Molecular Studies on Biofilm-associated-protein (Bap) in *Staphylococcus aureus*

Submitted by

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DECLARATION

I, hereby declare that the investigation presented in the thesis has been carried out by me. The work is original and has not been submitted earlier as a whole or in part for a degree/diploma at this or any other Institution/University.

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DEDICATIONS

I dedicate this thesis to my beloved wife, ***Namrata*** *and the sweetest daughter in the world,* ***Saanvi****. They have been a great source of motivation and inspiration. I also dedicate this thesis to my parents* ***Mrs. Geeta Shukla*** *and* ***Mr. Shashi Bhushan Shukla*** *for instilling the importance of hard work, sincerity and higher education. Without the support of my family, this thesis would not have been in existence.*

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1.1 Bacterial biofilms

Scientists primarily studied bacteria in its free living form i.e. planktonic form for over a century. Although studies on planktonic bacteria allowed us to understand many fundamentals of bacterial form, physiology, etc., understanding of bacterial life style was incomplete because those conditions do not represent the two different lifestyles of bacteria (planktonic and sessile). Since few decades, it was learned that bacteria can adhere to solid surfaces and form a slimy, slippery layers termed as biofilms (Zobell 1943). These bacterial biofilms are predominant on wet surfaces in nature including rivers, stagnant pools, man-made materials and biological matter and cause many environmental benefits and problems. These bacterial aggregates provide protection from a wide range of environmental factors such as fluctuating pH (Svensäter et al. 1997), exposure to UV light (Espeland and Wetzel 2001), dehydration and antimicrobial agents (Mah and O'Toole 2001, Stewart and Costerton 2001). Some reports claim the existence of bacterial biofilms, as old as 3.2-billion years (Rasmussen 2000). The study of bacteria in biofilms, largely pioneered by Costerton, opened up a completely novel and unexplored area of research about how bacteria live and interact with their environment (Costerton et al. 1978, Costerton et al. 1999).

1.2 Stages of bacterial biofilm development

Commonly, bacteria lives two kind of lifestyle; one a free-living planktonic lifestyle and another a static and sessile lifestyle known as biofilm (Monds and O'Toole 2009). A transition between these two different lifestyles involves many triggering factors such several environmental stimuli and a variety of internal factors such as lack of nutrients, etc. This whole life cycle is termed as biofilm life-cycle and it involves several stages as depicted in Figure. 1.1 (Shukla et al.

2014). Whenever a single motile bacterium comes in contact with a solid surface, the first stage of the new life cycle takes place in the form of **initial cellular adhesion** (Marshall 1985). This initial event is followed by a feeble and **reversible adhesion phase**. At this stage, different cues from the environment determine the production of additional adhesive molecules that would foster bacterial adhesion or bacteria will detach itself and settle at some other place (Davies et al. 1998, Marshall 1985). Further sensing of the local environment as a favourable one, the bacterial cells begin to divide and start the secretion of a sticky matrix called extracellular polymeric substances (EPS). The polymer complex comprises of polysaccharides, proteins, nucleic acids and lipids (Flemming and Wingender 2010). This stage of bacterial life cycle is known as **irreversible adhesion**. During this transformation, bacteria changes its phenotype and there is a change in the array of surface protein molecules and appendages (Stoodley et al. 2002). As long as conditions remain favourable, bacterial cells grow and divide along with secretion of EPS. There are formation of different types of unique structures at this stage of biofilm growth such as water channels and mushroom kind of structure. These fine structures allow a three dimensional architecture of the mature biofilms and this stage of biofilm growth is known as **biofilm maturation** (Costerton et al. 1999, Hall-Stoodley et al. 2004, Stoodley et al. 2002). After sensing some unfavourable conditions or augmented by various environmental cues such as nutrient depletion or oxidative stress (Fux et al., 2004, Boles et al. 2005, Hall-Stoodley and Stoodley, 2005), biofilm cells can switch over back to the planktonic life style and this process is called as **biofilm dispersal** (Boles and Horswill 2008, McDougald et al. 2012, Kiedrowski and Horswill 2011). At this stage inter-cellular communication, various dispersing

agents such as Dispersin B (Boles and Horswill 2008, Donelli et al. 2007), Phenol soluble modulins etc. play a critical role (Davies et al. 1998, Kiedrowski and Horswill 2011, Otto 2014, Stoodley et al. 2002). These reagents act as bio-surfactants, whereas few enzymes disrupt inter-bacterial interactions, assist in the active and passive dispersal of cells from the biofilm mass (Monds and O'Toole 2009).

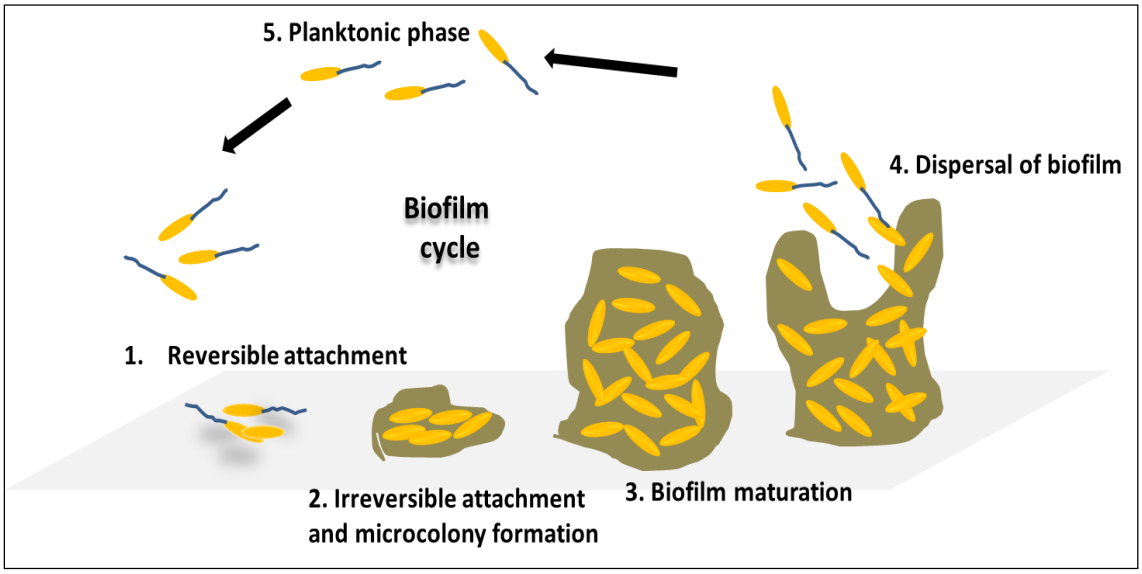


Figure 1.1: Illustration showing different developmental stages of bacterium biofilm cycle. (reproduced from Shukla et al. 2014)

1.3 Biofilm formation and persistent infections

Bacterial biofilms are ubiquitous and found in almost all kind of environments (Garrett et al. 2008). Industrial and clinical biofilms are of higher interest as they directly impact our lives. In clinical biofilms, *Staphylococcus aureus* biofilms are of particular interest as they grow in and on human host and are recognised as a major cause of persistent infections (Costerton et al. 1999, Gotz 2002, Otto 2008). *S. aureus* is a universal pathogen which causes mild to severely life threatening diseases (Cheesbrough 2006). Staphylococci are the

most common cause of infections that are associated with indwelling medical devices, such as catheters and prosthetic joints. The ability to form multicellular communities known as biofilms is crucial to the success of bacteria in device-related infections and probably also has a role in many other types of infection (Otto 2008, Otto 2009). Biofilm infections are clinically important because bacteria in biofilms cells are resistant to phagocytosis and, as some cells are in a dormant state, it is difficult to target them with antibiotics (Monds and O'Toole 2009, O'Toole et al. 2000).This bacterium also constitutes a major cause of hospital-acquired/healthcare-associated infections (HAIs). According to Center for Disease Control and prevention (CDC), *S. aureus* strains are associated with 15.6 % of the total HAIs reported between 2009 and 2010, (Sievert et al. 2013) and 12.3 % between 2011 and 2012 in Europe (Gravel et al. 2007).

1.4 The biofilm matrix

Microbial cells in biofilms are enclosed in self-produced polymeric matrix (Flemming and Wingender 2010). Although producing this matrix is not very economic but it renders the residing bacterial community a wide array of advantages such as protection from predation and harsh environments (Xavier and Foster 2007). The composition of biofilm matrix varies species to species and is dynamic in response to the environmental conditions (Mangwani et al. 2014). Over the years with huge amount of research done in this area, it is understood that there are a few classes of molecules that are typically present as the major components in the biofilm matrix, such as biofilm associated protein, adhesins, amyloid fibers, polysaccharides, extracellular DNA (eDNA), metal ions, etc., (Flemming and Wingender 2010, Sutherland 2001).

Table 1.1: Major components of extracellular polymeric substances and their function in bacterial biofilms (Flemming and Wingender, 2010)

EPS components	Functions in the context of biofilms
Polysaccharides	Adhesion, Aggregation of bacterial cells, Cohesion of biofilms, Protective barrier, Retention of water (by hydrophilic polysaccharide), Sorption of organic & Inorganic compounds, Sink for excess energy
Proteins	Enzymatic activity, Electron donor or acceptor, Adhesion, Aggregation of bacterial cells, Cohesion of biofilms, Protective barrier, Sorption of organic & Inorganic compounds, Sink for excess energy, Export of cell components
DNA	Exchange of genetic Information, Adhesion, Aggregation of bacterial cells, Cohesion of biofilms,
Divalent ions	Mechanical stability, Regulation of biofilm-associated proteins, Regulation of EPS production
Surfactants and lipids	Bacterial attachment and detachment, Hydrophobicity
Water	Provides hydrated environment, medium for movements for nutrients

A brief introduction of these macromolecules in context of bacterial biofilm is as follows.

1.4.1 Polysaccharides

Earlier exopolysaccharide was thought to be major component of the biofilm and constitutes 50-90% of the organic carbon in a biofilm that is found in the polysaccharide (Flemming and Wingender 2010, Xavier and Foster 2007).

However, a number of different polysaccharides are produced by different bacteria which are entirely different from each other. For example, *Staphylococcus aureus* produces a poly-cationic homo-polymer called as Polysaccharide Intercellular Adhesin (PIA) (Gotz 2002). Whereas *E. coli* and *Salmonella typhimurium* produce an uncharged, homo-polymeric cellulose (Zogaj et al. 2001). *Pseudomonas aeruginosa* produces at least three distinct exopolysaccharides viz., alginate Pel, and Psl (Chang et al. 2007, Franklin et al. 2011, Ryder et al. 2007). *Streptococcus thermophilus* produces three distinct heteropolymeric polysaccharides as part of its biofilm matrix (Vaningelgem et al. 2004). These polysaccharides can play many roles in the biofilm, including aiding in initial adherence to a surface (Ma et al. 2006), adding structural stability (Hentzer et al. 2001), serving as a nutrient storage reservoir (Laue et al. 2006), retaining water to prevent desiccation (Chang et al.2007), and protecting from the host immune system (Mishra et al. 2012).

1.4.2 eDNA

eDNA is also one of the major structural component of biofilms formed by many species (Whitchurch et al. 2002). The importance of this component in biofilms formed by many species can be seen in the fact that exogenous DNase addition can either inhibit or disperse biofilms of *E. coli* (Nijland et al. 2010), *P. aeruginosa* (Whitchurch et al.2002), *N. gonorrhoeae* (Steichen et al. 2011), *S. aureus* (Kaplan et al. 2012), and others. First of all, the importance of DNA in microbial biofilms was observed in *Rhodovulum*, a self-flocculating bacteria. *Rhodovulum* produces EPS that is comprised of polysaccharides, proteins and nucleic acids (Watanabe et al. 1998). Treatment of flocculated cells of *Rhodovulum* with nucleolytic enzymes resulted in deflocculation, whereas

polysaccharide-degrading and protein-degrading enzymes had no effect. Though, it has been identified as an important component, amount of eDNA produced by different bacterial biofilms can vary significantly, even between closely related species. For example, eDNA is a major structural component in the biofilm matrix of *S. aureus*, whereas it forms minor component of biofilms in *S. epidermidis* (Izano et al. 2008).In *P. aeruginosa* biofilms, eDNA functions as an intercellular connector (Yang et al. 2007), and *Bacillus cereus* uses eDNA as an adhesion molecule (Vilain et al. 2009). eDNA also aids in exchange of genetic information, aggregation of bacterial cells and determine the mechanical stability of biofilms. A recent study has shown that eDNA also facilitated the self-organization of cell inside bacterial biofilms (Gloag et al. 2013).

Origin of eDNA in biofilm matrix is not well understood. It is proposed that it comes from autolysis of a subpopulation of cells embedded within the biofilm matrix (Mann et al. 2009) or through a coordinated fratricidal mechanism lysing a subpopulation to release eDNA (Thomas et al. 2009). eDNA can also be generated without lysis such as through membrane vesicles by several gram negative bacteria, including *P. aeruginosa*, *E. coli*, *S. typhimurium*, and *Y. pestis* (Dorward and Garon 1990, Schooling et al. 2009) or by a type IV secretion system in *N. gonorrhoeae* (Hamilton et al. 2005).

1.4.3 Lipids and Biosurfactants

Extracellular polymeric substances (EPS) also contains substantial amount of lipids and alkyl group-linked polysaccharides, such as methyl and acetyl groups (Busalmen et al. 2002, Neu et al. 1992) which confers the hydrophobic property to EPS apart from highly hydrated hydrophilic molecules like polysaccharides, proteins and DNA. Many microbes produce surface-active

hydrophobic molecules which influence the surface tension at the air–water interface and called as bio-surfactants. These biosurfactant molecules are very critical for adhesion phenomenon and also play important role in dispersal phenomenon in biofilms (Sand and Gehrke 2006).

1.4.4 Proteins

Proteins are second most abundant component present in the biofilm matrix. In the last decade, several studies suggested that importance of extracellular and surface associated proteins in biofilm matrix in biofilm architecture and stability (Cucarella et al. 2001, Latasa et al. 2006). Broadly, they can be divided into two subcategories; Extracellular enzymes and structural proteins.

1.4.5 Extracellular Enzymes

Many extracellular enzymes have been reported in biofilms. In general, they also called as EPS modifying enzymes as these enzymes participate in the degradation of EPS (Flemming and Wingender 2010). EPS constituents act as a nutrient-store and when required, it is digested with the help of these extracellular enzymes (Kiedrowski and Horswill 2011). The substrates of these enzymes are biopolymers such as polysaccharides, cellulose, proteins and nucleic acids. Apart from this, they are also useful in the dispersal of biofilm and hence the release of microbial cells and resettlement on a new substratum occurs.

1.4.6 Structural Proteins/Protein adhesins

There are many different proteins in this category which are involved in surface adhesion, and interaction with host factors such as fibronectin, fibrinogen etc. In general, roles of surface proteins are highly redundant and

poorly understood. These structural proteins comprises either cell-wall associated proteins or extracellular and matrix associated proteins. Most of these structural proteins are classified Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs) that specifically recognize and interact with host factors to facilitate attachment of bacteria to surfaces within a host (Patti et al. 1994). These include proteins that bind to fibronectin, fibrinogen, collagen, and other host proteins (Deivanayagam et al. 2002, Keane et al. 2007). Amyloids fibres comes under matrix-associated proteins which are incredibly resilient fibers that were originally thought to be the result of protein folding error (Otzen and Nielsen 2008). Chapman et al., and others showed that amyloids can also have some functional role (Chapman et al. 2002, Chapman et al. 1999). A number of functional amyloids have been reported in the context of the biofilm matrix (Otzen and Nielsen 2008). Amyloids share unique folding properties though amyloid components of biofilms are highly diverse in different bacterial species. For example, *Bacillus subtilis* produces amyloids formed of the 261-residue TasA (Romero et al. 2010), while in *S. aureus*, amyloids are comprised of Phenol Soluble Modulin (PSM) peptides, which are as small as 20 residues long (Schwartz et al. 2012).

On the other hand, cell-wall associated proteins forms a large group which are associated with a number of functions such as adhesion, invasion and evasion (Foster et al. 2014). Structurally, they have vast differences, in *S. aureus*, which is based on structural similarity. Cell wall associated proteins are classified in to four major classes. A detail description is given in review of literature. Another group of extracellular proteins are biofilm-associated proteins (Bap) and

homologous Bap-like proteins (Latasa et al.2006). This family of proteins shares some common features like very high-molecular-mass, a core domain of tandem repeats and attachment with cell surface that promote biofilm formation. Apart from these, amyloids are the second most found proteinaceous components of the biofilm matrix. The amyloids have been detected ubiquitously in various types of environmental biofilms such as freshwater lakes, brackish water, drinking-water reservoirs and wastewater treatment plants (Otzen and Nielsen 2008).

1.5 *Staphylococcus aureus* biofilm regulation

As described in Figure 1, biofilm life cycle comprises of several phases which are tightly regulated at the molecular level and are in tune with the changing environment (Kostakioti et al. 2013, O'Toole et al. 2000, Stoodley et al. 2002). As biofilms are very diverse even within a species, it is obvious to expect their regulation to be differing from each other. In *S. aureus,* the most studied regulatory system is *ica*-operon that produces enzymes involved in the production of Polysaccharide Intercellular Adhesin (PIA). The *ica* genes were first discovered in a *S. epidermidis* (Mack et al. 1994) and later in *S. aureus* (Cramton et al. 1999).

The quorum-sensing system in *S. aureus* is known as Accessory Gene Regulator (*agr*) system (Yarwood et al. 2004) As quorum-sensing is often associated with biofilm formation, relationship between *agr* system and biofim formation has been heavily studied (Boles and Horswill 2008, Kiedrowski and Horswill 2011, Vuong et al. 2000). Relationship between *agr* system and biofilm formation in *S. aureus* has been found to be complex and dependent on the strains. As results of different studies have been mixed, with some studies showing *agr* mutations to inhibit, or have no effect, and some studies to showing increase in biofilm formation (Beenken et al. 2003, Vuong et al.2000). Similarly,

σ_B a stress response regulator in *S. aureus*, has been shown to up regulate as well as down regulate PIA production (Rachid et al. 2000, Valle et al. 2003). These observations suggest that PIA production in *S. aureus* is complex and dependent on strains, growth conditions (Yarwood et al. 2004). *ica* operon, thus PIA-dependent *S. aureus* biofilm formation is regulated by multiple genetic regulators (Tormo et al. 2005). Spx down regulates the PIA production (Pamp et al. 2006), whereas another global regulator, SarA (Staphylococcal Accessory Regulator), up regulates ica transcription (Valle et al. 2003). SarA also regulates expression of *agr*, however SarA mediated regulation of *ica* shown to be *agr*-independent (Beenken et al. 2010). Apart from genetic regulation mechanisms, several environmental conditions have been shown to regulate PIA production (Beenken et al. 2004, O'Gara 2007). Sub-inhibitory concentrations of certain antibiotics, including tetracycline, increase *ica* expression through poorly understood mechanism (Rachid et al. 2000).

Initially, the *ica* genes were thought to be indispensable and absolutely essential for biofilm formation in *S. aureus* (Cramton et al. 1999). Of late, few reports showed the existence of PIA-independent *S. aureus* biofilms (Cucarella et al. 2001, Otto 2008) and established the fact that few protein adhesins, such as biofilm-associated protein (Bap), were sufficient to establish biofilm formation, even if the *ica* operon was mutated (Beenken et al. 2004, Cucarella et al. 2001, Cucarella et al. 2004). In both cases, deletion of *sarA* abrogated biofilm formation, suggesting that SarA in plays a critical role in both PIA-dependent and PIA-independent biofilm development. Taken together, studies on PIA-independent *S. aureus* biofilm regulation indicates that PIA-independent biofilm development either involve up regulation of protein adhesins or down-regulate the production

of extracellular proteases (Boles and Horswill 2008, Karlsson et al. 2001, Kiedrowski and Horswill 2011, Luna-Velasco et al. 2013). In both the cases, net outcome is an increase in the abundance of extracellular protein adhesions, thereby emphasizing the importance of extracellular proteins in *S. aureus* biofilm development.

1.6 Importance of CWA proteins in *S. aureus* biofilm development

As discussed above, development of *S. aureus* biofilm is a complex process and depends on many factors. *S. aureus* biofilm cycle can also be divided into four phases; primary attachment, irreversible attachment, accumulation or maturation, and dispersal phase (Gotz 2002, O'Toole et al. 2000, Otto 2014). Primary attachment of Staphylococci cells can occur on inert such as the plastic, metal surface of an indwelling medical device etc., or biotic surfaces. Primary attachment to such surface particularly involve surface proteins/adhesins/components such as the accumulation-associated protein (Aap) (Conlon et al. 2014), autolysins AtlA (Houston et al. 2011, Bose et al. 2012) and AtlE (Rupp et al. 2001) or wall teichoic (WTA) and lipoteichoic acids (LTA) (Gross et al. 2001). Primary attachment to a biotic surface such as host tissues is mostly governed by cell wall-anchored (CWA) proteins including clumping factors A and B (ClfA and ClfB) (Deivanayagam et al.2002, Wertheim et al. 2008), Biofilm-associated protein (Bap) (Cucarellaet al.2001), and the fibronectin-binding proteins A and B (FnBPA and FnBPB) from *S. aureus* (Edwards et al. 2010, Peacock et al. 1999). Of late, several staphylococcal surface proteins are recognized that promote the accumulation phase in an *ica*-independent manner (Foster et al.2014). Thus, CWA proteins first mediate primary attachment and promote thereafter also participate in intercellular adhesion and biofilm

maturation (Figure 1.1). This is followed by the disintegration phase, in which the *S. aureus* biofilm is dispersed due to degradation of matrix protein components by proteolytic activity by extra-cellularly secreted protease through activation of *agr*- system (Boles and Horswill 2008), nucleases (Beenken et al. 2012, Kiedrowski and Horswill 2011, Sharma-Kuinkel et al. 2009), and a group of small amphiphilic α-helical peptides, known as phenol soluble modulins (PSMs) (Periasamy et al. 2012, Wang et al. 2011).

