914	0.495
2.63	0.5738	0.5089
2.83	0.5702	0.5229
3.03	0.558	0.5277
5.28	0.4811	0.5884
9.2	0.4252	0.652

FmtA WT 5 µM +WTA RN4220 5mg/mL		
time (hr)	**D-Ala Ribitiol**	**Free D-Ala**
0.03	0.9759	0.0174
0.23	0.9647	0.054
0.45	0.9479	0.0369
0.65	0.9418	0.069
0.83	0.9452	0.0823
1.05	0.9295	0.0908
1.9	0.9038	0.1203
7.95	0.8252	0.1974
8.1	0.8278	0.1989
23.4	0.758	0.2468
23.45	0.7544	0.2453

Appendix J

The effect of WTA concentration in the rate of removals of D-Ala from WTA (Figure 2.3.3.2) (integration: CH3 of Glc-NAc, 1) (Free D-Ala peak around 1.45 ppm)

WTA 5 mg/ml		
FmtA WT 10 µM+ WTA RN4220 5 mg/mL		
time (hr)	D-Ala - Ribitol	free D-Ala
0	0.9505	0.0426
0.5	0.8779	0.1101
1	0.8312	0.1704
1.5	0.7809	0.2052
2.05	0.7467	0.2439
2.55	0.715	0.2826
3.06	0.6827	0.3079
3.56	0.6612	0.3351
3.84	0.6526	0.3383
6.5	0.58	0.42
8.5	0.55	0.46
12.5	0.49	0.5
17.33	0.4477	0.5493
19.25	0.4336	0.5569
20.53	0.4166	0.5564
23.5	0.4123	0.5698
25.03	0.4051	0.5663

WTA 2.5mg/ml		
FmtA WT 10 µM+ WTA RN4220 2.5 mg/mL		
time (hr)	D-Ala - Ribitol	free D-Ala
0	0.9425	0.0631
1.03	0.7054	0.2837
2.51	0.5519	0.4614
3.53	0.4728	0.5363
4.55	0.4138	0.5603
5.56	0.3712	0.6205
6.6	0.349	0.6551
7.61	0.3254	0.6795
8.63	0.3023	0.6961
9.66	0.2816	0.7291
10.68	0.2621	0.7368
11.7	0.2536	0.7488
12.72	0.2385	0.757
13.75	0.2273	0.771
17.71	0.191	0.8032

WTA 1mg/ml		
FmtA WT 10 µM+ WTA RN4220 1 mg/mL		
time (hr)	D-Ala - Ribitol	free D-Ala
0	0.8769	0.1213
0.2	0.6993	0.2811
0.41	0.6115	0.3626
0.61	0.5491	0.4445
0.81	0.5007	0.4863
1.38	0.4116	0.5703
1.58	0.3782	0.5958
1.78	0.3579	0.6257
1.98	0.3395	0.6529
2.2	0.3242	0.6503
2.68	0.2914	0.6888
4.41	0.2142	0.7486
5.8	0.1957	0.7709
7.65	0.1634	0.8099
9.61	0.0995	0.8864

WTA 0.5mg/mL		
FmtA WT 10 µM+ WTA RN4220 0.5 mg/mL		
time (hr)	**D-Ala -Ribitol**	**free D-Ala**
0	0.8428	0.1388
0.53	0.5497	0.4465
0.75	0.4805	0.4995
0.95	0.4448	0.5251
1.15	0.3942	0.5731
1.35	0.3785	0.5918
3.06	0.2533	0.6775
4.65	0.1966	0.7233
5.13	0.1848	0.7321
5.58	0.1965	0.7338
5.81	0.1817	0.7577
6.15	0.1724	0.7635
7.51	0.1344	0.8074
9.5	0.1153	0.8337
11.51	0.1519	0.8699

WTA 7.5mg/mL		
FmtA WT 10 µM+ WTA RN4220 7.5 mg/mL		
time (hr)	**D-Ala -Ribitol**	**free D-Ala**
0	0.939	0.0419
0.13	0.9156	0.0649
1.55	0.7703	0.2222
3.41	0.6451	0.3711
4.88	0.5733	0.4227
5.86	0.3475	0.4561
7.55	0.3331	0.4889
7.61	0.3336	0.6845
9.35	0.3151	0.6983
11.05	0.3002	0.7064
12.61	0.2853	0.7262
15.73	0.2681	0.7417
16.78	0.2178	0.8035

Appendix K

Esterase activity of FmtA$^{\Delta 27}$ against p-NPA and p-NPB by continuous spectrophotometric assay (Figure 2.3.4.2)