1.7 Cell-wall associated (CWA) Proteins in Staphylococci

Those surface proteins which are associated with cell wall of staphylococcal cells by a covalent bond are termed as cell-wall associated or cell-wall anchored (CWA) proteins (Foster et al. 2014). Cell-wall anchored surface proteins share few common features such as a long ~40 amino acids amino-terminal signal sequence which is required for Sec- dependent protein secretion, and a wall-spanning region at carboxyl-terminal that is either rich in proline and glycine residues or composed of Ser–Asp dipeptide repeats, and an LPXTG motif and a hydrophobic membrane-spanning domain followed by a series of positively charged residues (Schneewind et al. 1995). Secretory signal sequences that are located at the amino termini are cleaved during secretion. Ligand-binding functions are often located in the amino-terminal domains (Patti et al.1994). Majority of these proteins are anchored to peptidoglycan by LPXTG motif with the help of enzymatic activity of sortase A (Foster et al. 2014). In contrast, few CWA proteins has different sorting signals with the motif NPQxN/P such as Isd proteins are recognised and anchored to the cell wall by sortase B of *S. aureus* and *S. lugdunensis* (Foster et al. 2014).

The precise repertoire of CWA proteins varies among strains and also altered by growth and environmental conditions. Based on available literature it is concluded that *S. aureus* can express up to 24 different CWA proteins whereas coagulase negative Staphylococci such as *S. epidermidis* and *S. lugdunensis* express a smaller number (Foster et al. 2014). Moreover, the expressions of CWA proteins depend on the environmental conditions such as presence of iron. IsdA expresses only under iron-limited conditions (Clarke et al. 2007). Such limited number of surface protein and studies on CWA proteins suggest that CWA proteins in *S. aureus* can carry out multiple functions. This can be explained in terms of evolutionary selective pressure and fitness. As CWA proteins are exposed on the surface of bacterial cells and are in direct contact with the host to carry out multiple functions related to the adhesion, colonization, and the evasion of host defenses machinery etc., they remain under a constant selective pressure. Due to such selective pressure CWA proteins have evolved for multiple roles. Apart from having multifunctional feature, these proteins also tend to show functional redundancy, which means multiple proteins perform similar functions. For example, at least five CWA proteins bind the plasma glycoprotein fibrinogen and involve in adhesion function. This functional redundancy also affect the studies related to any particular protein as a null mutant that affects one particular CWA protein's function might only be partially defective as the function, which is under investigation, can be complemented by other CWA proteins.

1.8 Classification of CWA proteins

Based upon structural and functional characteristics, Foster et al. (2014) proposed a new classification of CWA proteins. As per this new classification, all CWA proteins can be classified in four families.

1. **MSCRAMM family:** MSCRAMM stands for ‘microbial surface component recognizing adhesive matrix molecules’. MSCRAMM term was originally applied to a group of surface proteins of *S. aureus* that were found to be involved in attachment to components of the host ECM, such as fibrinogen, fibronectin and collagen (Arrecubieta et al. 2007), at a time when it was thought that bacteria primarily attached to glycol-conjugates present on cell surfaces (Patti et al. 1994). Under new classification all those proteins come in MSCRAMM family which share structural similarities and a common mechanism for ligand binding, which is mediated by two tandemly-linked IgG-like sub-domains in the N-terminal A region (Foster et al. 2014, Geoghegan et al. 2013). In the typical MSCRAMMs SdrG, ClfA, and ClfB, the N2 and N3 sub-domains are associated to ligand-binding by the dock, lock, and latch (DLL) mechanism (Donelli et al. 2007, Ponnuraj et al. 2003). However, Cna, another member of the family binds its ligand by ‘collagen hug’ mechanism (Zong et al. 2005). The N terminal A region connects with the c-terminal cell wall-anchoring domain by serine-aspartate dipeptide repeats of varying length in the case of the Clf–Sdr subfamily (Arrecubieta et al. 2007), or tandem repeats of fibronectin binding domain in the case of FnBPs (Foster et al. 2014). SdrC, SdrD and SdrE have two or more repeated domains called B_{SDR} domains, which comprise 110–113 residues that are located between the A region and the flexible SD region (Josefsson et al. 1998). The B_{SDR} repeats are folded separately and form a rigid rod that projects the A domain away from the cell surface and that is dependent on Ca^{2+} for structural integrity (Josefsson et al. 1998).

2. **NEAT motif family:** These are a small group of CWA proteins which contains ‘Near iron transporter’ (NEAT) motif which are involved in heme capture from hemoglobin and help bacteria to survive in the host, where the presence of iron is very limited (Grigg et al. 2010). The defining characteristic of Isd CWA proteins is the presence of one or more NEAT motifs, which bind either hemoglobin or heme (Hammer and Skaar 2011).

3. **Three helical bundle family:** Protein A is a ubiquitous and multifunctional protein in *S. aureus* CWA protein. At the N terminus, protein A contains four or five homologous modules (known as EABCD), each of which consists of single separately folded three-helical bundles that can bind to several distinct ligands. An Xr region composed of octa-peptide repeats located between this region and the cell surface. Number of octa-peptide is highly variable in number and it is followed by a constant region, known as Xc (Foster et al. 2014).

4. **G5-E repeat family:** This group proteins contain identical G5 domains in a tandem array that is separated by 50-residue sequences that are known as E regions (Conrady et al. 2013, Gruszka et al. 2012). G5 domains are characterized by five conserved glycine residues which adopt a β–triple helix–β-like fold that has no known ligand-binding function. Amino acid sequence of each G5 domain are identical, and it is known that proteins with highly identical domains in a tandem arrangement are highly prone to undergo mis-folding (Borgia et al. 2011). It is speculated that the presence of alternatively individually folded E regions prevents protein mis-folding. SasG in *S. aureus* (Gruszka et al. 2012) and Aap in *S. epidermidis* (Conlon et al. 2014, Rohde et al. 2005) are well studied proteins in this category.

5. **Structurally uncategorised protein adhesins:** This group of surface proteins are comprised of proteins which are structurally not well defined. For example; SasX, which plays a role in biofilm formation (Li et al. 2012); SasC (Schroeder et al. 2009) reported to promotes primary attachment and accumulation phases of biofilm formation; and a few putative LPXTG proteins such as SasB, SasD, SasF, SasJ, SasK and SasL identified from genome sequences analyses but with unknown structure or function are recognized (Roche et al. 2003).

Another example of such protein adhesion is biofilm-associated protein (Bap) that was discovered in certain *S. aureus* strains tend to belong to MSCRAMM family but not yet classified due to partially understood structure and function (Latasa et al. 2006). Further investigation revealed the presence of Bap-like homologs in *Enterococcus faecalis* (Tendolkar et al. 2004, Tendolkar et al. 2005), *E. coli* (Roux et al. 2005), *Salmonella enteritidis* (Latasa et al. 2005) and other species.

1.9 CWA proteins involved in Staphylococci biofilm formation

As discussed earlier, the accumulation of staphylococci during biofilm formation was attributed only to the PIA, encoded by the *ica* operon 102, but later it was recognized that staphylococcal surface proteins can promote accumulation in an *ica*-independent manner. The CWA proteins that participate in biofilm formation are biofilm-associated protein (Bap) (Cucarella et al. 2001, Cucarella et al. 2004), ClfB (Wertheim et al. 2008), FnBPs (Geoghegan et al. 2013, O'Neill et al. 2008), SasC (Schroeder et al. 2009), SasG (Corrigan et al. 2007) and protein A (Gómez et al. 2004). Among these proteins, Bap, SasG and SasC are less studied and all aspects of molecular understanding of their functioning have

not been explored. These proteins have been reported to be present only in subsets of isolates, whereas the others CWA proteins are widely distributed among the population. There is an urgent need to study and develop fundamental understanding of such CWA proteins which are reported to play roles in biofilm formation but the mechanistic basis is less well understood. Molecular studies on Bap or CWA etc., are of importance and relevance to Methicilin Resistant *Staphylococcus aureus* (MRSA) as both hospital-associated and community-associated strains depend on proteins rather than polysaccharide for biofilm formation (Geoghegan et al. 2013, Lauderdale et al. 2009, O'Neill et al. 2008).

1.10 Biofilm associated Protein

The first surface protein identified by transposon mutagenesis that could induce biofilm development was Biofilm associated Protein **(**Bap) discovered in a mastitis isolate *S. aureus* V329 (Cucarella et al. 2001, Cucarella et al. 2004). Bap is a large protein of 2276 amino acids and molecular weight of 238 kDa, whose core region consists of 13 identical tandem repeats of 86 residues (Figure 1.2). Bap promotes biofilm formation in staphylococcal strains isolated from mammary glands in ruminants suffering from mastitis (Cucarella et al., 2001; Arrizubieta et al., 2004, Latasa et al. 2006). It is unclear whether Bap–mediated aggregation and biofilm formation is the result of homophilic interactions between two identical molecules expressed on the surface of neighbouring cells or Bap binds to its ligand present on the surface of its neighbouring cells by heterophilic interactions.

1.11 Structure of Bap protein in *S. aureus*

Primary sequence analysis of Bap proteins shows very similar structural characteristics as of a typical cell-wall-anchored proteins. The N-terminus domain has an extracellular secretary signal sequence (first 44 amino acids). The

remaining amino terminal sequence up to amino acid 818 can be divided into two regions. Region A (amino acids 45–360) contains two short repeats of 32 amino acids separated by 26 amino acids. Region B (amino acids 361–819) is devoid of repetitions and exhibits significant similarity with the *Enterococcus faecalis* surface protein (Esp) (Tendolkar et al. 2004,Tendolkar et al. 2005). Domain A and B are connected with C terminal region with a C- region, which is comprised of a core consisting of 13 tandem repeats of 86 amino acids, identical even at the nucleotide level (Cucarella et al. 2001). Although the function of this region has not been discerned, it is hypothesized that it could have a structural role, maintaining the proper protein conformation on the cell surface. Earlier report by Cucarella et al. (2004) demonstrated that there was production of Bap proteins with different lengths throughout the course of a staphylococcal infection due to the homologous recombination events between identical repeats. Interestingly, the number of repeats found to have negligible effect on the protein functionality (Cucarella et al.2004). The C terminus region comprises three short repeats of 18 amino acids, designated as region D, and a cell-wall-anchoring region consisting of an LPXTG motif and a series of positively charged residues (Figure1.2). Primary sequence analysis also reveal the presence of four putative potential EF calcium binding motifs within the Bap sequence. Three of them are located N-terminal to the C repeat region, whereas the fourth is located at the C terminus close to the LPXTG motif (Arrizubieta, 2004). Studied showed that the presence of calcium inhibited *Bap*-dependent multi-cellular behaviour in *S. aureus.* However, role of calcium in regulation of *S. aureus* biofilm is not very well understood.

1.12 Role of Bap in the *S. aureus* biofilm formation

Cucarella et al. (2001) reported the existence of cooperation between PIA production and Bap expression during the second step of biofilm formation as it was found that there was an enhanced PIA production in Bap overproducing strains and decreased levels of PIA was reported in strains where *bap* gene had been mutated. This observation suggests that Bap protein is not only involved in the primary attachment step, but also, together with the polysaccharide PIA production, in cell-to-cell aggregation and thus biofilm maturation. Importance of Bap protein in biofilm formation can be understood by the fact that *S. aureus* strains harbouring the gene *bap* were able to produce biofilm even if the *ica* operon, responsible for PIA production was disrupted (Cucarella et al. 2004). Thus, expression of the Bap protein is sufficient to promote biofilm development on abiotic surfaces in a PIA–independent manner.

1.13 Genetic regulation of Bap expression

Synthesis of such a large protein like Bap, has to be strictly regulated and coordinated with the production of other elements of the biofilm matrix such as PIA etc., to reduce the metabolic effort. Results shown by Trotonda et al., established the fact that SarA is a global virulence regulator positively regulates the *bap* expression (Trotonda et al. 2005). SarA can modulate gene transcription through a direct interaction with the target gene promoter or activating the *agr* regulatory cascade (Chien et al. 1999). In the case of the *bap* gene, results concluded that SarA directly binds to the *bap* promoter region. Besides, it was also shown that the disruption of *agr* system did not have any effect on *bap* transcription, indicating that the mechanism through which SarA regulates *bap* is *agr*-independent (Trotonda et al. 2005). In another study, it was shown that

disruption of SarA downregulates the transcription of the *ica* operon and thus PIA-dependent biofilm formation in *S. aureus* (Beenken et al. 2003, Trotonda et al., 2005, Valle et al. 2003).

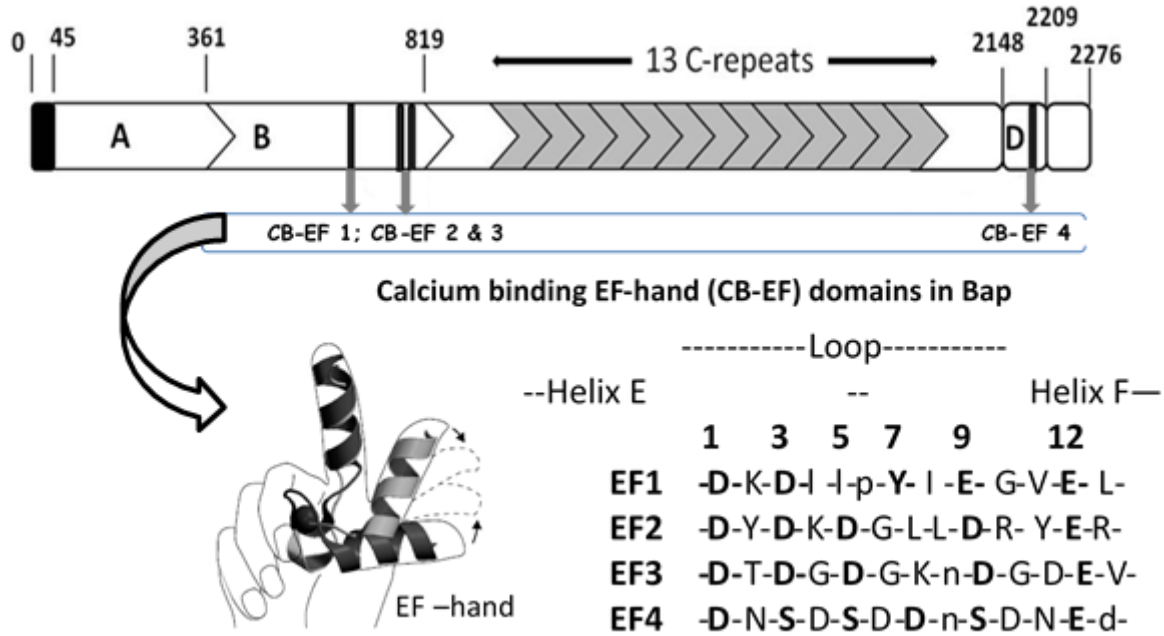


Figure 1.2: Schematic representation of Bap protein and EF hand motif. Bap length in *S. aureus* V329 is 2276 Amino acids and corresponding gene length is 6831 bp. Respective Ca^{2+} binding EF hand domains are also indicated in detail (Figure not on scale) A Symbolic representation of the EF-hand motif. Helix E winds down the index finger, whereas helix F winds up the thumb of a right hand. When the calcium ion binds, helix F moves from the closed (apoprotein, light grey) to the open (holoprotein, dark grey) conformation.

1.14 Rationale of this study

There are several reports on the role of surface proteins in *S. aureus* biofilm formation and its stability (Foster et al. 2014, Latasa et al. 2006, Whelan et al. 2013, Zhou et al. 2014). Among various surface proteins, biofilm-associated-

protein (Bap) was first reported as a large, multi-domain, cell surface anchored protein, which plays a crucial role in *S. aureus* biofilm development, architecture and in the pathogenesis of bovine mastitis (Cucarella et al.2001, Cucarella et al.2004, Stewart and Costerton 2001, Zuloaga et al. 2009). A recent study carried out in Brazil showed the presence of *bap* gene in all the coagulase-negative *Staphylococcus spp.* strains isolated from the nosocomial infections (Potter et al. 2009). Another recent report showed a higher frequency of occurrence of *bap* gene (56.6%) in *Staphylococcus* spp. (189 samples) isolated from bovine subclinical mastitis. Apart from this, frequency of *bap* gene occurrence was significantly higher in coagulase-negative strains as compared with coagulase-positive (Zuniga et al. 2015). The involvement of polysaccharide-intercellular adhesin (*ica*-dependent) component of the *S. aureus* biofilm matrix has been studied comprehensively (Kaplan et al. 2003). However, role of *ica*-independent mechanisms which is predominantly mediated by biofilm associated surface proteins (Bap, Aap, FnBPs, etc.) in the stability of staphylococci biofilm matrix is poorly understood (Whelan et al. 2013, Xiao et al. 2012, Yirui et al. 2009). Previous reports on *ica*-independent biofilm formation in *Staphylococci* showed a strong link between biofilm formation and cell wall associated proteins in particular, Bap, (Cucarella et al. 2001) the accumulation associated protein (Aap) (Yirui et al. 2009) and a Bap-homologue protein (Bhp) (Tormo et al.2005).

A better understanding of factors involved in primary attachment, intercellular communications, and their role in persistent infections of *S. aureus* are warranted to develop new vaccines and in providing effective treatment and control of *S. aureus*. For instance, a surface protein *of* BAP family in *A. baumannii,* BapA was found to be involved in early development of biofilm

architecture and its maturation (Loehfelm et al. 2008). In the recent past, an intensive *in silico* analysis of this protein has been done to identify antibody-triggering regions in BapA protein (Rahbar et al. 2010).This identification led to successful protection against *A. baumannii* infection via its functional deprivation of BapA (Fattahian et al. 2011) as well as enabled the researchers to develop a diagnostic test for precise identification of *A. baumannii* (Rahbar et al. 2012). Therefore, Bap and Bap-like surface proteins could be an important target to devise antibiofilm regimes. In broader perspective, thesis has following objectives to be accomplished:

- Determination of the functional role of different predicted domains in Bap protein.
- Cloning of the *bap* gene in a high expression vector.
- Investigation of intermolecular interaction between the Bap protein molecules and their role in the biofilm formation.
- Role of c-di-GMP, a secondary messenger molecules in phenotypic transformation, to be investigated in biofilm development of *Staphylococcus*.

Reference

Arrecubieta C, Lee M-H, Macey A, Foster TJ, Lowy FD. 2007. SdrF, a *Staphylococcus epidermidis* surface protein, binds type I collagen. J Biol Chem. 282:18767-18776.

Arrizubieta MJ, Toledo-Arana A, Amorena B, Penades JR, Lasa I. 2004. Calcium inhibits bap-dependent multicellular behavior in *Staphylococcus aureus*. J Bacteriol. 186:7490-7498.

Beenken KE, Blevins JS, Smeltzer MS. 2003. Mutation of sarA in *Staphylococcus aureus* limits biofilm formation. Infect Immun. 71:4206-4211.

Beenken KE, Dunman PM, McAleese F, Macapagal D, Murphy E, Projan SJ, Blevins JS, Smeltzer MS. 2004. Global gene expression in *Staphylococcus aureus* biofilms. J Bacteriol. 186:4665-4684.

Beenken KE, Mrak LN, Griffin LM, Zielinska AK, Shaw LN, Rice KC, Horswill AR, Bayles KW, Smeltzer MS. 2010. Epistatic relationships between sarA and agr in *Staphylococcus aureus* biofilm formation. Plos One. 5:5.

Beenken KE, Spencer H, Griffin LM, Smeltzer MS. 2012. Impact of extracellular nuclease production on the biofilm phenotype of *Staphylococcus aureus* under in vitro and in vivo conditions. Infect Immun. 80:1634-1638.

Boles B, Horswill A. 2008. Agr-mediated dispersal of *Staphylococcus aureus* biofilms. PLoS Pathog. 4.

Boles BR, Thoendel M, Singh PK. 2005. Rhamnolipids mediate detachment of *Pseudomonas aeruginosa* from biofilms. Mol Microbiol. 57: 1210–1223.

Borgia MB, Borgia A, Best RB, Steward A, Nettels D, Wunderlich B, Schuler B, Clarke J. 2011. Single-molecule fluorescence reveals sequence-specific misfolding in multidomain proteins. Nature. 474:662-665.

Bose JL, Lehman M, Fey PD, Bayles KW. 2012. Contribution of the *Staphylococcus aureus* Atl AM and GL murein hydrolase activities in cell division, autolysis, and biofilm formation. PloS one. 7:e42244.

Busalmen J, Vazquez M, De Sánchez S. 2002. New evidences on the catalase mechanism of microbial corrosion. Electrochimica Acta. 47:1857-1865.

Chang W-S, van de Mortel M, Nielsen L, de Guzman GN, Li X, Halverson LJ. 2007. Alginate production by *Pseudomonas putida* creates a hydrated

microenvironment and contributes to biofilm architecture and stress tolerance under water-limiting conditions. J Bacteriol. 189:8290-8299.

Chapman MR, Robinson LS, Pinkner JS, Roth R, Heuser J, Hammar M, Normark S, Hultgren SJ. 2002. Role of *Escherichia coli* curli operons in directing amyloid fiber formation. Science. 295:851-855.

Chapman PF, White GL, Jones MW, Cooper-Blacketer D, Marshall VJ, Irizarry M, Younkin L, Good MA, Bliss T, Hyman BT. 1999. Impaired synaptic plasticity and learning in aged amyloid precursor protein transgenic mice. Nat Neurosci. 2:271-276.

Cheesbrough M. 2006. District laboratory practice in tropical countries: Cambridge university press.

Chien Y-t, Manna AC, Projan SJ, Cheung AL. 1999. SarA, a Global Regulator of Virulence Determinants in *Staphylococcus aureus*, Binds to a Conserved Motif Essential for sar-dependent Gene Regulation. J Biol Chem. 274:37169-37176.

Clarke SR, Mohamed R, Bian L, Routh AF, Kokai-Kun JF, Mond JJ, Tarkowski A, Foster SJ. 2007. The *Staphylococcus aureus* surface protein IsdA mediates resistance to innate defenses of human skin. Cell Host Microbe. 1:199-212.

Conlon BP, Geoghegan JA, Waters EM, McCarthy H, Rowe SE, Davies JR, Schaeffer CR, Foster TJ, Fey PD, O'Gara JP. 2014. Role for the a domain of unprocessed Accumulation-Associated Protein (Aap) in the attachment phase of the *Staphylococcus epidermidis* biofilm phenotype. J Bacteriol. 196:4268-4275.

Conrady DG, Wilson JJ, Herr AB. 2013. Structural basis for Zn^{2+} dependent intercellular adhesion in staphylococcal biofilms. Proc Natl Acad Sci USA. 110:202-211.

Corrigan RM, Rigby D, Handley P, Foster TJ. 2007. The role of *Staphylococcus aureus* surface protein SasG in adherence and biofilm formation. Microbiology. 153:2435-2446.

Costerton JW, Geesey G, Cheng K. 1978. How bacteria stick. Scientific American. 86-95.

Costerton JW, Stewart PS, Greenberg E. 1999. Bacterial biofilms: a common cause of persistent infections. Science. 284:1318-1322.

Cramton SE, Gerke C, Schnell NF, Nichols WW, Götz F. 1999. The intercellular adhesion (ica) locus is present in *Staphylococcus aureus* and is required for biofilm formation. Infect Immun. 67:5427-5433.

Cucarella C, Solano C, Valle J, Amorena B, Lasa I, Penades JR. 2001. Bap, a *Staphylococcus aureus* surface protein involved in biofilm formation. J Bacteriol. 183:2888-2896.

Cucarella C, Tormo MA, Ubeda C, Trotonda MP, Monzon M, Peris C, Amorena B, Lasa I, Penades JR. 2004. Role of biofilm-associated protein *bap* in the pathogenesis of bovine *Staphylococcus aureus*. Infect Immun. 72:2177-2185.

Davies DG, Parsek MR, Pearson JP, Iglewski BH, Costerton J, Greenberg E. 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science. 280:295-298.

Deivanayagam CC, Wann ER, Chen W, Carson M, Rajashankar KR, Höök M, Narayana SV. 2002. A novel variant of the immunoglobulin fold in surface

adhesins of *Staphylococcus aureus*: crystal structure of the fibrinogen-binding MSCRAMM, clumping factor A. EMBO J. 21:6660-6672.

Donelli G, Francolini I, Romoli D, Guaglianone E, Piozzi A, Ragunath C, Kaplan J. 2007. Synergistic activity of dispersin B and cefamandole nafate in inhibition of staphylococcal biofilm growth on polyurethanes. Antimicrob. Agents Chemother. 51:2733-2740.

Dorward DW, Garon CF. 1990. DNA is packaged within membrane-derived vesicles of Gram-negative but not Gram-positive bacteria. Appl Environ Microbiol. 56:1960-1962.

Edwards AM, Potts JR, Josefsson E, Massey RC. 2010. *Staphylococcus aureus* host cell invasion and virulence in sepsis is facilitated by the multiple repeats within FnBPA. PLoS Pathog. 6:e1000964.

Espeland E, Wetzel R. 2001. Complexation, stabilization, and UV photolysis of extracellular and surface-bound glucosidase and alkaline phosphatase: implications for biofilm microbiota. Microb Ecol. 42:572-585.

Fattahian Y, Rasooli I, Gargari SLM, Rahbar MR, Astaneh SDA, Amani J. 2011. Protection against *Acinetobacter baumannii* infection via its functional deprivation of biofilm associated protein (Bap). Microb Pathog. 51:402-406.

Flemming H-C, Wingender J. 2010. The biofilm matrix. Nat Rev Microbiol. 8:623-633.

Foster TJ, Geoghegan JA, Ganesh VK, Höök M. 2014. Adhesion, invasion and evasion: the many functions of the surface proteins of *Staphylococcus aureus*. Nat Rev Microbiol. 12:49-62.

Franklin MJ, Nivens DE, Weadge JT, Howell PL. 2011. Biosynthesis of the *Pseudomonas aeruginosa* extracellular polysaccharides, alginate, Pel, and Psl. Front Microbiol. 2:167

Fux CA, Wilson S, Stoodley P. 2004. Detachment characteristics and oxacillin resistance of *Staphyloccocus aureus* biofilm emboli in an *in vitro* catheter infection model. J Bacteriol.186: 4486–4491.

Garrett TR, Bhakoo M, Zhang Z. 2008. Bacterial adhesion and biofilms on surfaces. Prog Nat Sci.18:1049-1056.

Geoghegan JA, Monk IR, O'Gara JP, Foster TJ. 2013. Subdomains N2N3 of fibronectin binding protein A mediate *Staphylococcus aureus* biofilm formation and adherence to fibrinogen using distinct mechanisms. J Bacteriol. 195:2675-2683.

Gloag ES, Turnbull L, Huang A, Vallotton P, Wang H, Nolan LM, Mililli L, Hunt C, Lu J, Osvath SR. 2013. Self-organization of bacterial biofilms is facilitated by extracellular DNA. Proc Natl Acad Sci USA. 110:11541-11546.

Gómez MI, Lee A, Reddy B, Muir A, Soong G, Pitt A, Cheung A, Prince A. 2004. *Staphylococcus aureus* protein A induces airway epithelial inflammatory responses by activating TNFR1. Nat Med.10:842-848.

Gotz F. 2002. Staphylococcus and biofilms. Mol Microbiol. 43:1367-1378.

Gravel D, Taylor G, Ofner M, Johnston L, Loeb M, Roth V, Stegenga J, Bryce E, Matlow A, Program CNIS. 2007. Point prevalence survey for healthcare-associated infections within Canadian adult acute-care hospitals. J Hosp Infect. 66:243-248.

Grigg JC, Ukpabi G, Gaudin CF, Murphy ME. 2010. Structural biology of heme binding in the *Staphylococcus aureus* Isd system. J Inorg Biochem.104:341-348.

Gross M, Cramton SE, Götz F, Peschel A. 2001. Key role of teichoic acid net charge in*Staphylococcus aureus* colonization of artificial surfaces. Infect Immun. .69:3423-3426.

Gruszka DT, Wojdyla JA, Bingham RJ, Turkenburg JP, Manfield IW, Steward A, Leech AP, Geoghegan JA, Foster TJ, Clarke J. 2012. Staphylococcal biofilm-forming protein has a contiguous rod-like structure. Proc Natl Acad Sci USA.109:E1011-E1018.

Hall-Stoodley L, Costerton JW, Stoodley P. 2004. Bacterial biofilms: from the natural environment to infectious diseases. Nat Rev Microbiol.2:95-108.

Hall-Stoodley L, Stoodley P. 2005. Biofilm formation and dispersal and the transmission of human pathogens. Trends Microbiol. 13: 7–10.

Hamilton HL, Domínguez NM, Schwartz KJ, Hackett KT, Dillard JP. 2005. *Neisseria gonorrhoeae* secretes chromosomal DNA via a novel type IV secretion system. Mol Microbiol. 55:1704-1721.

Hammer ND, Skaar EP. 2011. Molecular mechanisms of *Staphylococcus aureus* iron acquisition. Ann Rev Microbiol. 65.

Hentzer M, Teitzel GM, Balzer GJ, Heydorn A, Molin S, Givskov M, Parsek MR. 2001. Alginate overproduction affects *Pseudomonas aeruginosa* biofilm structure and function. J Bacteriol.183:5395-5401.

Houston P, Rowe SE, Pozzi C, Waters EM, O'Gara JP. 2011. Essential role for the major autolysin in the fibronectin-binding protein-mediated *Staphylococcus aureus* biofilm phenotype. Infect Immun. 79:1153-1165.

Izano EA, Amarante MA, Kher WB, Kaplan JB. 2008. Differential roles of poly-N-acetylglucosamine surface polysaccharide and extracellular DNA in *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms. Appl Environ Microbiol. 74:470-476.

Josefsson E, McCrea KW, Eidhin DN, O'Connell D, Cox J, Hook M, Foster TJ. 1998. Three new members of the serine-aspartate repeat protein multigene family of *Staphylococcus aureus*. Microbiology.144:3387-3395.

Josefsson E, O'Connell D, Foster TJ, Durussel I, Cox JA. 1998. The binding of calcium to the B-repeat segment of SdrD, a cell surface protein of *Staphylococcus aureus*. J Biol Chem. 273:31145-31152.

Kaplan JB, LoVetri K, Cardona ST, Madhyastha S, Sadovskaya I, Jabbouri S, Izano EA. 2012. Recombinant human DNase I decreases biofilm and increases antimicrobial susceptibility in staphylococci. J Antibiot. 65:73-77.

Kaplan JB, Ragunath C, Ramasubbu N, Fine DH. 2003. Detachment of Actinobacillus actinomycetemcomitans biofilm cells by an endogenous beta-hexosaminidase activity. J Bacteriol. Aug;185:4693-4698.

Karlsson A, Saravia-Otten P, Tegmark K, Morfeldt E, Arvidson S. 2001. Decreased amounts of cell wall-associated protein A and fibronectin-binding proteins in *Staphylococcus aureus* sarA mutants due to up-regulation of extracellular proteases. Infect Immun. Aug;69:4742-4748. Epub 2001/07/12.

Keane FM, Loughman A, Valtulina V, Brennan M, Speziale P, Foster TJ. 2007. Fibrinogen and elastin bind to the same region within the A domain of fibronectin binding protein A, an MSCRAMM of *Staphylococcus aureus*. Mol Microbiol. 63:711-723.

Kiedrowski MR, Horswill AR. 2011. New approaches for treating staphylococcal biofilm infections. In: Annals of the New York Academy of Sciences. 104-121.

Kostakioti M, Hadjifrangiskou M, Hultgren SJ. 2013. Bacterial biofilms: development, dispersal, and therapeutic strategies in the dawn of the postantibiotic era. Cold Spring Harbor perspectives in medicine. 3:a010306.

Latasa C, Roux A, Toledo-Arana A, Ghigo JM, Gamazo C, Penadés JR, Lasa I. 2005. BapA, a large secreted protein required for biofilm formation and host colonization of Salmonella enterica serovar Enteritidis. Mol Microbiol. 58:1322-1339.

Latasa C, Solano C, Penadés JR, Lasa I. 2006. Biofilm-associated proteins. C R Biol. 329:849-857.

Lauderdale KJ, Boles BR, Cheung AL, Horswill AR. 2009. Interconnections between Sigma B, agr, and proteolytic activity in *Staphylococcus aureus* biofilm maturation. Infect Immun. 77:1623-1635.

Laue H, Schenk A, Li H, Lambertsen L, Neu TR, Molin S, Ullrich MS. 2006. Contribution of alginate and levan production to biofilm formation by *Pseudomonas syringae*. Microbiology.152:2909-2918.

Li M, Du X, Villaruz AE, Diep BA, Wang D, Song Y, Tian Y, Hu J, Yu F, Lu Y. 2012. MRSA epidemic linked to a quickly spreading colonization and virulence determinant. Nat Med. 18:816-819.

Loehfelm TW, Luke NR, Campagnari AA. 2008. Identification and characterization of an *Acinetobacter baumannii* biofilm-associated protein. J Bacteriol.190:1036-1044.

Luna-Velasco MA, Esparza-García F, Cañízares-Villanueva RO, Rodríguez-Vázquez R. 2007. Production and properties of a bioemulsifier synthesized by phenanthrene-degrading *Penicillium* sp. Process Biochem. 42:310-314.

Ma L, Jackson KD, Landry RM, Parsek MR, Wozniak DJ. 2006. Analysis of *Pseudomonas aeruginosa* conditional psl variants reveals roles for the psl polysaccharide in adhesion and maintaining biofilm structure post attachment. J Bacteriol.188:8213-8221.

Mack D, Nedelmann M, Krokotsch A, Schwarzkopf A, Heesemann J, Laufs R. 1994. Characterization of transposon mutants of biofilm-producing *Staphylococcus epidermidis* impaired in the accumulative phase of biofilm production: genetic identification of a hexosamine-containing polysaccharide intercellular adhesin. Infect Immun.62:3244-3253.

McDougald D, Scott AR, Nicolas B, Peter DS, Staffan K. 2011. Should we stay or should we go: mechanisms and ecological consequences for biofilm dispersal. Nat Rev Microbiol. 10: 39-50.

Mah T-FC, O'Toole GA. 2001. Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol.9:34-39.

Mangwani N, Shukla SK, Rao TS, Das S. 2014. Calcium-mediated modulation of *Pseudomonas mendocina* NR802 biofilm influences the phenanthrene degradation. Colloids Surf B. 114:301-309.

Mann EE, Rice KC, Boles BR, Endres JL, Ranjit D, Chandramohan L, Tsang LH, Smeltzer MS, Horswill AR, Bayles KW. 2009. Modulation of eDNA release and degradation affects *Staphylococcus aureus* biofilm maturation. PloS one.4:e5822.

Marshall KC. 1985. Mechanisms of bacterial adhesion at solid-water interfaces. In: Bacterial adhesion. Springer. 133-161.

Mishra M, Byrd MS, Sergeant S, Azad AK, Parsek MR, McPhail L, Schlesinger LS, Wozniak DJ. 2012. *Pseudomonas aeruginosa* Psl polysaccharide reduces neutrophil phagocytosis and the oxidative response by limiting complement-mediated opsonization. Cell Microbiol. 14:95-106.

Monds RD, O'Toole GA. 2009. The developmental model of microbial biofilms: ten years of a paradigm up for review. Trends Microbiol.17:73-87.

Neu T, Dengler T, Jann B, Poralla K. 1992. Structural studies of an emulsion-stabilizing exopolysaccharide produced by an adhesive, hydrophobic *Rhodococcus* strain. J Gen Microbiol. 138:2531-2537.

Nijland R, Hall MJ, Burgess JG. 2010. Dispersal of biofilms by secreted, matrix degrading, bacterial DNase. PLoS One.5:e15668.

O'Gara JP. 2007. ica and beyond: biofilm mechanisms and regulation in *Staphylococcus epidermidis* and *Staphylococcus aureus*. FEMS Microbiol Lett. 270:179-188.

O'Neill E, Pozzi C, Houston P, Humphreys H, Robinson DA, Loughman A, Foster TJ, O'Gara JP. 2008. A novel *Staphylococcus aureus* biofilm phenotype mediated by the fibronectin-binding proteins, FnBPA and FnBPB. J Bacteriol. 190:3835-3850.

O'Toole G, Kaplan HB, Kolter R. 2000. Biofilm formation as microbial development. Annu Rev Microbiol.54:49-79.

Otto M. 2008. Staphylococcal biofilms. In: Bacterial biofilms. Springer. p. 207-228.

Otto M. 2009. *Staphylococcus epidermidis*—the 'accidental' pathogen. Nat Rev Microbiol. 7:555-567.

Otto M. 2014. Phenol-soluble modulins. Int J Med Microbiol. 304:164-169.

Otzen D, Nielsen PH. 2008. We find them here, we find them there: functional bacterial amyloid. Cellular and Molecular Life Sciences.65:910-927.

Pamp SJ, Frees D, Engelmann S, Hecker M, Ingmer H. 2006. Spx is a global effector impacting stress tolerance and biofilm formation in *Staphylococcus aureus*. J Bacteriol.188:4861-4870.

Patti JM, Allen BL, McGavin MJ, Hook M. 1994. MSCRAMM-mediated adherence of microorganisms to host tissues. Ann Rev Microbiol. 48:585-617.

Peacock SJ, Foster TJ, Cameron BJ, Berendt AR. 1999. Bacterial fibronectin-binding proteins and endothelial cell surface fibronectin mediate adherence of *Staphylococcus aureus* to resting human endothelial cells. Microbiology.145:3477-3486.

Periasamy S, Joo H-S, Duong AC, Bach T-HL, Tan VY, Chatterjee SS, Cheung GY, Otto M. 2012. How *Staphylococcus aureus* biofilms develop their characteristic structure. Proc Natl Acad Sci USA. 109:1281-1286.

Ponnuraj K, Bowden MG, Davis S, Gurusiddappa S, Moore D, Choe D, Xu Y, Hook M, Narayana SV. 2003. A “dock, lock, and latch” structural model for a staphylococcal adhesin binding to fibrinogen. Cell.115:217-228.

Potter A, Ceotto H, Giambiagi-Demarval M, dos Santos KR, Nes IF, Bastos Mdo C. 2009. The gene bap, involved in biofilm production, is present in *Staphylococcus* spp. strains from nosocomial infections. J Microbiol. 47:319-326.

Rachid S, Ohlsen K, Wallner U, Hacker J, Hecker M, Ziebuhr W. 2000. Alternative Transcription Factor ςB Is Involved in Regulation of Biofilm Expression in a *Staphylococcus aureus* Mucosal Isolate. J Bacteriol.182:6824-6826.

Rachid S, Ohlsen K, Witte W, Hacker J, Ziebuhr W. 2000. Effect of subinhibitory antibiotic concentrations on polysaccharide intercellular adhesin expression in biofilm-forming *Staphylococcus epidermidis*. Antimicrob Agents Chemother. 44:3357-3363.

Rahbar MR, Rasooli I, Gargari SLM, Amani J, Fattahian Y. 2010. In silico analysis of antibody triggering biofilm associated protein in *Acinetobacter baumannii*. J Theor Biol. 266:275-290.

Rahbar MR, Rasooli I, Gargari SLM, Sandstrom G, Amani J, Fattahian Y, Jahangiri A, Jalali M. 2012. A potential in silico antibody–antigen based diagnostic test for precise identification of *Acinetobacter baumannii*. J Theor Biol. 294:29-39.

Rasmussen B. 2000. Filamentous microfossils in a 3,235-million-year-old volcanogenic massive sulphide deposit. Nature.405:676-679.

Roche FM, Massey R, Peacock SJ, Day NP, Visai L, Speziale P, Lam A, Pallen M, Foster TJ. 2003. Characterization of novel LPXTG-containing proteins of *Staphylococcus aureus* identified from genome sequences. Microbiology.149:643-654.

Rohde H, Burdelski C, Bartscht K, Hussain M, Buck F, Horstkotte MA, Knobloch JKM, Heilmann C, Herrmann M, Mack D. 2005. Induction of *Staphylococcus epidermidis* biofilm formation via proteolytic processing of the accumulation-associated protein by staphylococcal and host proteases. Mol Microbiol. 55:1883-1895.

Romero D, Aguilar C, Losick R, Kolter R. 2010. Amyloid fibers provide structural integrity to Bacillus subtilis biofilms. Proc Natl Acad Sci USA. 107:2230-2234.

Roux A, Beloin C, Ghigo J-M. 2005. Combined inactivation and expression strategy to study gene function under physiological conditions: application to identification of new *Escherichia coli a*dhesins. J Bacteriol.187:1001-1013.

Rupp ME, Fey PD, Heilmann C, Götz F. 2001. Characterization of the importance of *Staphylococcus epidermidis* autolysin and polysaccharide intercellular adhesin in the pathogenesis of intravascular catheter-associated infection in a rat model. J Infect Dis. 183:1038-1042.

Ryder C, Byrd M, Wozniak DJ. 2007. Role of polysaccharides in *Pseudomonas aeruginosa* biofilm development. Curr Opin Microbiol. 10:644-648.

Sand W, Gehrke T. 2006. Extracellular polymeric substances mediate bioleaching/biocorrosion via interfacial processes involving iron (III) ions and acidophilic bacteria. Res Microbiol.157:49-56.

Schneewind O, Fowler A, Faull KF. 1995. Structure of the cell wall anchor of surface proteins in *Staphylococcus aureus*. Science.268:103-106.

Schooling SR, Hubley A, Beveridge TJ. 2009. Interactions of DNA with biofilm-derived membrane vesicles. J Bacteriol.191:4097-4102.

Schroeder K, Jularic M, Horsburgh SM, Hirschhausen N, Neumann C, Bertling A, Schulte A, Foster S, Kehrel BE, Peters G. 2009. Molecular characterization of a novel *Staphylococcus aureus* surface protein (SasC) involved in cell aggregation and biofilm accumulation. PloS one.4:e7567.

Schwartz K, Syed AK, Stephenson RE, Rickard AH, Boles BR. 2012. Functional amyloids composed of phenol soluble modulins stabilize *Staphylococcus aureus* biofilms. PLoS Pathog.8:e1002744-e1002744.

Sharma-Kuinkel BK, Mann EE, Ahn J-S, Kuechenmeister LJ, Dunman PM, Bayles KW. 2009. The *Staphylococcus aureus* LytSR two-component regulatory system affects biofilm formation. J Bacteriol. 191:4767-4775.