	1mM pNPB in DMSO, 50 mM Sodium phosphate buffer pH,7.0				
	Ave	**std dev**	**std error**	**trial1**	**trail2**
Blank	0.00285	0.00021213	0.00015	0.0027	0.003
FmtA WT+ pNPB	0.00385	7.0711E-05	5E-05	0.0039	0.0038
PBP4 + pNPB	0.0028	0.00014142	1E-04	0.0027	0.0029
PBP2a+ pNPB	0.0029	0.00014142	0.0001	0.0028	0.003

	1mM pNA in DMSO, 50 mM Sodium phosphate buffer pH,7.0				
	Ave	**Std Dev**	**Std Error**	**Trial1**	**Trail2**
Blank	0.0067	0.00014142	1E-04	0.0066	0.0068
FmtA WT+ pNPA	0.0085	0.00014142	0.0001	0.0086	0.0084
PBP4 5uM + pNPA	0.00695	7.0711E-05	5E-05	0.0069	0.007
PBP2a 5uM + pNPA	0.00685	7.0711E-05	5E-05	0.0069	0.0068

Appendix L

Assessment of esterase activity of $FmtA^{\Delta 27}$ by NMR (Figure 2.3.5.2)

1 mM P nitrophenyl acetate-Blank	
time(hr)	peak 8.35 ppm
1.53	10.9
2.21	10.57
3.25	6.8
4.06	5.09
4.73	5.57
5.48	4.58
6.21	4.26
6.91	5.29
6.58	5.11
8.83	4.5
10.06	4.89
12.38	3.56
14.63	2.86
16.88	2.71
18.11	2.62
19.13	2.23
21.45	1.77
23.73	1.91
23.71	1.91
27.72	1.8
45.45	1.03

p-NPA 1mM+ FmtA WT 5 µM	
time(hr)	peak 8.35ppm
0.93	9.19
1.56	6.66
2.25	5.61
3.4	3.67
4.15	3.61
4.76	3.78
5.53	3.66
6.26	3.18
6.96	2.98
7.38	3.06
8.86	2.72
10.11	2.58
12.43	2.25
14.81	1.82
16.91	1.65
18.06	1.55
19.16	1.47
21.51	1.26
23.75	1.17
25.75	1
27.75	1.01
46.48	0.62

pNPA 1 mM + 5 µM PBP4	
time(hr)	peak 8.35ppm
0.6	20.96
1.11	16.56
1.9	14.59
2.7	12.95
3.41	7.53
4.3	6.24
5.41	5
6.31	4.62
6.15	4.91
8.5	4.15
9.85	3.89
11.26	3.73
12.73	3.53
14.4	2.92
15.9	2.77
18.38	2.48
20.91	1.96
23.35	2.01
25.25	1.89
26.61	1.72

1 mM pNitrophenyl Butyrate-Blank	
time(hr)	peak1 8.35 ppm
2.15	17.18
3.13	10.8
4.83	9.68
6.16	7.99
8.61	6.06
10	5.9
11.28	5.1
12.61	5.09
14.11	4.51
15.48	4.29
17.88	4.62
22.65	3.91
24.73	2.41
26.6	2.58

1 mM pNB+5 µM FmtA WT	
time(hr)	peak 8.35 ppm
0.58	0.06
0.91	0.05
1.78	0.08
2.63	0.1
3.61	0.1
4.81	0.14
5.83	0.23
6.83	0.3
7.85	0.28
9.76	0.29
11.66	0.41
13.58	0.46
15.5	0.48
17.41	0.51
20.28	0.63
20.783	0.62
24.63	0.7

1 mM pNB + 5 µM PBP4	
time(hr)	peak 8.35 ppm
3.33	10.73
4	8.56
4.68	13.12
5.45	8.93
6.18	7.04
6.88	7.54
7.51	6.71
8.78	6.14
10.03	5.8
11.35	5.41
14.6	4.32
16.85	4.83
17.96	3.76
19.08	3.6
21.41	3.83
23.7	3.5
25.66	3.34
27.7	3.18
46.43	1.91

Trial1 :1 mM D-ala -Methyl Ester blank	
time(hr)	**peak 1.53 ppm**
0.25	29.9
0.53	25.34
1.48	15.62
2.21	11.61
3.16	9.63
3.86	7.99
4.61	7.12
5.41	6.09
6.2	5.47
7.43	4.74
8.71	3.95
10.06	3.51
11.4	3.06
12.68	2.75
14.15	2.38
15.55	2.13
17.96	1.88
19.85	1.7
22.71	1.43
24.78	1.33
26.63	1.19