Shukla SK, Mangwami N, Rao TS, Das S. 2014. 8 Biofilm-Mediated Bioremediation of Polycyclic Aromatic Hydrocarbons. Microbial Biodegradation and Bioremediation. 203.

Sievert DM, Ricks P, Edwards JR, Schneider A, Patel J, Srinivasan A, Kallen A, Limbago B, Fridkin S. 2013. Antimicrobial-resistant pathogens associated with healthcare-associated infections: summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2009–2010. Infect Control Hosp Epidemiol. 34:1-14.

Steichen CT, Cho C, Shao JQ, Apicella MA. 2011. The Neisseria gonorrhoeae biofilm matrix contains DNA, and an endogenous nuclease controls its incorporation. Infect Immun. 79:1504-1511.

Stewart PS, Costerton JW. 2001. Antibiotic resistance of bacteria in biofilms. The lancet. 358:135-138.

Stoodley P, Sauer K, Davies DG, Costerton JW. 2002. Biofilms as complex differentiated communities. Annu Rev Microbiol. 56:187-209.

Sutherland IW. 2001. The biofilm matrix–an immobilized but dynamic microbial environment. Trends Microbiol. 9:222-227.

Svensäter G, Larsson UB, Greif E, Cvitkovitch D, Hamilton I. 1997. Acid tolerance response and survival by oral bacteria. Oral Microbiol Immun. 12:266-273.

Tendolkar PM, Baghdayan AS, Gilmore MS, Shankar N. 2004. Enterococcal surface protein, Esp, enhances biofilm formation by *Enterococcus faecalis*. Infect Immun. 72:6032-6039.

Tendolkar PM, Baghdayan AS, Shankar N. 2005. The N-terminal domain of enterococcal surface protein, Esp, is sufficient for Esp-mediated biofilm enhancement in *Enterococcus faecalis*. J Bacteriol. 187:6213-6222.

Thomas VC, Hiromasa Y, Harms N, Thurlow L, Tomich J, Hancock LE. 2009. A fratricidal mechanism is responsible for eDNA release and contributes to biofilm development of *Enterococcus faecalis*. Mol Microbiol. 72:1022-1036.

Tormo MA, Knecht E, Gotz F, Lasa I, Penades JR. 2005. Bap-dependent biofilm formation by pathogenic species of Staphylococcus: evidence of horizontal gene transfer? Microbiology. Jul;151:2465-2475.

Trotonda MP, Manna AC, Cheung AL, Lasa I, Penadés JR. 2005. SarA positively controls bap-dependent biofilm formation in *Staphylococcus aureus*. J Bacteriol. 187:5790-5798.

Valle J, Toledo-Arana A, Berasain C, Ghigo JM, Amorena B, Penadés JR, Lasa I. 2003. SarA and not σB is essential for biofilm development by *Staphylococcus aureus*. Mol Microbiol. 48:1075-1087.

Vaningelgem F, Zamfir M, Mozzi F, Adriany T, Vancanneyt M, Swings J, De Vuyst L. 2004. Biodiversity of exopolysaccharides produced by *Streptococcus thermophilus* strains is reflected in their production and their molecular and functional characteristics. Appl Environ Microbiol. 70:900-912.

Vilain S, Pretorius JM, Theron J, Brözel VS. 2009. DNA as an adhesin: Bacillus cereus requires extracellular DNA to form biofilms. Appl Environ Microbiol .75:2861-2868.

Vuong C, Saenz HL, Gotz F, Otto M. 2000. Impact of the agr quorum-sensing system on adherence to polystyrene in *Staphylococcus aureus*. J Infect Dis. 182:1688-1693.

Wang R, Khan BA, Cheung GY, Bach T-HL, Jameson-Lee M, Kong K-F, Queck SY, Otto M. 2011. *Staphylococcus epidermidis* surfactant peptides promote biofilm maturation and dissemination of biofilm-associated infection in mice. J Clin Invest. 121:238.

Watanabe M, Sasaki K, Nakashimada Y, Kakizono T, Noparatnaraporn N, Nishio N. 1998. Growth and flocculation of a marine photosynthetic bacterium *Rhodovulum* sp. Appl Microbiol Biotechnol. 50:682-691.

Wertheim H, Walsh E, Choudhurry R, Melles DC, Boelens H, Miajlovic H, Verbrugh HA, Foster T, van Belkum A. 2008. Key role for clumping factor B in *Staphylococcus aureus* nasal colonization of humans. PLoS Med. 5:e17.

Whelan DR, Bambery KR, Puskar L, McNaughton D, Wood BR. 2013. Quantification of DNA in simple eukaryotic cells using Fourier transform infrared spectroscopy. J biophotonics.6:775-784.

Whitchurch CB, Tolker-Nielsen T, Ragas PC, Mattick JS. 2002. Extracellular DNA required for bacterial biofilm formation. Science. 295:1487-1487.

Xavier JB, Foster KR. 2007. Cooperation and conflict in microbial biofilms. Proc Natl Acad Sci, USA 104:876-881.

Xiao J, Klein MI, Falsetta ML, Lu B, Delahunty CM, Yates 3rd J, Heydorn A, Koo H. 2012. The exopolysaccharide matrix modulates the interaction between 3D architecture and virulence of a mixed-species oral biofilm. PLoS Pathog. 8:e1002623.

Yang L, Barken KB, Skindersoe ME, Christensen AB, Givskov M, Tolker-Nielsen T. 2007. Effects of iron on DNA release and biofilm development by *Pseudomonas aeruginosa*. Microbiology.153:1318-1328.

Yarwood JM, Bartels DJ, Volper EM, Greenberg EP. 2004. Quorum sensing in *Staphylococcus aureus* biofilms. J Bacteriol. 186:1838-1850.

Yirui W, Tengteng H, ZHONG M, ZHANG Y, Enmin L, HUANG T, Zhong H. 2009. Isolation of marine benzo [a] pyrene-degrading *Ochrobactrum sp*. BAP5 and proteins characterization. J Environ Sci. 21:1446-1451.

Zhou L, Xia S, Zhang Z, Ye B, Xu X, Gu Z, Wang X. 2014. Effects of pH, temperature and salinity on extracellular polymeric substances of *Pseudomonas aeruginosa* biofilm with N-(3-Oxooxtanoyl)-L-Homoserine Lactone addition. J Water Sustain. 4:91-100.

Zobell CE. 1943. The effect of solid surfaces upon bacterial activity. J Bacteriol. 46:39.

Zogaj X, Nimtz M, Rohde M, Bokranz W, Römling U. 2001. The multicellular morphotypes of *Salmonella typhimurium* and *Escherichia coli* produce cellulose as the second component of the extracellular matrix. Mol Microbiol. 39:1452-1463.

Zong Y, Xu Y, Liang X, Keene DR, Höök A, Gurusiddappa S, Höök M, Narayana SV. 2005. A ‘Collagen Hug’model for *Staphylococcus aureus* CNA binding to collagen. EMBO J. 24:4224-4236.

Zuloaga O, Prieto A, Usobiaga A, Sarkar S, Chatterjee M, Bhattacharya B, Bhattacharya A, Alam MA, Satpathy K. 2009. Polycyclic aromatic hydrocarbons in intertidal marine bivalves of Sunderban mangrove wetland,

India: an approach to bioindicator species. Water, air, and soil pollution. 201:305-318.

Zuniga E, Melville PA, Saidenberg AB, Laes MA, Gonsales FF, Salaberry SR, Gregori F, Brandão PE, dos Santos FG, Lincopan NE. 2015. Occurrence of genes coding for MSCRAMM and biofilm-associated protein Bap in *Staphylococcus* spp. isolated from bovine subclinical mastitis and relationship with somatic cell counts. Microb pathog. 89:1-6.

CHAPTER 2 : *In silico* molecular characterization of Biofilm-Associated-Proteins

2.1 Introduction

Biofilm formation by *Staphylococcus aureus* has been recognised as a major factor for increased colonization and persistence in infection leading to higher rates of body device related problems (Costerton et al. 1999, Gotz 2002). Biofilms are highly structured microbial communities attached to a surface wherein microbial cells are embedded in self-produced exopolymeric substances (Flemming et al. 2007). These embedded bacterial cells in biofilms exhibit very different behaviour from their planktonic counterparts in terms of morphology and physiology. Some of the studies in last one decade showed that bacterial surface motility structures such as pilli, flagella and fimbri, not only participate in the initiation of primary bacterial adhesion and but also stabilize the subsequent biofilm development and its structure (O'Toole and Kolter 1998, Tolker-Nielsen et al. 2000). Earlier, exopolysaccharide was alone thought to play a critical role in fortifying and maintenance of the biofilm integrity (Danese et al. 2000, Gracia et al. 1997). Of late, the role of surface proteins in intercellular adhesion and in the accumulation of cell clusters was identified (Cucarella et al. 2001, Schroeder et al. 2009). Thereafter, a group of surface proteins with several common structural and functional features emerged as an important factor in the biofilm formation process. This family of proteins is called as Biofilm-Associated-Proteins (BAP) family (Latasa, et al. 2006). BAP-family proteins share some common features such as; their presence on the bacterial surface, very high molecular weight and a core domain of tandem repeats (Lasa and Penadés 2006, Latasa et al. 2006). Some protein members identified in this group are Bap (Cucarella et al. 2001), Esp (Shankar et al. 2002), LapA (Hinsa et al. 2003), BapA (Latasa et al. 2006) and BapA in *Acinetobacter baumannii* (Loehfelm et al. 2008). Among the various

proteins the role of biofilm associated protein of *S. aureus* in early adhesion is highly prevalent, Among other studies biofilm persistence and biofilm architecture have been well established (Arrizubieta et al. 2004, Cucarella et al. 2001 and 2004, Shukla and Rao 2013a, Shukla and Rao 2013b, Valle et al. 2012). More recently, it has been shown that Bap interact with GP96 host receptor and prevents cellular internalization thereby conferring its resistance to immune response of the host (Valle et al. 2012).

A better understanding of factors involved in primary attachment, intercellular communications, and their role in persistent infections of *S. aureus* are warranted to develop new vaccines and for providing effective treatment and control of *S. aureus*. For instance, a surface protein of BAP family in *A. baumannii,* Bap-A was found to be involved in early development of biofilm architecture and its maturation (Loehfelm et al. 2008). In the recent past, an intensive *in silico* analysis of this protein has been done to identify antibody-triggering regions in Bap-A protein(Rahbar et al. 2010). This identification led to successful protection against *A. baumannii* infection via its functional deprivation of Bap-A (Fattahian et al. 2011) as well as enabled the researchers to develop a diagnostic test for precise identification of *A. baumannii* (Rahbar et al. 2012).

Of late, researchers have been targeting biofilm associated proteins to develop a potential vaccine against pathogens. Soluble and hydrophilic region of cell-anchored proteins are known to be more potent B-cell antigens than hydrophobic proteins (Skeiky et al. 1994) and presence of tandem repeats containing proteins are easily recognised by the B-cell (Kemp et al. 1987, Reeder and Brown 1996). Therefore, Bap of *S. aureus* might be a good candidate to

target for the development of some *S. aureus* infection combating strategies. In the present study, several bioinformatic tools were chosen to develop better understanding and characterizing the Biofilm Associated Protein (Bap) of *S. aureus.* The study was designed *in silico* to identify and select the potential immuno-dominant regions of Bap as effective antigenic agents.

2.2 Methods

2.2.1 Sequence availability and similarity search

A Domain Enhanced Lookup Time Accelerated BLAST (Larkin et al.) was performed against non-redundant protein database for Bap protein sequence (Accession No. AAK38834.2) using BLOSUM 80 matrix at www.blast.ncbi.nlm.nih.gov. DELTA-BLAST is a more sensitive algorithm for proteins that contain well-conserved domains therefore it is a useful program for the detection of remote protein homologs (Boratyn et al. 2012). BLOSUM 80 scoring matrix was chosen to collect less divergent homologous sequences due to its strict existence-extension scoring matrix for gap costs (10:1).

2.2.2 Alignments and trees

All alignments were generated in MUSCLE and CLUSTALW (Larkin et al. 2007). Multiple sequence alignment was performed on 81 DELTA-BLAST sequences in COBALT (Constraint-based Multiple Alignment Tool) at www.ncbi.nlm.nih.gov. The closest 11 sequences were selected and were used to perform alignments in MUSCLE (Edgar 2004)

2.2.3 Primary sequence analysis

Xstream online software was used as a practical algorithm for identification and architecture modelling of tandem repeats in biofilm-associated protein

sequences at http://jimcooperlab.mcdb.ucsb.edu/xstream/about.jsp (Newman and Cooper 2007). Various physicochemical parameters such as amino acid composition, molecular weight and theoretical pI of biofilm-associated proteins and selected regions were determined using PratParam software (Gasteiger et al. 2005). VICMpred was used to classify all proteins into four broad functional classes viz.; virulence factor, metabolism, cell process and information storage in the web site http://www.imtech.res.in/raghava/vicmpred(Saha and Raghava 2006). The presence of potential functional motif in Bap protein sequence was predicted at PROSCAN software platform (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_proscan.html) using PROSITE database (Hulo et al. 2008).

2.2.4 Topology prediction

Trans-membrane regions in all selected proteins were determined by using TMHMM (http://www.cbs.dtu.dk/services/TMHMM) (Server). For the classification of protein as secretory or non-secretory Bap sequence was analysed using SRTpred program at http://www.imtech.res.in/raghava/srtpred(Garg and Raghava 2008). Sub-cellular localization of all proteins were predicted by support vector machine in PSLpred (Bhasin et al. 2005) at http://www.imtech.res.in/raghava/pslpred and CELLO (Yu et al. 2006) at http://cello.life.nctu.edu.tw.

2.2.5 Secondary and tertiary structure prediction

Secondary structures of selected Bap proteins were predicted at GOR software platform (http://npsa-pbil.ibcp.fr/cgi-bin/secpred_gor4.pl) (Garnier et al. 1996). Further secondary structures of tandem repeats were evaluated at

PSIpred (http://www.bioinf.cs.ucl.ac.uk/psipred (Marsden et al. 2002). In order to determine the homologous 3D structures the selected amino acid sequence were run at Phyre2 (Protein Homology/analogY Recognition Engine) software (http://www.sbg.bio.ic.ac.uk/phyre2/html) (Kelley et al. 2015, Kelley and Sternberg 2009).

2.2.6 Identification of functionally and structurally important residues

Functionally and structurally important amino acids (AAs) residues in 86 AAs repeat modules of Bap proteins were determined by ConSeq (Berezin et al. 2004) at http://conseq.tau.ac.il. CSI-BLAST for 3 iterations with MUSCLE alignment against Uniprot database with *E*-value of 0.0001 and maximum likelihood (ML) as a method of calculating amino acid conservation score, were set as the software parameters. Combfunc program at http://www.sbg.bio.ic.ac.uk/combfunc/ was used for annotating functional residues of Bap sequence in twilight zone (Wass et al. 2012).

2.2.7 Immuno-informatic analysis

Segments within tandem repeats in Bap sequence that are likely to be antigenic were predicted using Bcepred at http://www.imtech.res.in/raghava/bcepred with accuracy of 58.7% on the basis of four amino acid properties viz.; hydrophylicity, flexibility, polarity and exposed surface (Saha and Raghava 2004). ABCpred at http://www.imtech.res.in/raghava/abcpred, is a recurrent neural network based software, which was employed to predict B-cell epitopes within four segments of choice with 65.93% accuracy (Saha and Raghava 2006). Predicted antigenic peptide based on Kolsar and Tongaonkar methods with 75% accuracy was run

at https://www.predictprotein.org. The later server was used to predict average antigenic propensity of Bap and selected regions. Probability of solvent accessibility, continuous B-cell epitopes, solubility upon over-expression in *E.coli* and antigenicity was predicted using ACCpro, COBEpro, SOLpro and ANTIGENpro at a sequence based, alignment free and pathogen independent server, http://scratch.proteomics.ics.uci.edu (Magnan and Baldi 2014, Sweredoski and Baldi 2009).

2.3 Results and Discussion

2.3.1 Primary sequence analysis and homology search

DELTA-BLAST using *S. aureus* Bap (AAK38834.1) as a query was analysed. 81 sequences of higher scores were selected to build multiple sequence alignments (*E*-value=0, bit score >600). After CLUSTALW alignment of 81 sequences the phylogenetic tree was constructed (for 19 sequences) based on Neighbourhood joining analysis (see Figure 2.1), the closest 11 sequences were selected for further analyses (Table 2.1). As Table 2.1 indicates all sequences were from Staphylococci and reported to be biofilm-associated protein except gram-positive signal peptide protein (WP_002501035.1) and gram-positive anchor protein (WP_002476148.1).

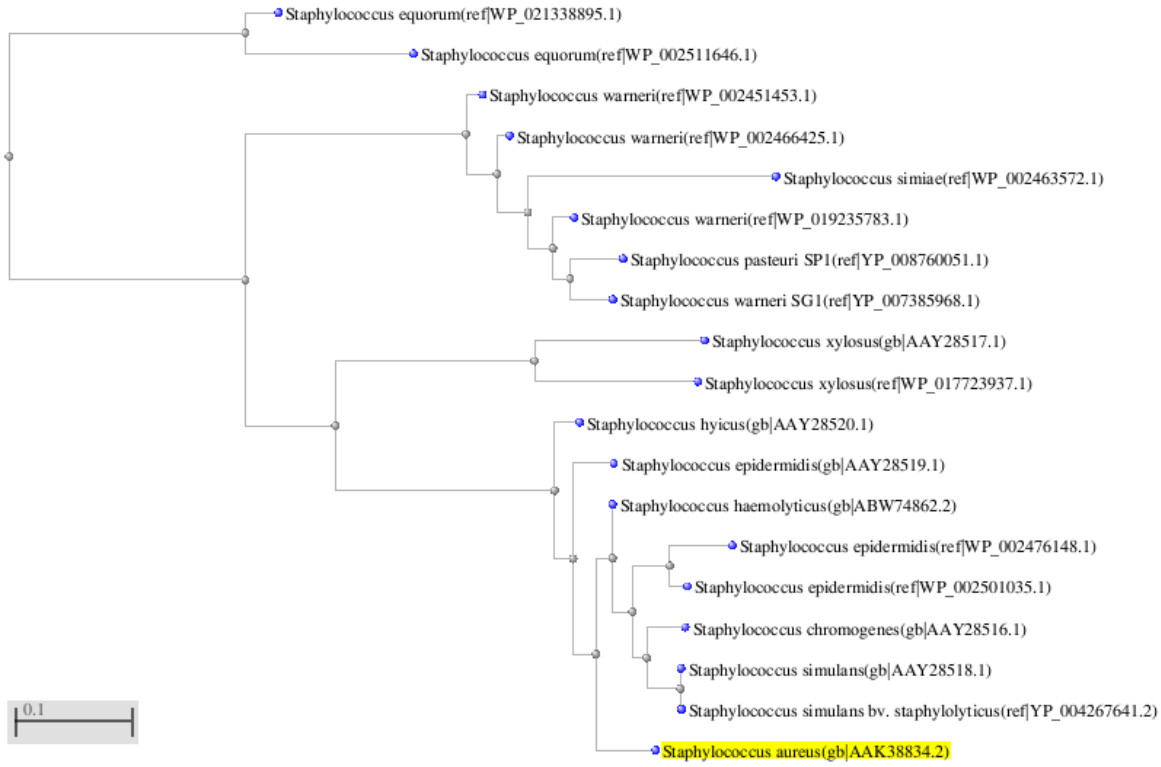


Figure 2.1: Phylogenetic analysis of 18 closest biofilm associated proteins based on DELTA –BLAST score against non-redundant protein database for Bap protein (Accession No. AAK38834.2) using BLOSUM 80 matrix.

Table 2.1: The 11 closest sequences with highest relevance to Bap. These sequences were chosen to carry out further bioinformatics analysis.

Sequence name	Accession no.	Organism
biofilm-associated surface protein (Bap)	AAK38834.2	*Staphylococcus aureus*
biofilm-associated protein	AAY28520.1	*Staphylococcus hyicus*
Gram-positive signal peptide protein	WP_002501035.1	*Staphylococcus epidermidis*
Hypothetical protein	WP_017723937.1	*Staphylococcus xylosus*
biofilm-associated protein	AAY28516.1	*Staphylococcus chromogenes*
biofilm-associated protein	ABW74862.2	*Staphylococcus haemolyticus*
biofilm-associated protein	AAY28517.1	*Staphylococcus xylosus*
biofilm-associated protein	AAY28519.1	*Staphylococcus epidermidis*
biofilm-associated protein	AAY28518.1	*Staphylococcus simulans*
gram positive anchor	WP_002476148.1	*Staphylococcus epidermidis*
putative biofilm-associated protein	YP_004267641.1	*Staphylococcus simulans bv. staphylolyticus*

The primary sequence analysis of these selected proteins revealed the prevalence of Thr, Asn, Asp and Ser in all the sequences and absence of Cys. All proteins are predicted to have very low iso-electric pH (3.64 to 4.34) and high molecular mass (101 to 338.1 kDa).

Further analysis showed the presence of tandem repeats (TR) of 86 amino acids in all protein sequences except two sequences from *S. epidermidis* (WP_002501035.1) and *S. haemolyticus* (ABW74862.2) (Table 2.2 and Table 2.3). It should be noted that these two proteins were deposited in NCBI database as partial sequences, therefore, the presence of tandem repeats in the full sequence of the proteins cannot be ruled out. Interestingly, in each protein the tandem repeats were 100% identical to each other even at the gene level. Prevalence of negatively charged amino acid in TR, as well as a direct correlation between number of TR and lower pI, suggested that TR contribute to the acidic nature of these proteins. DELTA-BLAST which is a domain based BLAST showed most proteins (81 selected in this study) with higher homology in TR region. This observation suggested that TR domain might be of more importance in function and structural terms of Bap proteins. Further analysis using VCIMpred indicated most of the Bap proteins as virulence factors. Virulence and biofilm formation are related characteristics of a pathogenic bacterium.

Table 2.2: Physicochemical properties of primary sequences of biofilm-associated proteins in *Staphylococcus* sp.

Proteins Sequence id	Size	Most abundant residue	Theoretical mol. Wt. (KDa)	pI	Positions of tandem repeats	Highest Copy #	Lowest Copy #	Longest Period	Shortest Period
AAK38834.2	2276	Asp (11.86), Asn (9.62), Ser (8.44), Thr (14.76)	238.5	3.89	911-2144, 2147-2208	14.33	3	86	18
AAY28520.1	3278	Asp (11.9), Gly (8.2), Thr (17.9), Val (8.0)	338.1	3.64	899-3182, 3167-3204	26.53	2.11	86	18
WP_002501035.1	1334	Asn (9.1), Asp (9.9), Ser (8.4), Thr (13.1)	142.1	4.15	No repeats found	-	-	-	-
WP_017723937.1	1366	Ala (8.1), Asp (8.1), Ser (8.1), Thr 14.1), Val (8.7)	143.4	4.12	1193-1364	2	2	84	-
AAY28516.1	1530	Asp (10.9), Asn (10.1), Thr (12.75), Ser (9.15)	161.6	4.03	853-1393, 1402-1466	6.27	3.61	86	18
ABW74862.2	926	Asp (9.4), Ser (9.29), Thr (9.61), Asn (10.3)	101	4.34	No repeats found	-	-	-	-
AAY28517.1	3271	Asp (8.32), Glu (9), Gly (8.8), Thr (20)	336.7	3.69	1107-3052, 3089-3189, 122-131	22.63	2.00	86	5
AAY28519.1	2742	Thr (16), Asp (11.9), Asn (9.4), Ser(8.6)	284.5	3.74	928-2569, 2577-2670, 2669-2683	19.06	2.14	86	7
AAY28518.1	1674	Thr (13.1), Asp (11.2), Asn (9.68), Ser(9.2)	176.8	4.02	928-1537, 1545-1605	7.08	3.39	86	18
WP_002476148.1	1551	Thr (12.5), Asn (11.2), Asp (11.3), Ser (10.1),	163.5	4.0	832-1342, 1350-1481	7.33	5.94	86	18
YP_004267641.1	1562	Asp(11.3), Asn (9.8), Thr (13.3), Ser(9.15)	165	4.01	816-1425, 1433-1493	7.08	3.39	86	18

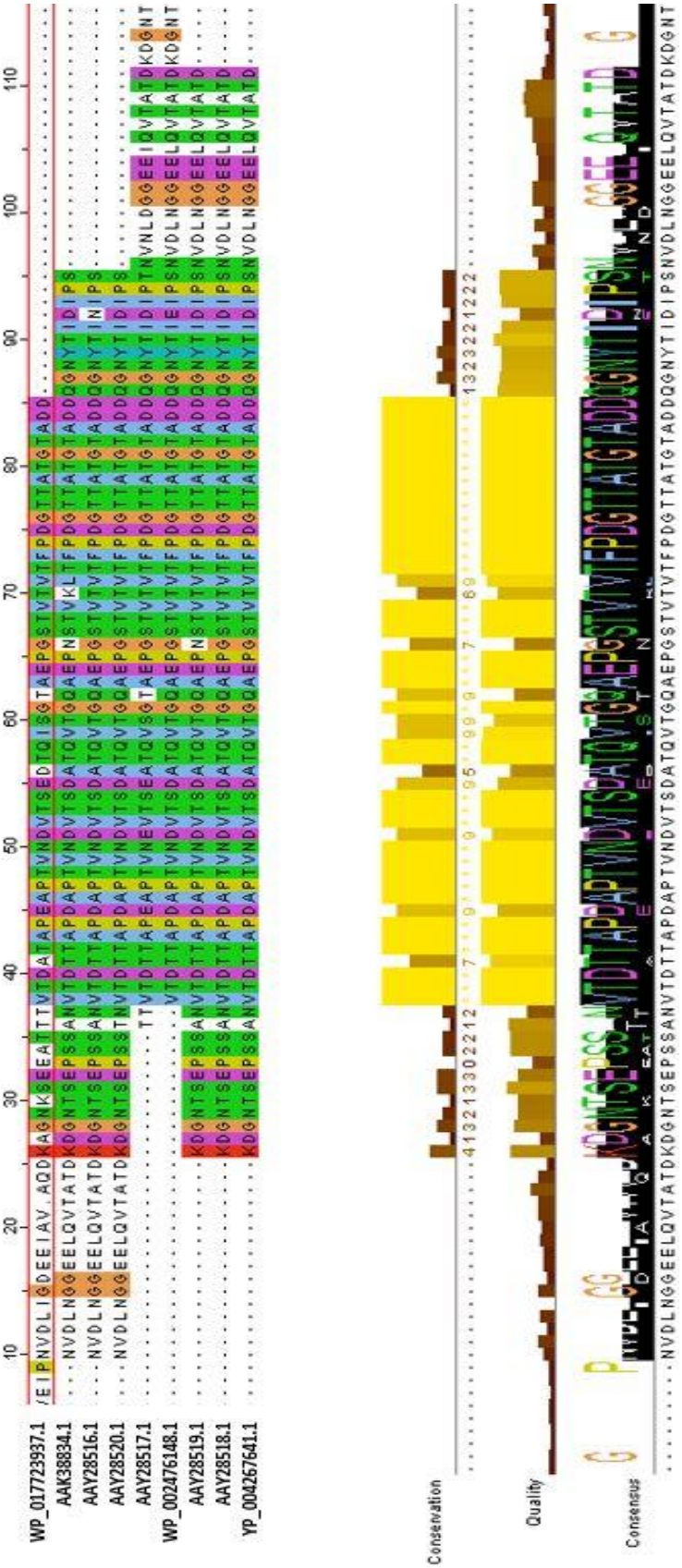


Figure 2.2: Graphical display of alignments of repeat modules.

Table 2.3: Amino acid sequence in 86 amino acids tandem repeats in different proteins used in this study.

Proteins	***Sequence in tandem repeats of 86 amino acids***
AAK388834.2	[1]NVDLNGGEELQVTATDKDGNTSEPSSANVTDTTAPDAPTVNDV TSDATQVTGQAEPNSTVKLTFPDGTTATGTADDQGNYTIDIPS[86]
AAY28520.1	[1]NVDLNGGEELQVTATDKDGNTSEPSSTNVTDTTAPDAPTVNDVT SDATQVTGQAEPGSTVTVTFPDGTTATGTADDQGNYTIDIPS[86]
WP_017723937.1	[1]QGNFSVEIPNVDLIGDEEIAVAQDKAGNKSEEATTTVTDATAPE APTVNDVTSEDTQISGTAEPGSTVTVTFPDGTTATGTADD[84]
AAY28516.1	[1]NVDLNGGEELQVTATDKDGNTSEPSSTNVTDTTAPDAPTVNDV TSDATQVTGQAEPGSTVTVTFPDGTTATGTADDQGNYTIDIPS[86]
AAY28517.1	[1]TTVTDTTAPEAPTVNEVTSEATQVSGTAEPGSTVTVTFPDGTTA TGTADDQGNYTIDIPTNVNLDGGEEIQVTATDKDGNTSSEAT[86]
AAY28519.1	[1]KDGNTSEPSSANVTDTTAPDAPTVNDVTSDATQVTGQAEPNST VTVTFPDGTTATGTADDQGNYTIDIPSNVDLNGGEELQVTATD[86]
AAY28518.1	[1]KDGNTSEPSSANVTDTTAPDAPTVNDVTSDATQVTGQAEPGST VTVTFPDGTTATGTADDQGNYTIDIPSNVDLNGGEELQVTATD[86]
WP_002476148.1	[1]VTDTTAPDAPTVNDVTSDATQVTGQAEPGSTVTVTFPDGTTAT GTADDQGNYTIEIPSNVDLNGGEELQVTATDKDGNTSEPSSAN[86]
YP_004267641.1	[1]KDGNTSEPSSANVTDTTAPDAPTVNDVTSDATQVTGQAEPGST VTVTFPDGTTATGTADDQGNYTIDIPSNVDLNGGEELQVTATD[86]

Earlier studies reported the presence of putative calcium binding EF domain in Bap (AAK38834.1) and its consequence on biofilm formation in the presence of calcium (Arrizubieta et al., 2004). When other proteins in this study were analysed for the presence of potential calcium binding EF-hand motif at >80% homology to consensus D-{W}-[DNS]-{ILVFYW}-[DENSTG]-[DNQGHRK]-{GP}-[LIVMC]-[DENQSTAGC]-x(2)-[DE]-[LIVMFYW] pattern at PROSCAN using PROSITE database, they showed the presence of minimum 2 EF-hand motifs with 91 and 89% homology and maximum 5 EF-hand motifs (Table 2.4). These EF-hand motifs with certain shared % homology were found to be 100% identical to each other. Table 2.4 also indicates that at least 3 EF-hand motif were located before the TR region of Bap proteins and one EF-hand motif after the TR region.

PSI-BLAST against Bap (AAK38834.1) with DYDKDGLLDRYEr (91% homology) and DTDGDGKnDGDEV (89% homology) produced 38 sequences (E-Score > $3e^{-162}$) and 26 sequences (E-Score > $2e^{-146}$) in NCBI database. Relatively conserved locations of predicted EF-hand motifs and significant homology in this study, suggest a clear functional role in biofilm-associated proteins. Previous studies have shown that the presence of calcium inhibits the biofilm formation in Bap expressing *S. aureus* and modulate its architecture (Arrizubieta et al., 2004, Shukla and Rao 2013b). This bioinformatics study suggests that calcium may regulate the biofilm formation in a number of staphylococci, which expresses homologous Bap proteins.

Table 2.4: Presence of putative calcium binding EF-hand domains and their location in respective proteins. [A= DKDllpYIEGVEL (80% homology); B = DYDKDGLLDRYEr (91% homology); C = DTDGDGKnDGDEV (89% homology); D = DNSDSDDnSDNEd (80% homology)]

PROTEINS SEQUENCE ID	**PRESENCE OF PUTATIVE EF HAND DOMAINS AND THEIR LOCATION IN RESPECTIVE PROTEINS**			
	A	**B**	**C**	**D**
AAK38834.2	598 - 610	729 - 741	752 - 764	2198 - 2210
AAY28520.1	586 - 598	717 - 729	740 - 752	3200 - 3212
WP_002501035.1	598 - 610	729 - 741	752 - 764	1073 - 1085
WP_017723937.1	594 - 606	725 - 737	748 - 760	--
AAY28516.1	540 - 552	671 - 683	694 - 706	1452 - 1464
ABW74862.2	598 - 610	729 - 741	752 - 764	--
AAY28517.1	594 - 606	725 - 737	748 - 760	--
AAY28519.1	598 - 610	729 - 741	752 - 764	2664 - 2676
AAY28518.1	598 - 610	729 - 741	752 - 764	1596 - 1608
WP_002476148.1	489 - 501	620 - 632	643 - 655	1473 - 1485
YP_004267641.1	486 - 498	617 - 629	640 - 652	1484 - 1496

2.3.2 Topology analysis of Bap proteins

Topological analysis was performed to predict the post-translation location of the proteins (Table 2.5). Analysis done at SRTpred indicated that all proteins had secretary signal peptide except ABW74862.2 from *S. haemolyticus*, which mean these proteins are exported outside the cell post-translation. Secretory signal sequences that are located at the amino termini direct the translated proteins to the secretory (Sec) apparatus in the membrane and are cleaved during secretion. This assumption is also supported by the aliphatic side chain containing amino acids, which are favorable residues in SecA chaperon binding site (Knoblauch et al. 1999), thus indicating that folding might occur during secretion (Rahbar et al. 2010). PSLpred and CELLO scores identify Bap proteins as extracellular proteins or outer membrane proteins. TMHMM analysis was done using a hybrid approach, which uses amino acid composition, physicochemical properties and dipeptide composition based algorithm. TMHMM analysis predicts the presence of trans-membrane helices at C-terminal in all proteins except ABW74862.2 from *S. haemolyticus*. Some proteins also showed the presence of trans-membrane helix at the N-terminal side of the proteins. The presence of C-terminal trans-membrane helix suggested that secretion of these proteins can only occur after translation (Delepelaire 2004).

2.3.3 Secondary structure analysis and patterns specific analysis

Secondary structural analysis using GOR platform suggest that biofilm associated proteins are comprised of random coil, extended strand and alpha helix as main structural contents (Table 2.7). Figure 2.3 indicate that Bap proteins share the similar secondary structures. The results (Figure 2.3 and Table 2.7)

also show that N-terminal regions of Bap proteins were dominated by alpha helices (blue color), whereas C-terminal TR were mainly comprised of coils (red).

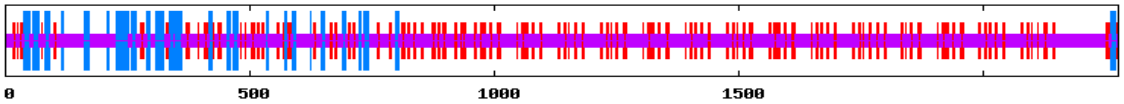
Table 2.5: Localization of biofilm associated proteins as predicted by different bioinformatics tools. [S= secretary protein; NS = Non secretary protein; OMP= outer membrane protein; ECP= Extracellular protein]

SEQUENCE ID	ORGANISM TYPE		SCORE	LOCALIZATION PSLPRED	LOCALIZATION CELLO
AAK38834.2	*S. aureus*	S	0.95575	OMP	Extracellular, Cell wall, membrane
AAY28520.1	*S. hyicus*	S	1.07560	OMP	Extracellular, Cell wall, membrane
WP_002501035.1	*S. epidermidis*	S	0.92002	OMP	Extracellular
WP_017723937.1	*S. xylosus*	S	0.99359	ECP	Extracellular, Cell wall membrane
AAY28516.1	*S. chromogenes*	S	0.91460	OMP	Extracellular
ABW74862.2	*S. haemolyticus*	NS	-0.5282	ECP	Extracellular
AAY28517.1	*S. xylosus*	S	1.13235	OMP	Extracellular, Cell wall membrane
AAY28519.1	*S. epidermidis*	S	0.99988	OMP	- do -
AAY28518.1	*S. simulans*	S	0.90689	OMP	- do -
WP_002476148.1	*S. epidermidis*	S	0.87125	ECP	Extracellular
YP_004267641.1	*S. simulans bv. Staphylolyticus*	S	0.90970	OMP	Extracellular, Cell wall, membrane

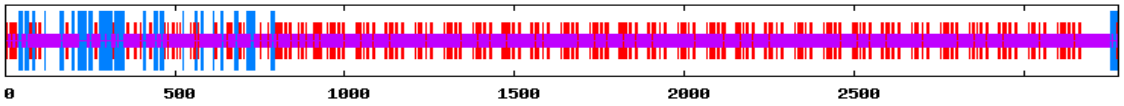
Table 2.6: TMHMM Analysis prediction of transmembrane helices in proteins.

Sequence ID	Organism	Inside	TM helix	Outside	TM helix	Inside
AAK38834.2	*S. aureus*			1 2248	2249-2266	2267-2276
AAY28520.1	*S. hyicus*	1-19	20-39	40-3247	3248-3267	3268-3278
WP_002501035.1	*S. epidermidis*	1-19	20-39	40-1334		
WP_017723937.1	*S. xylosus*	-	-	1-1366	-	-
AAY28516.1	*S. chromogenes*	1-19	20-39,	40-1499	1500-1519	1520-1530
ABW74862.2	*S. haemolyticus*	-	-	1 -926	-	-
AAY28517.1	*S. xylosus*			1 -3243	3244-3261	3262-3271
AAY28519.1	*S. epidermidis*	1-19	20-39	40-2714	2715-2732	2733-2742
AAY28518.1	*S. simulans*			1-1646	1647-1664	1665-1674
WP_002476148.1	*S. epidermidis*			1-1523	1524-1541	1542-1551
YP_004267641.1	*S. simulans bv. Staphylolyticus*			1-1534	1535-1552	1553-1562

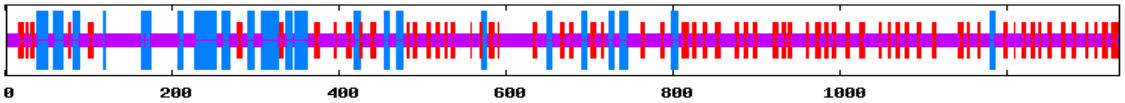
***S. aureus* (AAK38834.2)**



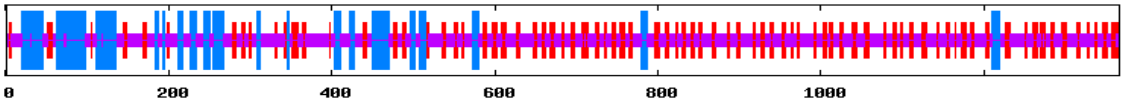
***S. hyicus* (AAY28520.1)**



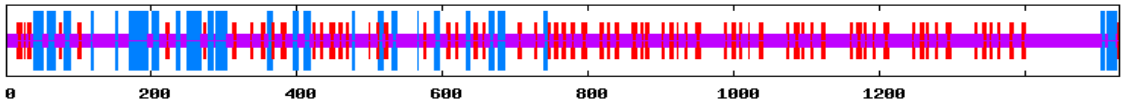
***S. epidermidis* (WP_002501035.1)**



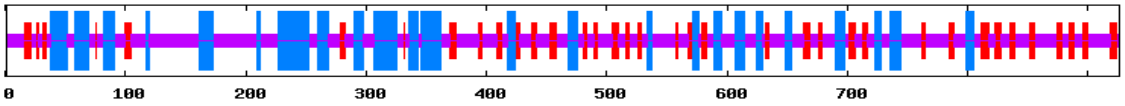
***S. xylosus* (WP_017723937.1)**



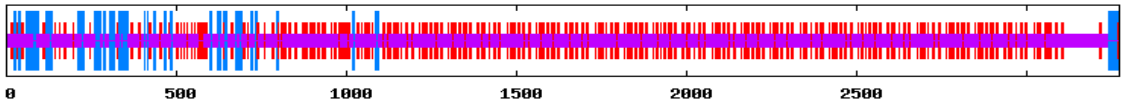
***S. chromogenes* (AAY28516.1)**



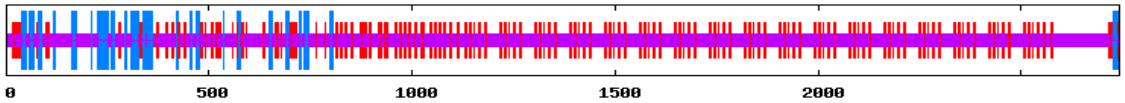
***S. haemolyticus* (ABW74862.2)**



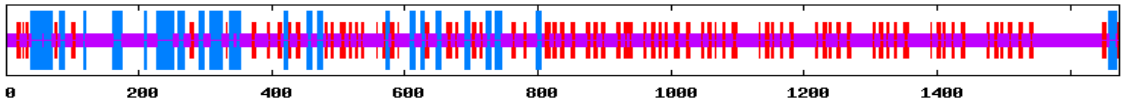
***S. xylosus* (AAY28517.1)**



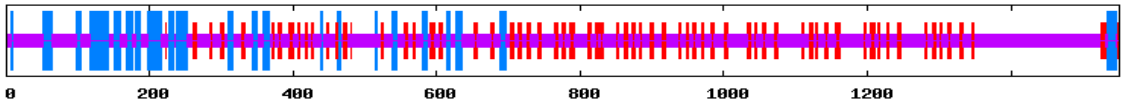
***S. epidermidis* (AAY28519.1)**



***S. simulans* (AAY28518.1)**



***S. epidermidis* (WP_002476148.1)**



***S. simulans by.* (YP_004267641.1)**

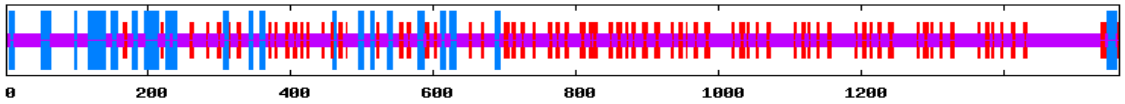


Figure 2.3: Graphical results for secondary structure predictions of biofilm associated proteins. Extended strand: purple, Coil: red, Helix: blue.

Table 2.7: Percentage of secondary structure elements of Biofilm associated proteins.

Sequence id	Organism	Alpha helix (blue)	Random coil (orange)	Extended strand (red)
AAK38834.2	*S. aureus*	10.33	68.76%	20.91%
AAY28520.1	*S. hyicus*	7.32%	69.71%	22.97%
WP_002501035.1	*S. epidermidis*	16.04%	61.47%	22.49%
WP_017723937.1	*S. xylosus*	15.59%	57.98%	26.43%
AAY28516.1	*S. chromogenes*	14.25%	66.01%	19.74%
ABW74862.2	*S. haemolyticus*	24.84%	57.99%	17.17%
AAY28517.1	*S. xylosus*	8.28%	64.38%	27.33%
AAY28519.1	*S. epidermidis*	8.02%	70.13%	21.85%
AAY28518.1	*S. simulans*	13.92%	65.29%	20.79%
WP_002476148.1	*S. epidermidis*	13.02%	67.83%	19.15%
YP_004267641.1	*S. simulans bv. Staphylolyticus*	12.61%	66.39%	21.00%

This drastic difference in structure in N-terminal and C-terminal may have different functional and structural implications. As shown in Table 2.4, three putative EF domain present in N-terminal of the proteins might play a calcium-mediated regulatory role whereas C-terminal could play a structural role. However, the presence of repeat domain with high homology in all proteins suggests that TR may have some functional role as well.