Trail 1 :1 mM D-ala ME +5 uM FmtA WT	
time(hr)	**peak 1.53 ppm**
0.31	17.18
0.6	20.59
0.53	13.81
2.3	10.45
3.21	7.93
3.9	7.16
4.65	6.71
5.45	5.36
7.46	4.3
6.23	4.8
8.75	3.74
10.11	3.11
11.45	2.73
12.73	2.31
14.28	2.03
15.58	1.99
18	1.63
19.9	1.52
22.76	1.27
24.83	1.21
26.66	1.13

Trial 1:1 mM D-ala ME +5 uM PBP4	
time(hr)	**peak 1.53 ppm**
0.35	23.31
0.63	22.41
1.56	14.51
2.36	11.6
2.25	9.36
3.95	7.76
4.68	6.91
5.51	5.83
6.28	5.12
7.51	4.76
8.8	4.11
10.15	3.48
11.51	3.04
12.81	2.81
14.38	2.44
15.65	2.25
18.08	1.99
19.96	1.74
21.8	1.5
24.86	1.38
26.71	1.27

Trial 2 :1 mM D-Ala -Methyl Ester blank	
time(hr)	peak 1.53 ppm
0.31	21.47
0.66	20.16
1.18	15.14
1.96	12.11
2.76	9.57
3.53	8.21
4.38	6.76
5.5	5.77
6.4	4.94
7.21	4.38
8.58	3.9
9.96	3.42
11.36	3.03
12.86	2.64
14.3	2.36
15.98	2.13
18.5	1.86
21	1.59
23.43	1.41
25.3	1.28
26.66	1.23

Trail 2 :1 mM D-Ala ME +5 µM FmtA WT	
time(hr)	peak 1.53 ppm
0.38	15.95
0.68	12.57
1.21	10.6
2.5	8.67
2.8	7.76
3.56	6.52
4.41	5.39
5.55	4.96
6.43	4.16
7.26	3.97
8.61	3.35
10.03	2.94
11.41	2.64
12.91	2.48
14.55	2.17
16.05	1.95
18.53	1.72
21.06	1.47
23.46	1.32
25.31	1.22
26.68	1.12

Trial 2:1 mM D-Ala ME +5 µM PBP4	
time(hr)	peak 1.53 ppm
0.4	21.1
0.68	18.77
1.23	14.51
2.01	10.37
2.81	8.9
3.56	7.57
4.45	6.53
5.58	5.62
6.46	4.87
8.38	3.79
10.06	3.41
11.5	2.94
13	2.67
14.58	2.37
16.08	2.16
18.58	1.83
21.11	1.6
23.5	1.42
25.33	1.31
26.7	1.23

Appendix M

Screening for potential esterase activity of FmtA$^{\Delta 27}$on cephalothin (Figure 2.3.5.3)

Blank: Cephalothin 3 mM	
Time ,hr	**Acetate peak 1.9ppm**
0	0.102
0.2	0.1051
0.416	0.1053
0.6166	0.1056
0.81	0.1071
1.03	0.1073
1.23	0.1093
1.65	0.1101
1.75	0.11
1.81	0.112
4.9	0.1193

FmtA WT 40 µM+Cephalothin 3 mM	
Time ,hr	**Acetate peak 1.9ppm**
0.2	0.1313
0.61	0.1346
0.81	0.1372
1	0.1381
3.71	0.1472
8.78	0.1562

Appendix N

Active site (SXXK motif) of FmtA$^{\Delta 27}$ WT for esterase activity (Figure 2.3.6) (integration: CH3 of Glc-NAc 1) (Free D-Ala peak around 1.45 ppm)

FmtA WT 10 µM+ WTA RN4220 5 mg/mL		
time (hr)	**D-Ala -Ribitol**	**free D-Ala**
0	1.0519	0.0265
0.2	1.1035	0.0577
0.41	0.9995	0.0811
0.61	1.002	0.1021
0.81	0.98	0.1224
1.2	0.9658	0.1574
2.23	0.9121	0.2088
3.75	0.8435	0.2618
4.61	0.8405	0.2783
5.36	0.8167	0.3026
6.55	0.8064	0.3206
8.75	0.7796	0.3564
8.78	0.7732	0.3534
10.3	0.7598	0.37
18	0.72	0.414
22.66	0.7085	0.4254
24.31	0.7221	0.4316
24.38	0.6831	0.4287
25.13	0.6812	0.4293
27.55	0.6627	0.4354