Multiple sequence alignment shows that some amino acid sequences are highly conserved (Figure 2.2). A recent report shows that repeated domains contain an amyloidogenic peptide motif -STVTVTF- derived from the biofilm-associated protein from *S. epidermidis* C533 isolate (GenBank: AAY28519.1),

which was responsible for cell–cell interaction through hydrophobic interaction (Lembre et al. 2014).

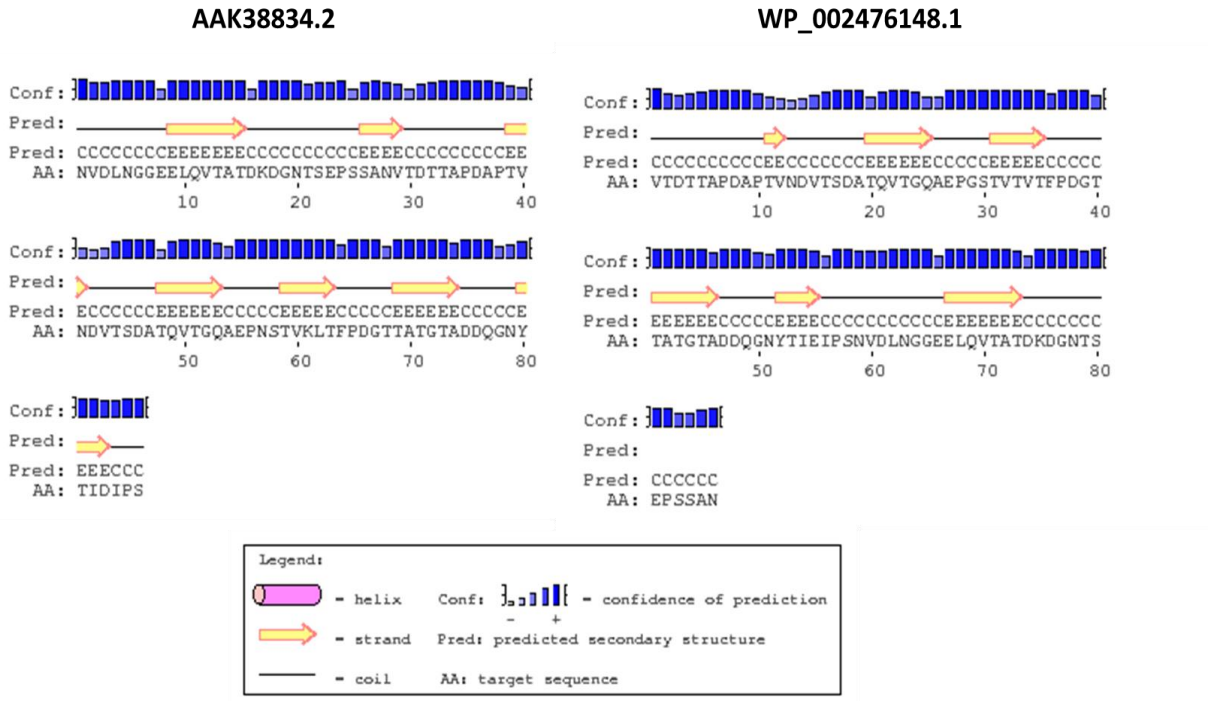


Figure 2.4: Colored scheme of conserved functional and structural residues in AAK38834.2 and WP_002476148.1. The important functionally and structurally conserved amino acid residues in 86 AA repeat modules are located upstream to amyloidogenic heptapeptide.

When all proteins in this study were investigated for the presence of amyloidogenic heptapeptide motif -STVTVTF-, it was found to be present in all the proteins except AAK38834.1. AAK38834.1 had only one -STVTVTF- in primary sequence whereas the rest of the repeats have slightly different heptapeptide (-STVKLTF-). TANGO, AGGRESCAN and PASTA analysis was performed to reconfirm that 86 amino acid peptide sequence manifest aggregation propensity due to the presence of heptapeptide. These tools are designed based on a simple statistical mechanics algorithm. This algorithm is based on the physicochemical principles of secondary structure formation which

is based on the assumption that the core regions of an aggregate are fully buried. PSIpred analysis predicts heptapeptide -STVTVTF-, which is hypothesized to facilitate protein –protein interaction, to be present on β-strands and exposed towards the surface (Figure 2.4).

2.3.4 Tertiary structure homology/analogy recognition

The secondary structure analysis suggest that TRs may participate in aggregation and aid in protein-protein interaction due to the hydrophobic nature of the heptapeptide, tertiary structure analysis was done at Phyre2 server. Analysis at Phyre2 server generates a number of homologous potential models with different ranking depending on the confidence score. Figure 2.5 shows predicted homologous domains for WP_002476148.1, which showed homologous structures with the highest confidence at Phyre2 server. All proteins in these study, showed highest structural homology with giant Ca^{2+}-binding adhesin SiiE in *Salmonella enterica* (Griessl et al., 2013) with confidence score of 98.4 to 99.3, followed by a bacterial immunoglobulin-like domain of ice-binding adhesin from the *Marinomonas primoryensis* (Guo et al., 2013). However, it can be speculated that these predicted domains might be structurally similar but tend to be functionally different as calcium binding EF domains are not located in TRs.

In these predicted homologous models, amyloidogenic heptapeptide –STVTVTF was found to be present on β-strands in all the predicted domains which confers ability to participate in the domain-domain interaction. This observation reconfirms the secondary structural analysis at PSIpred and suggests similar folding topology. Earlier, TRs were hypothesized to be HYR domain kind of structures (Latasa et al. 2006). High homology of predicted module with Figure 2.5A and 5E suggest the folding pattern of that TR modules

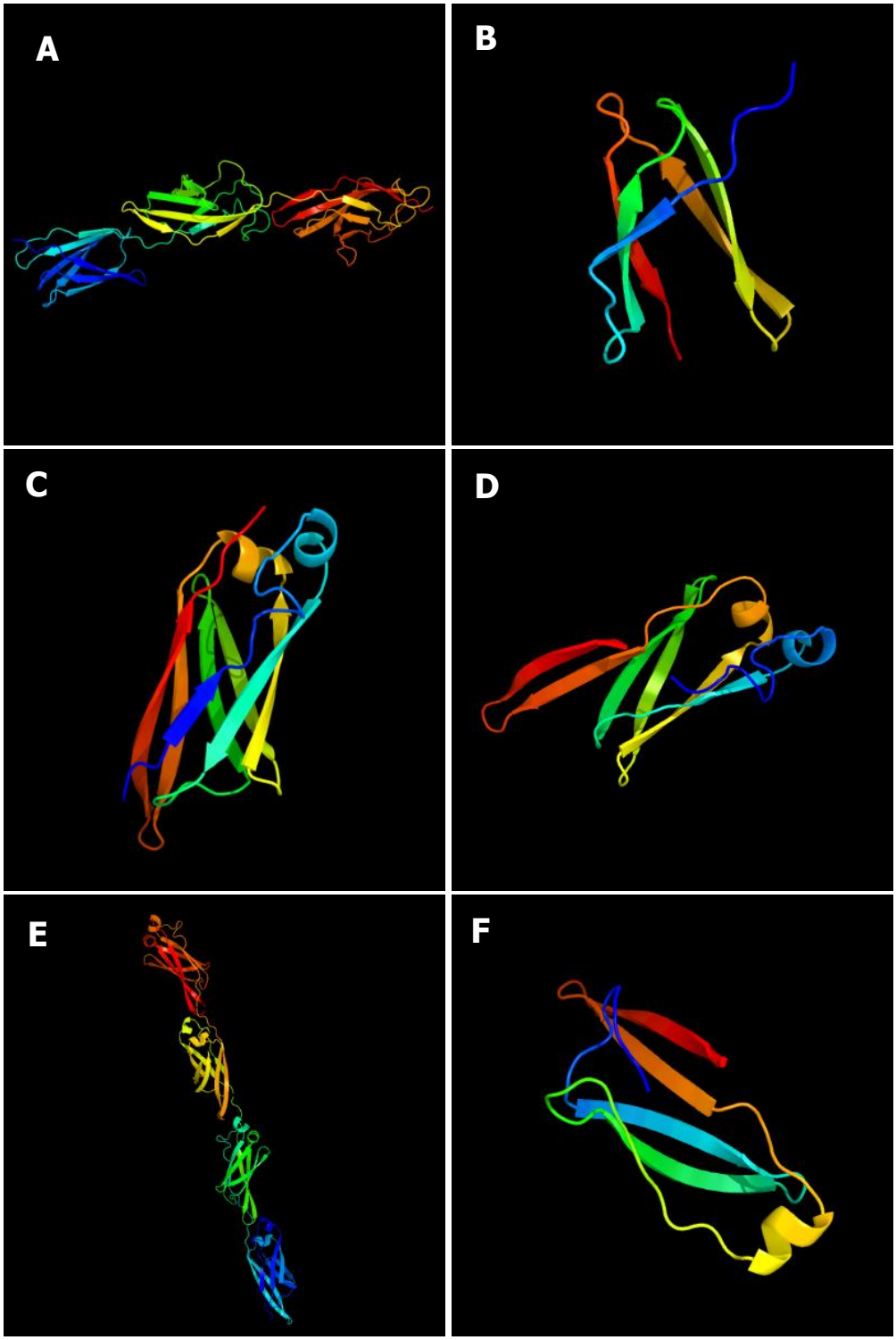


Figure 2.5: Predicted homologous domains for WP_002476148.1 which showed homologous structure with highest confidence at Phyre2 server. (A), (C) and (E) are submitted PDB structure of SiiE (Griessl et al. 2013), Ice binding adhesin (Guo et al. 2013) and Ca^{2+} stabilized Ice binding adhesin (Vance et al. 2014). (B), (D) and (F) are homologous structures to (A) (C) and (E) with confidence score of 99.3, 99.0 and 98.1 respectively.

are very similar to seven-stranded sandwich proteins within the immunoglobulin-like fold super family (PFAM accession no. CL0159), which includes the HYR domain as a family member (Callebaut et al. 2000). This suggests that Bap forms a “beaded-filament” kind of tertiary structure (Sanz-Aparicio et al. 1997).

2.3.5 Functionally and structurally important residues

Further investigation for the functionally and structurally important residues were carried out at ConSurf server. Figure 2.6 shows that most functional and conserved residues are reported to be located in upstream of the heptapeptide. The highest number of functionally and structurally conserved amino acid residues in TR sequence were located at 15-28 amino acid upstream of the amyloidogenic heptapeptide. Such distribution arrangement of the functionally and structurally conserved amino acid residues suggest that the conserved regions might function to maintain a common structural motif, while amyloidogenic heptapeptide might aid in inter-protein interaction and adhesion.

2.3.6 Antigenic propensity and surface accessibility

To have a better antigenic propensity, it is imperative to focus on protein sequences that are comparatively conserved. Other important factors are repetition, hydrophilicity and accessibility. Thus, TR in Bap are potential target to search for immunogenic epitopes, therefore, were selected for immunogenic studies. BcePred analysis with accuracy of 56% using four amino acid properties viz.; hydrophylicity, flexibility, polarity and exposed surface identifies TVTVTFPD that overlaps amyloidogenic heptapeptide as a major B-cell epitope (Table 2.8). Whereas AntigenPro predicted DVTSDATQVTGQAEPNST,just upstream to amyloidogenic heptapeptide to be B-cell epitope with antigenic propensity index

in the range of 0.69 to 0.83 as shown in Table 7. As other analyses have indicated in this study, if amyloidogenic heptapeptide indulges into domain-domain interaction it would not be easily accessible as B-cell epitope.

Figure 2.6: Identification of conserved structural and functional residues in repeat modules of Bap proteins.

Whereas the sequence as per the AntigenPro that is upstream to amyloidogenic heptapeptide and part of the random coil (Figure 2.6) has higher antigenicity and accessibility. These epitopes were also predicted to have good

solubility ranging from 0.55 to 0.83 when over-expressed in *E. coli.* ConSurf analysis also showed this upstream sequence to be conserved as shown in Figure 2.6.

Table 2.8: Predicted antigenic propensity and predicted solubility upon overexpression in *E. coli.* **A** = Predicted Probability of Antigenicity; **B** = Predicted Solubility upon Overexpression

Proteins	Predicted Antigenic Propensity	A	B
AAK38834.2	NVDLNGGEELQVTATDKDGNTSEPSSANVTDTTAPDAPTVNDVTSDATQVTGQAEPNSTVKLTFPDGTTATGTADDQGNYTIDIPS	0.79	0.70
AAY28520.1	NVDLNGGEELQVTATDKDGNTSEPSSTNVTDTTAPDAPTVNDVTSDATQVTGQAEPGSTVTVTFPDGTTATGTADDQGNYTIDIPS	0.78	0.70
WP_017723937.1	QGNFSVEIPNVDLIGDEEIAVAQDKAGNKSEEATTTVTDATAPEAPTVNDVTSEDTQISGTAEPGSTVTVTFPDGTTATGTAD	0.83	0.83
AAY28516.1	NVDLNGGEELQVTATDKDGNTSEPSSANVTDTTAPDAPTVNDVTSDATQVTGQAEPGSTVTVTFPDGTTATGTADDQGNYTINIPS	0.78	0.84
AAY28517.1	TTVTDTTAPEAPTVNEVTSEATQVSGTAEPGSTVTVTFPDGTTATGTADDQGNYTIDIPTNVNLDGGEEIQVTATDKDGNTSSEAT	0.69	0.55
AAY28519.1	KDGNTSEPSSANVTDTTAPDAPTVNDVTSDATQVTGQAEPNSTVTVTFPDGTTATGTADDQGNYTIDIPSNVDLNGGEELQVTATD	0.72	0.66
AAY28518.1	KDGNTSEPSSANVTDTTAPDAPTVNDVTSDATQVTGQAEPGSTVTVTFPDGTTATGTADDQGNYTIDIPSNVDLNGGEELQVTATD	0.73	0.73
WP_002476148.1	VTDTTAPDAPTVNDVTSDATQVTGQAEPGSTVTVTFPDGTTATGTADDQGNYTIEIPSNVDLNGGEELQVTATDKDGNTSEPSSAN	0.73	0.74
YP_004267641.1	KDGNTSEPSSANVTDTTAPDAPTVNDVTSDATQVTGQAEPGSTVTVTFPDGTTATGTADDQGNYTIDIPSNVDLNGGEELQVTATD	0.73	0.73

2.4 Conclusion

This study establishes that Bap like proteins in staphylococci are cell wall anchored proteins which can be exported using Type I or III secretory system. Bap proteins have two distinct parts; N-terminal part, which contains calcium binding EF-hand domain, that plays a regulatory role in Bap functioning and C-

terminal part, which predominantly comprised of tandem repeats plays a structural and functional role. Amyloidogenic heptapeptide is conserved and hypothesized to participate in domain-domain interaction via hydrophobic forces. TR domains show the highest homology with giant Ca^{2+} binding adhesin SiiE in *Salmonella enterica* and acquire a beaded-chain like structure. Amino acid sequence “DVTSDATQVTGQAEPNST” in TR domain is predicted to be a potential antigenic region, which can be used in immunogenic tests.

References

Arrizubieta MJ, Toledo-Arana A, Amorena B, Penades JR, Lasa I. 2004. Calcium inhibits bap-dependent multicellular behavior in *Staphylococcus aureus*. J Bacteriol. 186:7490-7498.

Berezin C, Glaser F, Rosenberg J, Paz I, Pupko T, Fariselli P, Casadio R, Ben-Tal N. 2004. ConSeq: the identification of functionally and structurally important residues in protein sequences. Bioinformatics. 20:1322-1324.

Bhasin M, Garg A, Raghava GP. 2005. PSLpred: prediction of subcellular localization of bacterial proteins. Bioinformatics. 21:2522-2524.

Boratyn GM, Schaffer A, Agarwala R, Altschul SF, Lipman DJ, Madden TL. 2012. Domain enhanced lookup time accelerated BLAST. Biol Direct.7:12.

Callebaut I, Gilges D, Vigon I, Mornon J-P. 2000. HYR, an extracellular module involved in cellular adhesion and related to the immunoglobulin-like fold. Protein Sci. 9:1382-1390.

Costerton JW, Stewart PS, Greenberg EP. 1999. Bacterial biofilms: a common cause of persistent infections. Science. 284:1318-132

Cucarella C, Solano C, Valle J, Amorena B, Lasa I, Penades JR. 2001. Bap, a *Staphylococcus aureus* surface protein involved in biofilm formation. J Bacteriol. 183:2888-2896.

Cucarella C, Tormo MA, Ubeda C, Trotonda MP, Monzon M, Peris C, Amorena B, Lasa I, Penades JR. 2004. Role of biofilm-associated protein bap in the pathogenesis of bovine *Staphylococcus aureus*. Infect Immun. 72:2177-2185.

Danese PN, Pratt LA, Kolter R. 2000. Exopolysaccharide production is required for development of *Escherichia coli* K-12 biofilm architecture. J Bacteriol 182:3593-3596.

Delepelaire P. 2004. Type I secretion in gram-negative bacteria. BBA-Mol Cell Res. 1694:149-161.

Edgar RC. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32:1792-1797

Fattahian Y, Rasooli I, Gargari SLM, Rahbar MR, Astaneh SDA, Amani J. 2011. Protection against *Acinetobacter baumannii* infection via its functional deprivation of biofilm associated protein (Bap). Microb Pathog. 51:402-406.

Flemming HC, Neu TR, Wozniak DJ. 2007. The EPS matrix: The "House of Biofilm Cells". J Bacteriol 189:7945-7947.

Garg A, Raghava GP. 2008. A machine learning based method for the prediction of secretory proteins using amino acid composition, their order and similarity-search. In silico biology. 8:129-140.

Garnier J, Gibrat JF, Robson B. 1996. GOR method for predicting protein secondary structure from amino acid sequence. Methods Enzymol. 266:540-553.

Gasteiger E, Hoogland C, Gattiker A, Wilkins MR, Appel RD, Bairoch A. 2005. Protein identification and analysis tools on the ExPASy server: Springer.

Gotz F. 2002. *Staphylococcus* and biofilms. Mol Microbiol. 43:1367-1378.

Gracia E, Fernandez A, Conchello P, Laclériga A, Paniagua L, Seral F, Amorena B. 1997. Adherence of *Staphylococcus aureus* slime-producing strain variants to biomaterials used in orthopaedic surgery. Int Orthop. 21:46-51.

Hinsa SM, Espinosa-Urgel M, Ramos JL, O'Toole GA. 2003. Transition from reversible to irreversible attachment during biofilm formation by *Pseudomonas fluorescens* WCS365 requires an ABC transporter and a large secreted protein. Mol Microbiol. 49:905-918.

Hulo N, Bairoch A, Bulliard V, Cerutti L, Cuche BA, de Castro E, Lachaize C, Langendijk-Genevaux PS, Sigrist CJ. 2008. The 20 years of PROSITE. Nucleic Acids Res. 36:D245-249.

Kelley LA, Sternberg MJ. 2009. Protein structure prediction on the Web: a case study using the Phyre server. Nat Protoc. 4:363-371.

Kelley LA, Mezulis S, Yates CM, Wass MN, Sternberg MJE. 2015. The Phyre2 web portal for protein modeling, prediction and analysis. Nat Protoc. 10:845-858.

Kemp D, Coppel R, Anders R. 1987. Repetitive proteins and genes of malaria. Ann Rev Microbiol. 41:181-181.

Knoblauch NT, Rüdiger S, Schönfeld H-J, Driessen AJ, Schneider-Mergener J, Bukau B. 1999. Substrate specificity of the SecB chaperone. J Biol Chem. 274:34219-34225.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, et al. 2007. Clustal W and Clustal X version 2.0. Bioinformatics. 23:2947-2948

Lasa I, Penadés JR. 2006. Bap: a family of surface proteins involved in biofilm formation. Res Microbiol. 157:99-107.

Latasa C, Solano C, Penades JR, Lasa I. 2006. Biofilm-associated proteins. C R Biol. 329:849-857

Lembré P, Vendrely C, Di Martino P. 2014. Identification of an amyloidogenic peptide from the Bap protein of *Staphylococcus epidermidis*. Protein Peptide Lett. 21:75-79.

Loehfelm TW, Luke NR, Campagnari AA. 2008. Identification and characterization of an *Acinetobacter baumannii* biofilm-associated protein. J Bacteriol. 190:1036-1044.

Magnan CN, Baldi P. 2014. SSpro/ACCpro 5: almost perfect prediction of protein secondary structure and relative solvent accessibility using profiles, machine learning and structural similarity. Bioinformatics. 30:2592-2597.

Marsden RL, McGuffin LJ, Jones DT. 2002. Rapid protein domain assignment from amino acid sequence using predicted secondary structure. Protein Sci. 11:2814-2824.

Newman AM, Cooper JB. 2007. XSTREAM: a practical algorithm for identification and architecture modeling of tandem repeats in protein sequences. BMC bioinformatics. 8:382.

O'Toole GA, Kolter R. 1998. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. Mol Microbiol. 28:449-461.