FmtA S63 10 µM+ WTA RN4220 5 mg/mL		
time (hr)	**D-Ala -Ribitol**	**free D-Ala**
0	1.0437	0.0177
0.61	1.0391	0.0193
1.23	1.041	0.0224
2.05	1.0375	0.0298
3.06	1.0226	0.0314
4.08	1.0306	0.0369
5.11	1.0257	0.0417
5.48	1.0147	0.0474
6.13	1.0182	0.0515
6.51	1.011	0.0491
7.53	1.012	0.0573
8.56	1.0108	0.06
9.58	1.0079	0.0609
10.25	1.0102	0.0619
13.03	0.9973	0.0686
14.63	0.9992	0.0772

FmtA S127 10 µM+ WTA RN4220 5 mg/mL		
time (hr)	**D-Ala Ribitol**	**free D-Ala**
0	1.0672	0.0401
0.91	1.0589	0.0469
3.6	1.0127	0.0648
5.8	0.9806	0.0784
8.03	0.9551	0.0989
9.48	0.9535	0.1137

FmtA K130 10 µM+ WTA RN4220 5 mg/mL		
time (hr)	**D-Ala Ribitol**	**free D-Ala**
0	1.0699	0.0101
0.4	1.0771	0.0354
4.58	1.0334	0.0497
5.31	1.026	0.0578
6.79	1.0188	0.0731
8.7	1.0068	0.0865
10.58	0.9994	0.0981

Appendix O

Inhibitors studies for esterase activity of FmtA$^{\Delta 27}$ WT (Figure 2.3.7)

FmtA WT 10 µM+ WTA RN4220 5 mg/mL		
time (hr)	**D-Ala - Ribitol**	**free D-Ala**
0	0.9505	0.0426
0.5	0.8779	0.1101
1	0.8312	0.1704
1.5	0.7809	0.2052
2.05	0.7467	0.2439
2.55	0.715	0.2826
3.06	0.6827	0.3079
3.56	0.6612	0.3351
3.84	0.6526	0.3383
6.5	0.58	0.42
8.5	0.55	0.46
12.5	0.49	0.5
17.33	0.4477	0.5493
19.25	0.4336	0.5569
20.53	0.4166	0.5564
23.5	0.4123	0.5698
25.03	0.4051	0.5663

NaF 1 mM+ FmtA WT 10 µM+ WTA RN4220 5 mg/mL		
time (hr)	**D-Ala - Ribitol**	**free D-Ala**
0	1.0576	0.032
0.18	1.0318	0.0578
0.38	1.0093	0.0828
0.58	0.9988	0.1086
0.6	0.995	0.1053
2.4	0.8976	0.2122
2.6	0.902	0.2262
3.28	0.8638	0.2574
4.03	0.8372	0.2851
5.3	0.8222	0.309
5.38	0.8174	0.3242
7.45	0.7861	0.3512
8.95	0.7596	0.3635
21.33	0.7061	0.4392
23.75	0.6914	0.4406
23.8	0.6931	0.4432
26.33	0.6894	0.4514

Eserine 1 mM+ FmtA WT 10 µM+ WTA RN4220 5 mg/mL		
time (hr)	**D-Ala - Ribitol**	**free D-Ala**
0	1.0492	0.1894
0.15	1.0235	0.2082
0.36	1.0075	0.2488
0.56	0.9837	0.2537
0.76	0.965	0.297
0.96	0.9515	0.3072
1	0.939	0.3156
2.3	0.8664	0.3853
3.03	0.8355	0.4222
4.4	0.7938	0.4713
6.43	0.747	0.5116
7.91	0.7253	0.537
20.36	0.6437	0.6124
22.81	0.6346	0.6314
25.4	0.6346	0.6449

Trial 1

FmtA WT 10 µM+ PMSF 1mM +WTA RN4220 5 mg/mL		
time (hr)	**D-Ala -Ribitol**	**free D-ala**
0	0.9348	0.0347
0.5	0.8455	0.1124
1	0.7879	0.1514
1.5	0.7184	0.1712
2	0.6872	0.2109
2.5	0.6601	0.2411
3.05	0.627	0.2549
3.56	0.6161	0.2623
4.06	0.595	0.279
4.56	0.57	0.3043
5.1	0.5529	0.3097
5.6	0.5425	0.3072
6.11	0.5228	0.3374
6.63	0.5183	0.3347
7.13	0.5098	0.3508
7.63	0.4949	0.3468
8.16	0.4954	0.3624
8.67	0.4777	0.3674
9.7	0.4625	0.3722
10.18	0.4637	0.3721
13.05	0.4243	0.3903
16.46	0.4153	0.3973
18.45	0.3964	0.4066
19.8	0.3861	0.4282
21	0.3769	0.4282
23.13	0.3744	0.4382
25.1	0.3308	0.4622