Rahbar MR, Rasooli I, Gargari SLM, Amani J, Fattahian Y. 2010. In silico analysis of antibody triggering biofilm associated protein in *Acinetobacter baumannii*. J Theor Biol. 266:275-290.

Rahbar MR, Rasooli I, Gargari SLM, Sandstrom G, Amani J, Fattahian Y, Jahangiri A, Jalali M. 2012. A potential in silico antibody–antigen based diagnostic test for precise identification of *Acinetobacter baumannii.* J Theor Biol. 294:29-39.

Reeder JC, Brown GV. 1996. Antigenic variation and immune evasion in *Plasmodium falciparum* malaria. Immunol Cell Biol. 74:546-554.

Saha S, Raghava G. 2004. BcePred: prediction of continuous B-cell epitopes in antigenic sequences using physico-chemical properties. In: Artificial immune systems. Springer. p. 197-204.

Saha S, Raghava G. 2006. VICMpred: an SVM-based method for the prediction of functional proteins of Gram-negative bacteria using amino acid patterns and composition. Genomics Proteomics Bioinformatics. 4:42-47.

Sanz-Aparicio J, Hermoso J, Grangeiro TB, Calvete JJ, Cavada BS. 1997. The crystal structure of *Canavalia brasiliensis* lectin suggests a correlation

between its quaternary conformation and its distinct biological properties from Concanavalin A. FEBS lett. 405:114-118.

Schroeder K, Jularic M, Horsburgh SM, Hirschhausen N, Neumann C, Bertling A, Schulte A, Foster S, Kehrel BE, Peters G, et al. 2009. Molecular characterization of a novel *Staphylococcus aureus* surface protein (SasC) involved in cell aggregation and biofilm accumulation. PLoS One. 4:e7567

Server TMHMM. v. 2.0 http://www. cbs. dtu. dk/services. In: TMHMM.

Shankar N, Baghdayan AS, Gilmore MS. 2002. Modulation of virulence within a pathogenicity island in vancomycin-resistant *Enterococcus faecalis*. Nature. 417:746-750.

Shukla SK, Rao TS. 2013. Effect of calcium on *Staphylococcus aureus* biofilm architecture: A confocal laser scanning microscopic study. Colloids Surf B. 103:448-454.

Shukla SK, Rao TS. 2013. Dispersal of Bap-mediated *Staphylococcus aureus* biofilm by proteinase K. J Antibiot. 66:55-60.

Skeiky Y, Benson DR, Elwasila M, Badaro R, Burns J, Reed SG. 1994. Antigens shared by *Leishmania* species and *Trypanosoma cruzi:* immunological comparison of the acidic ribosomal P0 proteins. Infect Immun. 62:1643-1651**.**

Sweredoski MJ, Baldi P. 2009. COBEpro: a novel system for predicting continuous B-cell epitopes. Protein Eng Des Sel. 22:113-120.

Tolker-Nielsen T, Brinch UC, Ragas PC, Andersen JB, Jacobsen CS, Molin S. 2000. Development and dynamics of *Pseudomonas* sp. biofilms. J Bacteriol. 182:6482-6489.

Valle J, Latasa C, Gil C, Toledo-Arana A, Solano C, Penadés JR, Lasa I. 2012. Bap, a biofilm matrix protein of *Staphylococcus aureus* prevents cellular internalization through binding to GP96 host receptor. PLoS Pathog. 8:e1002843.

Wass MN, Barton G, Sternberg MJ. 2012. CombFunc: predicting protein function using heterogeneous data sources. Nucleic Acids Res. 40:W466-W470.

Yu CS, Chen YC, Lu CH, Hwang JK. 2006. Prediction of protein subcellular localization. Proteins. 64:643-651.

CHAPTER 3 : Biofilm development by *S. aureus* isolates in the presence of calcium

3.1 Introduction

Biofilm development is a multi-step process involving an initial adhesion phase, followed by micro-colony formation and subsequently resulting in a mature biofilm (Stoodley et al. 2002). In the recent past biofilms have been recognized as main cause of nosocomial infections (Costerton et al. 1995; Donlan 2001; Stoodley et al. 2002). Among the various infection causing bacteria, *Staphylococcus aureus* is predominant and it colonizes the host tissue by binding to extracellular matrix (Donlan 2001). The pronounced ability of *S. aureus* to develop biofilms in a variety of environments has drawn the attention of scientific community (Lowy 1998). Prevention and control of *S. aureus* infection is cumbersome since it is associated with biofilm development which per se confers tolerance to antimicrobial agents (Sutra and Poutrel 1990; Lowy 1998; Donlan 2001). The poor success rate in treatment of *staphylococci* is also due to ill-discerned fundamentals of biofilm development by *S. aureus.* Thus it is worthwhile to study the details of *S. aureus* biofilm establishment and development which can pave way to design strategies for treatment of *S. aureus* infections.

In biological systems, Ca^{2+} is recognized as intracellular and extracellular messenger molecule (Brown et al. 1995; Maurer et al. 1996). When Ca^{2+} acts as a secondary messenger, it regulates many vital functions like cell cycle and cell division, competence, pathogenesis, motility, chemotaxis, and quorum sensing (Norris et al. 1991, 1996; Smith, 1995; Holland et al. 1999; Michiels et al. 2002). On the other hand extracellular Ca^{2+} plays mainly a structural role where it maintains the integrity of the cell wall (Michiels 2002). Mastitis is an infection in milk producing mammary gland caused by *S. aureus* (Bramley et al, 1989). Milk

contains Ca^{2+} in the range of 5 to 32 mM and it has not been studied whether presence of Ca^{2+} can influence *S. aureus* biofilm development or inhibition. Stanley et al. (1983) observed that there was an increased adhesion of *Pseudomonas aeruginosa* to stainless steel surface when Ca^{2+} concentration was increased from 0.1 mM to 10 mM. In an interesting report it was shown that S*treptococcus thermophillus* shows maximum initial adhesion rate at 1 mM Ca^{2+} and a reduction in adhesion rate below or above 1 mM Ca^{2+} concentration (van Hoogmoed et al. 1997). The authors could not explain the unusual behaviour of *S. thermophillus,* whether it is based on electrostatic interactions etc. If it is electrostatic interaction between *S. thermophillus* and Ca^{2+}, it would have been either directly proportional or vice-versa. Slime producing staphylococci strains like *Staphylococcus epidermidis* showed enhanced adhesion to hydrophobic polystyrene surface in the presence of 128 µM Ca^{2+}. Variants of *S. epidermidis* that did not produce slime also exhibited similar adhesion property in presence of Ca^{2+}. These results suggest that exo-polysaccharides do not participate in Ca^{2+} dependent enhancement of bacterial adhesion to hydrophobic surfaces (Dunne and Burd 1992). Various studies have explained that all bacteria do not show enhanced adhesion with increasing Ca^{2+} concentration. Therefore, with current understanding (Stanley et al. 1983, Dunne and Burd 1992 and van Hoogmoed et al. 1997) it is hypothesized that the mechanism by which Ca^{2+} influences cell adherence to surfaces is a complex phenomenon. Thomas et al. (1993) reported that *S. aureus and S. epidermidis* showed Ca^{2+} dependent enhanced adhesion to sub-maxillary gland mucin. This study postulated two mechanisms for the adherence of *S. aureus to* mucin: one involving surface proteins that bind Ca^{2+} and the other involving surface proteins that interact with mucin independent of

Ca^{2+}. To establish primary attachment to surfaces many bacteria use their pili or flagella (Virji et al. 1993). There are also reports that surface proteins play an important role in primary adherence and in biofilm development (Cucarella et al. 2001; Eaton and Gasson 2002; Shankar et al. 2002; Tendolkar et al. 2004). Apart from surface proteins other biomolecules such as polysaccharides also participate in bacterial adhesion via specific or nonspecific interactions with solid surfaces (McNab et al. 1996, 1998; Whittaker et al. 1996). It was proposed that polymer bridging might be one of the mechanisms for anchoring the bacterial cell without making any intimate contact with the surface (Marshal et al. 1971; Fletcher 1988).

Recently, a large surface protein named Bap from *S. aureus* V329 was found to be involved exclusively in initial adhesion phase and biofilm formation (Cucarella et al. 2001). This protein has been reported to have four potential calcium binding EF domains (Arrizubieta et al. 2004). Calcium binding EF hand motif was discovered from the crystal structure of parvalbumin (Kretsinger et al. 1973) and since then it remained as the most commonly found calcium binding motif in nature. EF hand motif comprises of two nearly perpendicular α-helices separated by a 12-residue loop (Figure 1.1). Most of the EF-hand motif containing calcium-binding proteins are recognized by homology in their Ca^{2+}-binding loops where amino acid residues 1, 3, 5, 7, 9 and 12 of the loop provide the ligands, the complexion property of Ca^{2+} (Michiels et al. 2002).

Arrizubieta et al. (2004) reported that Ca^{2+} inhibits Bap-dependent multi-cellular behaviour in *S. aureus* but does not affect the Bap expression. However, the authors did not highlight the possible influence of the intrinsic Ca^{2+} in tryptic soy broth (TSB) medium and gave more emphasis on multi-cellular behaviour of

S. aureus V329 in presence of Ca^{2+}. In the present study, the influence of Ca^{2+} on biofilm development of four isolates of *S. aureus* (see Table 3.1), was investigated. The putative role of Bap in biofilm development in presence of Ca^{2+} was studied. Confocal laser scanning microscopy (CSLM) was used to analyze the topography of *S. aureus* biofilm in response to Ca^{2+} concentration.

3.2 Materials and Methods

3.2.1 Microorganisms and culture conditions

Five *S. aureus* isolates were used in the study. *S. aureus* V329 was procured from Prof. InigoLasa, Spain, which is a strong biofilm forming strain. Three clinical isolates of *S. aureus* were procured from Department of Microbiology, Karnataka veterinary college, Bengaluru, and were designated as SA7, SA10 and SA33 respectively. A *bap*-isogenic mutant, *S. aureus* M556 procured from Prof. José R Penadés, Spain, was also included in the study. M556 strain was generated by transposon insertion in *bap* gene of *S. aureus* V329 (Cucarella et al., 2001).

Before each experiment the inoculums from the stock cultures were added to the freshly prepared media and allowed to grow overnight in an incubator at 37^{o}C. Two ml of overnight grown *S. aureus* cultures were inoculated in 50 ml of tryptic soy broth (TSB) supplemented with 0.25% glucose (w/v) in 250 ml Erlenmeyer's flasks and were incubated at 37^{o}C in an incubator at 150 rpm. Mid log phase cells were harvested, washed two times with phosphate buffer saline (PBS) and used for the biofilm studies after adjusting the absorbance (O.D.) to 0.5 at 600 nm.

Table 3.1: Details of *S. aureus* strains used in this study

Strain	Details	Source
***S. aureus* V329**	V329, *bap*-positive mastitis isolates	Prof. I. Lasa, Spain
***S. aureus* M556**	M556, *bap*-isogenic mutant of V329	Prof. J.R. Penades, Spain
***S. aureus* 7**	SA7, mastitis isolate	Department of Microbiology, Karnataka Veterinary College, India
***S. aureus* 10**	SA10, mastitis isolate	-do-
***S. aureus* 33**	SA33, mastitis isolate	-do-

TRIS-G minimal media supplemented with 1% glucose (w/v) was used in some of the experiments. Constituents of the one liter TRIS medium were as follows; Tris (hydroxymethyl)aminomethan 6.057 g, Tris HCl 7.88 g, NaCl (4.675 g), KCl (1.4912 g), NH_4Cl (1.0698 g), Na_2SO_4 (4.261g), $MgCl_2.6H_2O$ (2.033 g), $CaCl_2.2H_2O$ (0.294 g), $NaH_2PO_4.2H_2O$ (40.mg), Ferrus ammonium-citrate (4.8 mg/l) and 100 µl of trace elements stock solution [5 mM Zinc sulphate heptahydrate, 5 mM manganese chloride-Tetrahydrate, 10 mM Boric acid, 1 mM Nickel chloride hexahydrate, 1,5 mM sodium molybdate- dehydrate]. In addition, culture media were supplemented with filter sterilized $CaCl_2$, $MgCl_2$, NaCl and EDTA at various concentrations as per the experimental design.

3.2.2 Bioinformatic analysis

Prediction of probable site and signature sequences present in Bap protein was done by searching against Prosite data base by using PROSCAN (http://npsa-

pbil.ibcp.fr/cgi-bin/npsa_automat.pl?Page=/NPSA/npsa_proscan.html) (Combet et al. 2000).

3.2.3 PCR for *bap* gene detection

Bacterial cultures were grown overnight and genomic DNA was isolated from 3 ml mid log phase culture using a Qiagen DNA isolation kit according to manufacturer's protocol except that the bacterial cells were lysed by 5 µl of a 10 mg/ml solution of lysostaphin (Sigma) and incubated at 37°C for 2 h. For the detection of the *bap* gene, a set of previously published primers were selected (Table 3.2) (Potter et al., 2009; Vautor et.al. 2008). This ensures specificity and minimum bias in PCR amplification. Each primer set was targeted to different region of *bap* gene. A very low stringent condition for PCR like annealing temperature was kept at 45^{o}C to rule out the possibility of sequence variations. PCR program was set up as follows; an initial denaturation at 94^{o}C for 5 min was followed by 40 cycles of 94^{o}C for 1 min, 45^{o}C for 30 sec, and 72^{o}C for 1 min with a final step at 72^{o}C for 5 min. The size of the PCR products was analyzed by electrophoresis on 1% (w/v) agarose gel.

3.2.4 Dot blotting and hybridization for the *bap* gene

Briefly, 50 ng of denatured DNA was spotted onto Hybond N^{+} nylon membrane and DNA was UV-cross-linked to the membrane according to the manufacturer's instructions (Amersham Biosciences, India). The PCR product amplified with the primers sabF and sabR (Table 3.2), specific for the *bap* gene (971 bp long) from the V329 *S. aureus* strain, was used as the probe. This PCR product was purified with a QIAquick PCR purification kit (Qiagen, India). Purified PCR product was labeled using random-primed DNA labeling method with digoxigenin-dUTP as per the manufacturer's instructions (Roche Applied

Science, India). The hybridization with labelled probe was done at 60°C overnight. The hybridized probe was detected by the DIG DNA labeling and detection kit (Roche Applied Science, India) as per the manufacturer's instructions.

3.2.5 Estimation of Ca^{2+} in TSB medium

Ca^{2+} concentration in TSB was estimated using an inductively coupled plasma-atomic emission spectrometer (ICP-AES, model: Ultima-2, Horiba Jovin-Yvon, France). Sodium ion interference in ICP-AES analysis was reduced by dilution. Three dilutions of TSB; 1:5, 1:10 and 1:20 were used to estimate the concentration of Ca^{2+}. The mean value of the three dilutions was taken in to account.

3.2.6 Auto-aggregation assay

This assay was performed as per the standard procedure detailed by Beloin et al. (2006). The *S. aureus* stock cultures were sub cultured; the overnight grown broth (2 ml) was inoculated in 50 ml of TSB medium. Mid log phase cells were harvested and washed three times in PBS. Final OD was set to 1.0 in the PBS at 600 nm. The cell suspension was incubated at 37°C for 4 h Filter sterilized Calcium chloride was added to achieve different concentration of Ca^{2+}. After 4h of incubation absorbance was measured at 600 nm and percentage of auto-aggregation was calculated by using the following formula:

$$\% \text{ Autoaggregation} = \frac{ODo - ODt}{ODo} \text{X}100$$

Where, OD_0 is OD at zero hour and OD_t is OD at t hours (i.e., given time).

3.2.7 Microtitre plate assay for biofilm quantification

Staphylococcus aureus V329, M556, SA7, SA10 and SA33 biofilms were formed on pre-sterilized 96 well flat bottom polystyrene micro-titre plates in

triplicates. A micro-titre plate based crystal violet assay is an indirect method of biofilm quantification and was first described by Christensen et al. (1985). Since then several modifications have been made to increase its accuracy (O'Toole and Kolter, 1998; Stepanovic et al., 2000). However, micro-titre plate based assays share the issue of “edge effect”. The “edge effect” occurs mainly due to two reasons; first, peripheral wells are more ventilated thus can provide more O_2 for bacterial growth. Secondly, water evaporates quickly from peripheral wells thereby providing the planktonic cells to stick to the walls, which in turn binds the crystal violet dye and gives a false reading as biofilm biomass. The “edge effect” poses serious concerns when determination of antimicrobial or anti-biofilm efficacy of compounds has to be tested since evaporation increases the concentration of “testing compound” and the experiment end up with wrong crystal violet absorbance values.

In this study, some improvisation was made in the crystal violet assay to reduce water loss from the peripheral wells and to reduce the edge effect. The improved method showed a significant reduction in edge effect and minimized the error in crystal violet assay. A 10 µl of cell suspension having 0.5 $O.D_{600}$ was inoculated in 190 µl TSB medium in each well and autoclaved distilled water was added in peripheral wells to reduce the water loss. Then micro-titre plate was incubated for 16 h at 37^{o}C. After aspiration of planktonic cells biofilms were fixed with 99% methanol. Plates are washed twice with phosphate buffer saline or sterile saline water and air-dried. Then, 200 µl of crystal violet solution (0.2%) was added to all wells. After 5 min, the excess crystal violet was removed and plates were washed twice and air dried. Finally, the cell bound crystal violet was dissolved in 33% acetic acid. Biofilm growth was monitored in terms of $O.D_{570}$ nm

using micro plate reader (Multiskan, Thermo Labsystems). The plate assay was also used to observe the interaction of Bap protein with Mg^{2+} and in NaCl gradient of 0.1 - 0.5 % in presence of Ca^{2+}.

3.2.8 Planktonic growth studies

The experiments were carried out with overnight grown bacterial cultures by harvesting the cells and rinsing them twice with PBS and OD of each culture was set to 0.5 at 600 nm wherein the cell concentration was $1x10^8$ cfu /ml. 100 µl of each *S. aureus* culture was inoculated in 1900 µl of TSB medium supplemented with 0.25% glucose. Cultures were incubated at 37^{o}C and 150 rpm. Absorbance of each culture was recorded at different time intervals after vortexing for 5 sec, to re-suspend the settled cells.

3.2.9 Confocal laser scanning microscopy (CSLM) studies and image analysis

Biofilms grown on pre-sterilized microscopic glass slides were studied using CSLM (Saravanan et al. 2006). A 2 ml of 0.5 OD cultures of *S. aureus* was inoculated in sterile petri plates containing 20 ml of TSB medium supplemented with 0.25% glucose. Sterile glass slides were immersed into the medium as substratum for biofilm growth. The petri-plates were incubated at 80 rpm for 15 min on a shaker for proper mixing of the $CaCl_2$ and EDTA and incubated for 16 h at 37^{o}C. The glass slides were gently washed with PBS to remove loosely attached cells and stained with 0.2 % acridine orange for 5 mins, thereafter the slide is thoroughly washed with PBS. A thin cover slide was mounted over the stained biofilm and observed by keeping the slide upside down on objective lens of the confocal laser scanning microscope (TCS SP2 AOBS) equipped with DM IRE 2-inverted microscope (Leica Microsystems, Germany). Image stacks were

collected from 20 random points of the biofilms in order to get an accurate mean value of the biofilm parameters. Quantification of the biofilm parameters (average thickness, maximum thickness, total biomass, surface to biovolume ratio and roughness coefficient) was done by COMSTAT program written as a script in MATLAB 5.2 software (Heydorn et al. 2000). Each experiment was repeated three times to have statistically significant data.

3.2.10 Statistical Analysis

All data are expressed as mean standard deviation (SD) of the triplicate experimental data. A two-tailed Student's t-test was used to determine the differences in biofilm formation between the control and each group. The P value of < 0.05 was taken as significant.

3.3 Results

3.3.1 Improvisation in classical crystal violet assay method

Micro-titre plate results showed that when *S. aureus* biofilms are grown in 96 well micro-titre plates for more than 12 h, it is observed that biofilms grown in peripheral wells were thicker (with higher crystal violet absorbance at 570 nm) (Figure 3.1). However, when autoclaved distilled water was added in peripheral wells the results showed a significant reduction in the edge effect as shown in Figure 3.2. The results showed a relative homogeneity in biofilm formation in 96 well micro-titre plates with reduced standard deviation in crystal violet absorption values.

3.3.2 *In silico* analysis of Bap protein

Bap protein sequence analysis was done by searching against Prosite data base using PROSCAN (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_proscan.html) programme. The analysis showed the presence of

four putative EF hand motifs with 80% homology with consensus sequence of EF hand motif "D-{W}-[DNS]-{ILVFYW}-[DENSTG]-[DNQGHRK]-{GP}-[LIVMC]-DENQSTAGC]-x(2)-[DE]-[LIVMFYW]" (Prosite accession no. PS00018). Presences of putative sites of EF hand motif are shown in Table 3.3 and corresponding locations are shown in Figure 1.1, schematically.