Trial 2

FmtA WT 10 µM+ PMSF 1 mM+ WTA RN4220 5 mg/mL		
time (hr)	**D-Ala -Ribitol**	**free D-ala**
0	0.9241	0.0541
0.4	0.8119	0.1175
1.58	0.647	0.2373
2.93	0.5311	0.3235
4.95	0.4223	0.3785
6.03	0.3945	0.4055
9.58	0.329	0.4599
9.73	0.3303	0.4652
10.9	0.321	0.474
11.88	0.3011	0.491
12.88	0.3036	0.5034
14.86	0.2664	0.5255
16.85	0.2566	0.5388
18.81	0.254	0.5508
20.8	0.2295	0.5632
22.26	0.2121	0.5871

Appendix P

Inorganic phosphate assay for WTA (Table 2) (Absorbance at 620 nm)

	blank	std	RN4220	CM2	CM2+IPTG	FmtA del
Num	1	2	3	4	5	6
Sample1	0.14	0.502	0.269	0.285	0.241	0.247
Sample2	0.123	0.496	0.26	0.286	0.242	0.248
Sample3	0.123	0.491	0.262	0.286	0.251	0.249

Appendix Q

Determination of whole-cell surface charge (Absorbance at 530 nm) (Figure 2.3.10)

	OD 530							
	Trial1	**Trial2**	**Trial3**	**Trial4**	**Trial5**	**Trial6**	**Trial7**	**Trial8**
CytC	0.4	0.402	0.44	0.481	0.43	0.45	0.421	0.429
RN4220	0.2996	0.24	0.34	0.18	0.29	0.28	0.276	0.322
fmtA-CM	0.1868	0.09	0.14	0.113	0.17	0.16	0.148	0.203
fmtA-CM+IPTG100			0.25		0.29	0.25	0.294	
fmtA-CM+IPTG 500	0.1345	0.19	0.29	0.139	0.29	0.29	0.272	0.316
FmtA Del	0.1398	0.16	0.09	0.1	0.18	0.18	0.155	0.212
dltA	0.089	0.07	0.04	0.027	0.03	0.03	0.037	0.027
	% of applied protein							
	Trial1	**Trial2**	**Trial3**	**Trial4**	**Trial5**	**Trial6**	**Trial7**	**Trial8**
CytC	100	100	100	100	100	100	100	100
RN4220	74.9	59.75	76.9	37.5	67.7	62	65.51	75.17
fmtA-CM	46.7	22.42	31.4	23.44	39.4	35.5	35.13	47.24
fmtA-CM+IPTG100			56.2		67.8	56.1	69.92	
fmtA-CM+IPTG 500	33.625	47.32	65.3	28.93	67.6	64.2	64.63	73.65
FmtA Del	34.95	39.85	21.1	20.86	42.1	39.7	36.74	49.53
dltA	22.25	17.43	8.58	5.51	6.9	5.87	8.69	6.25

	Mean % of applied protein	**Std dev**	**std error**
CytC	100	0	0
RN4220	64.93125	12.7822147	4.51919534
fmtA-CM	35.15125	9.33708189	3.30115696
fmtA-CM+IPTG100	62.4975	7.37138329	3.68569165
fmtA-CM+IPTG 500	55.66838	16.8352611	5.95216363
FmtA Del	35.60763	9.99944381	3.53533726
dltA	10.185	6.20616744	2.19421154

Appendix R

Removal of D-Ala of LTA by FmtA$^{\Delta 27}$ (Figure 2.3.11.1)

LTA from sigma 5mg/mL + FmtA WT 10 µM		
time	**peak around 1.62 ppm**	**peak around 1.46 ppm**
0.08	1.24	0.1198
0.7	1.31	0.1697
1.31	1.09	0.2536
1.93	1.02	0.2841
2.71	1.16	0.3303
4.56	0.914	0.4073
6.6	0.8523	0.4704
8.65	0.8726	0.5648
10.68	0.762	0.5464
12.71	0.7277	0.5673
16.46	0.7177	0.6816
23	0.6198	0.6539