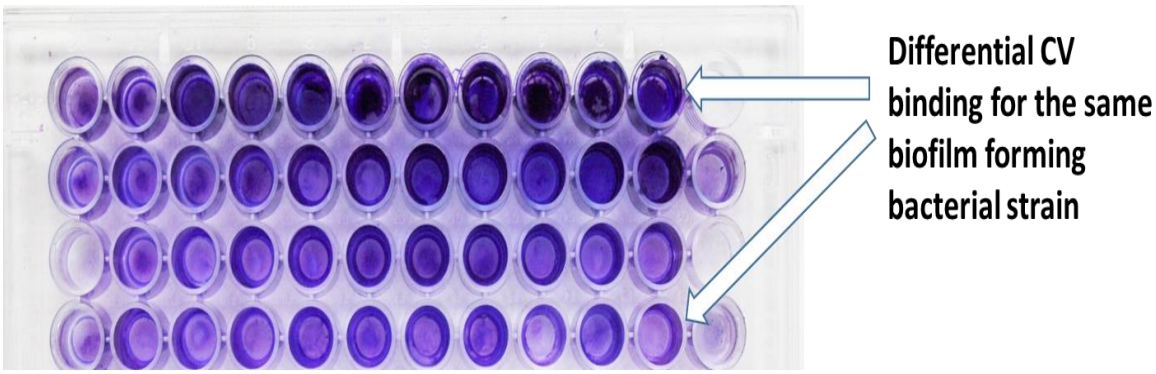


Figure 3.1: Image showing non-homogenously formed *S. aureus* biofilm in 96 well micro-titre plate due to edge effect. Apparently peripheral wells have more crystal violet binding to biofilm compared to inner wells.

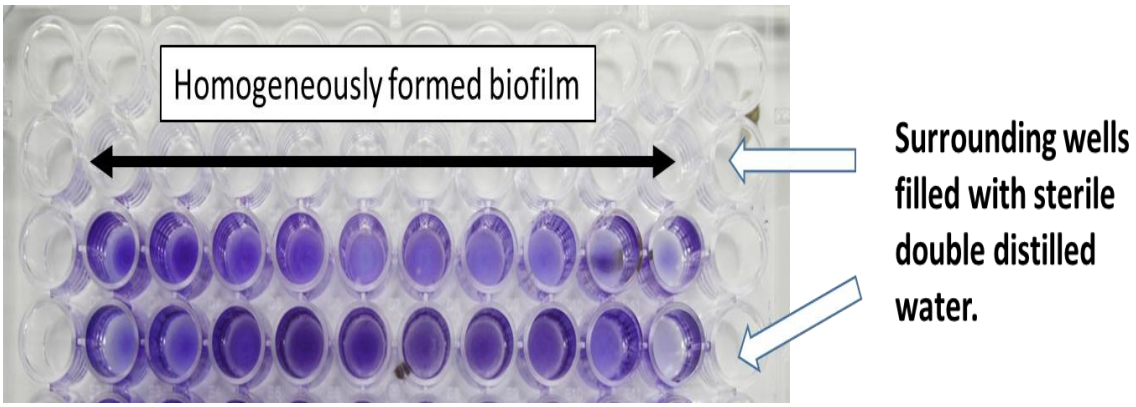


Figure 3.2: Image showing the homogeneity in *S. aureus* biofilm formation when side wells were filled with sterilized distilled water.

3.3.3 PCR mediated detection of *bap* gene in *S. aureus* isolates

All *S. aureus* strains were screened for the presence of *bap* gene, *S. aureus* V329 a *bap*-positive strain was used as positive control. PCR mediated search for *bap* gene resulted in the design of six sets *bap*-specific primers (Table 3.3) targeting different regions of *bap* was performed. Figure 3.3 shows that *bap* gene was present in V329 and M556 but no amplification band was found by any set of primers in SA7, SA10 and SA33. Therefore, all three *S. aureus* strains were considered as natural *bap*-negative strains. Figure 3.3 shows the corresponding sizes of the anticipated amplicons were present in V329 and M556 (as shown in Table 3.3). M556 strain showed the presence of *bap* gene, since it was constructed by insertion of transposon in to downstream of *bap* gene, resulting in the production of truncated protein lacking cell wall anchoring region.

***Table 3.2*:** Primers used to detect *bap* gene in the study.

Primers	Sequence (5'-3')	Amplified Product (bp)	Reference
Bap1 F	ATGGGAAATAAACAAGGTTTTTTACC	694	Potter et. al., 2009
Bap1 R	CTTCTTGTTGTTTATCTGGCTC		
Bap2 F	GAGCCAGATAAACAACAAGAAG	598	-do-
Bap2 R	CATGCTCAGCAATAATTGGATC		
Bap3 F	GATCCAATTATTGCTGAGCATG	574	-do-
Bap3 R	CACCTTCGATATATGGTAGTAAGTC		
Bap4 F	GACTTACTACCATATATCGAAGGTG	631	-do-
Bap4 R	CCTCTGCATTAATTACTTTAGC		
Bap5 F	GTTCCTCTTAAAGAAGGTGCAG	407	-do-
Bap5 R	CTAGCTGTTGAAGTTAATACTG		
sab F	CCCTATATCGAAGGTGTAGAATTGCAC	971	Vautor et.al., 2008
sab R	GCTGTTGAAGTTAATACTGTACCTGC		

Table 3.3: Presence of putative calcium binding EF-hand domains and their location in respective Bap protein sequences. [A= DKDllpYIEGVEL (80% homology); B = DYDKDGLLDRYEr (91% homology); C = DTDGDGKnDGDEV (89% homology); D = DNSDSDDnSDNEd (80% homology)]. (Chapter 2; Table 2.3)

PROTEINS SEQUENCE ID	PRESENCE OF PUTATIVE EF-HAND DOMAINS AND THEIR LOCATION IN RESPECTIVE PROTEINS			
	A	**B**	**C**	**D**
AAK38834.2	598 - 610	729 - 741	752 - 764	2198 - 2210
AAY28520.1	586 - 598	717 - 729	740 - 752	3200 - 3212
WP_002501035.1	598 - 610	729 - 741	752 - 764	1073 - 1085
WP_017723937.1	594 - 606	725 - 737	748 - 760	--
AAY28516.1	540 - 552	671 - 683	694 - 706	1452 - 1464
ABW74862.2	598 - 610	729 - 741	752 - 764	--
AAY28517.1	594 - 606	725 - 737	748 - 760	--
AAY28519.1	598 - 610	729 - 741	752 - 764	2664 - 2676
AAY28518.1	598 - 610	729 - 741	752 - 764	1596 - 1608
WP_002476148.1	489 - 501	620 - 632	643 - 655	1473 - 1485
YP_004267641.1	486 - 498	617 - 629	640 - 652	1484 - 1496

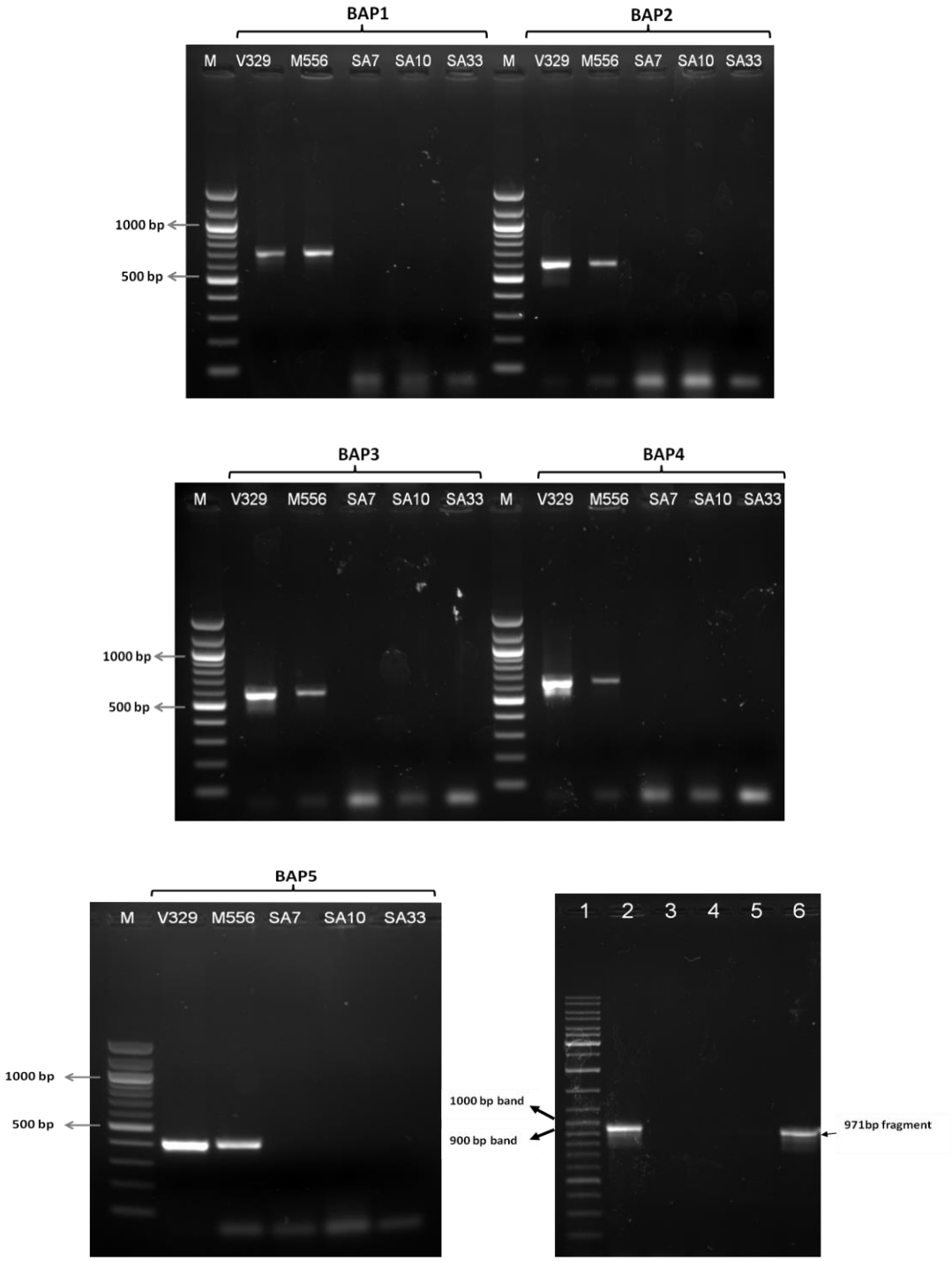


Figure 3.3: Detection of *bap* gene by six sets of *bap* specific primers in different strains. For Bap1 to Bap6 set of primers lanes are marked respectively in gel images. For sab F/R primers; Lane 1 -DNA ladder, lane 2- *S. aureus V329*, lane 3- SA 7, lane 4- SA10, lane 5- SA33, lane 6-M556.

3.3.4 Detection of *bap* gene by hybridization with *bap*-specific DIG-labeled probe

Random mutations or deletions in the primer pairing regions of the *bap* gene could have affected PCR mediated approach for the detection of *bap* gene. Dot blotting of isolated genomic DNA on nylon membrane and hybridization with *bap*-specific DIG-labeled probe followed by antibody mediated detection showed the presence of *bap* gene in V329 and M556 (Figure 3.4). Other *S. aureus* clinical isolates i.e. SA7, SA10 and SA33 were found to be *bap*- negative since there was no color development on genomic DNA blots respectively. Since similar results were noticed with PCR study, the dot blot assay compliment the findings of PCR mediated approach for the detection of *bap* gene.

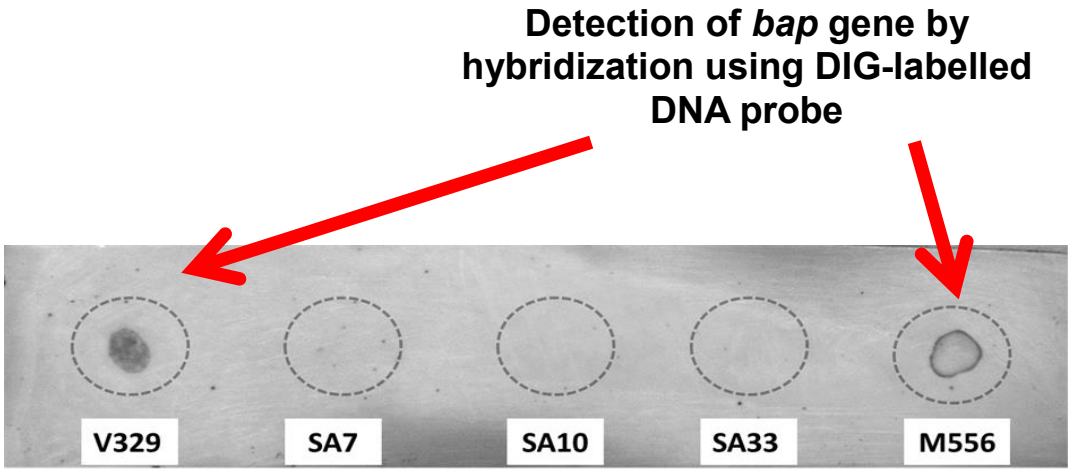


Figure 3.4: Detection of *bap* gene by dot blotting and hybridisation with *bap* specific probe (~1 kb) from *S. aureus* strains; (1) *S. aureus* V329 (positive control), (2) SA7 (3) SA10 (4) SA33 (5) M556.

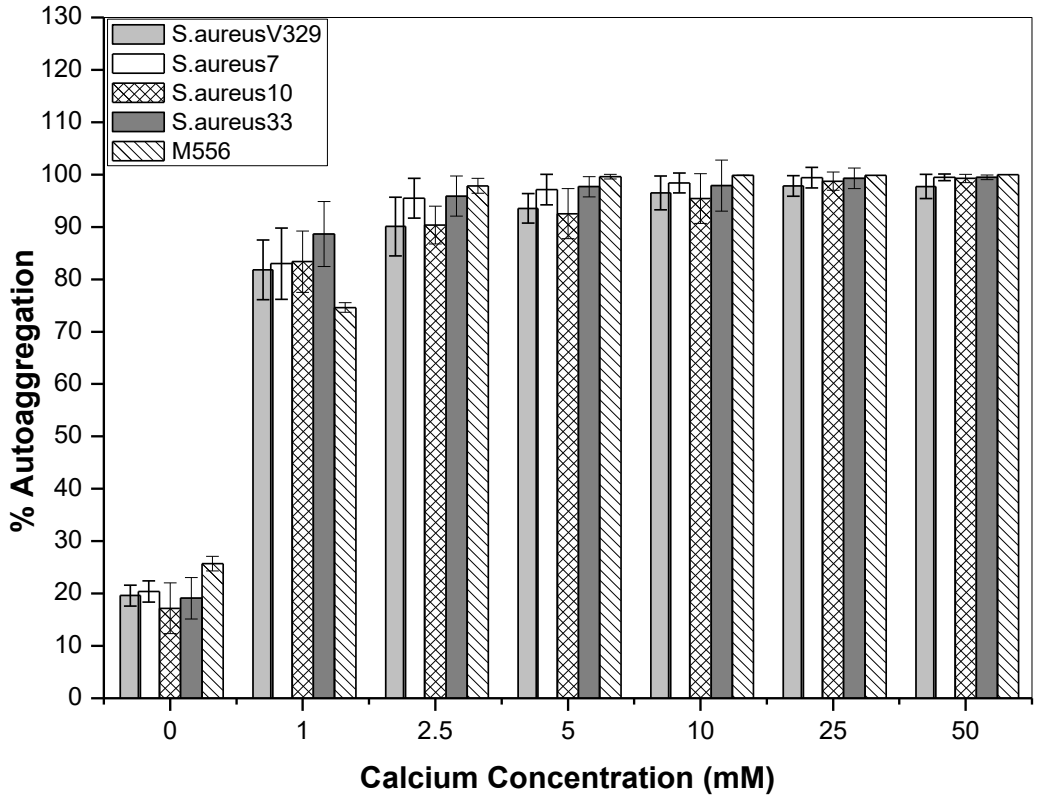


Figure 3.5: Effect of different concentration of Ca^{2+} on auto-aggregation behavior of different *S. aureus* strains. Error bars at some points are not visible due to very small variation.

3.3.5 Effect of Ca^{2+} on auto-aggregation in *S. aureus*

To study the effect of Ca^{2+} on auto-aggregation milimolar (mM) concentrations of Ca^{2+} were added and tested in *S. aureus* cultures. Ca^{2+} enhanced the auto-aggregation in all *S. aureus* strains, irrespective of the presence of *bap* gene. Figure 3.5 indicates that the percentage of auto-aggregation ranged from 80 to 99% with all the strains. Thus, it can be inferred that Bap may not be responsible for the Ca^{2+} mediated aggregation behaviour of *S. aureus* since *bap*-isogenic mutant M556 as well as other *bap*-negative *S. aureus* isolates also showed auto-aggregation.

3.3.6 Effect of Ca^{2+} on *S. aureus* biofilm development and planktonic growth

The effect of milimolar concentration of Ca^{2+} on biofilm growth of *bap*-positive V329, *bap* mutant M556 and the three *bap*-negative strains of *S. aureus* was monitored by micro-titre plate assay. The inhibition of V329 biofilm development in presence of Ca^{2+} was visually observed by the decrease in thickness of the biofilm growth ring formed at the surface of culture broth when grown in the glass test tubes (Figure 3.6). The biofilm assay (Figure 3.7) showed that Ca^{2+} significantly inhibited biofilm growth in *bap*-positive V329 strain in a dose dependent manner. However, it did not affect the biofilm growth of *bap*-negative SA7 and SA33 even up to 50 mM concentration while SA10 showed reduced biofilm formation at >12.5 mM Ca^{2+}. Strain V329 initially showed a gradual decrease in biofilm formation up to 6.25 mM, later significant reduction was observed. On the other hand *bap*-mutant M556 formed very weak biofilm and the presence of Ca^{2+} did not alter its biofilm forming capacity positively or negatively. Although SA7 showed an initial decrease in biofilm growth it gradually increased from 0.75 – 50 mM Ca^{2+}. Strains SA33 and M556 almost showed similar growth pattern in presence of Ca^{2+}. Growth studies (Figure 3.8) showed that Ca^{2+} did not decrease the growth of the cells in any of the *S. aureus* strains except SA10 where planktonic growth was hampered at $\geq$5 mM Ca^{2+}, which resulted in inhibition of biofilm formation. This also proves that biofilm inhibition of SA10 strain in presence of Ca^{2+} is essentially Bap independent.

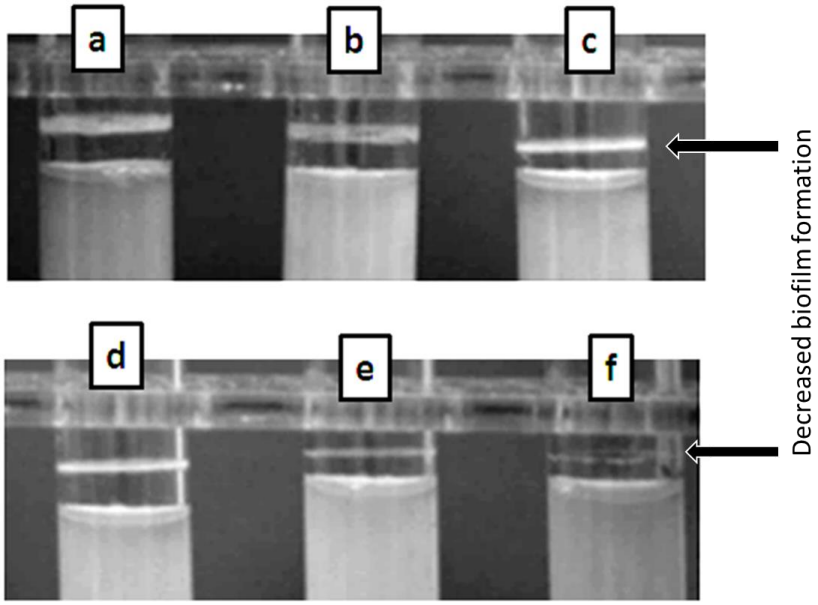


Figure 3.6: Effect of Ca^{2+} on biofilm forming capacity of a 24 h culture on the surface of the glass test tube (visual observation). a) Control; b) Ca^{2+} 0.5 mM; c) Ca^{2+} 1 mM; d) Ca^{2+} 2 mM; e) Ca^{2+} 5 mM; f) Ca^{2+} 10 mM.

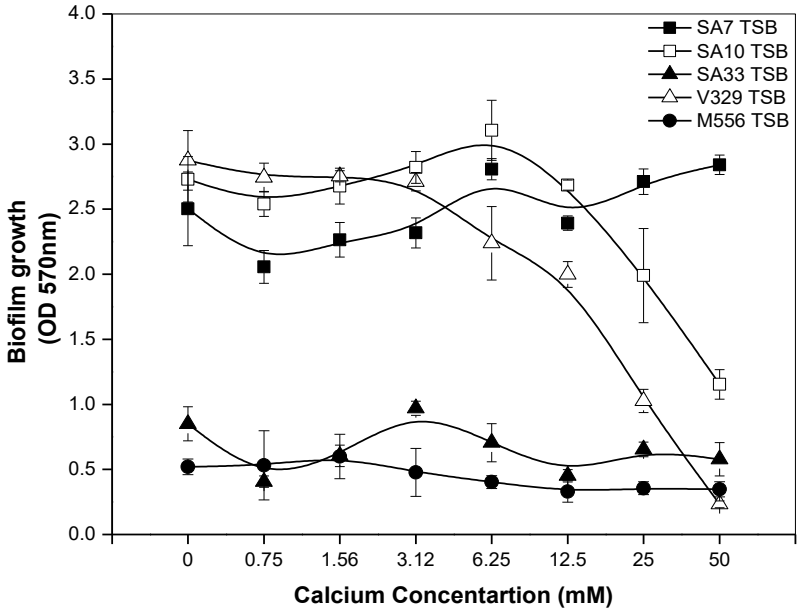


Figure 3.7: Comparative study of biofilm forming capacity among *bap*-positive and *bap*-negative strains of *S. aureus* in the presence of different concentration of Ca^{2+}. Biofilm was evaluated after 16 h of growth. Error bars at some points are not visible due to very small variation.

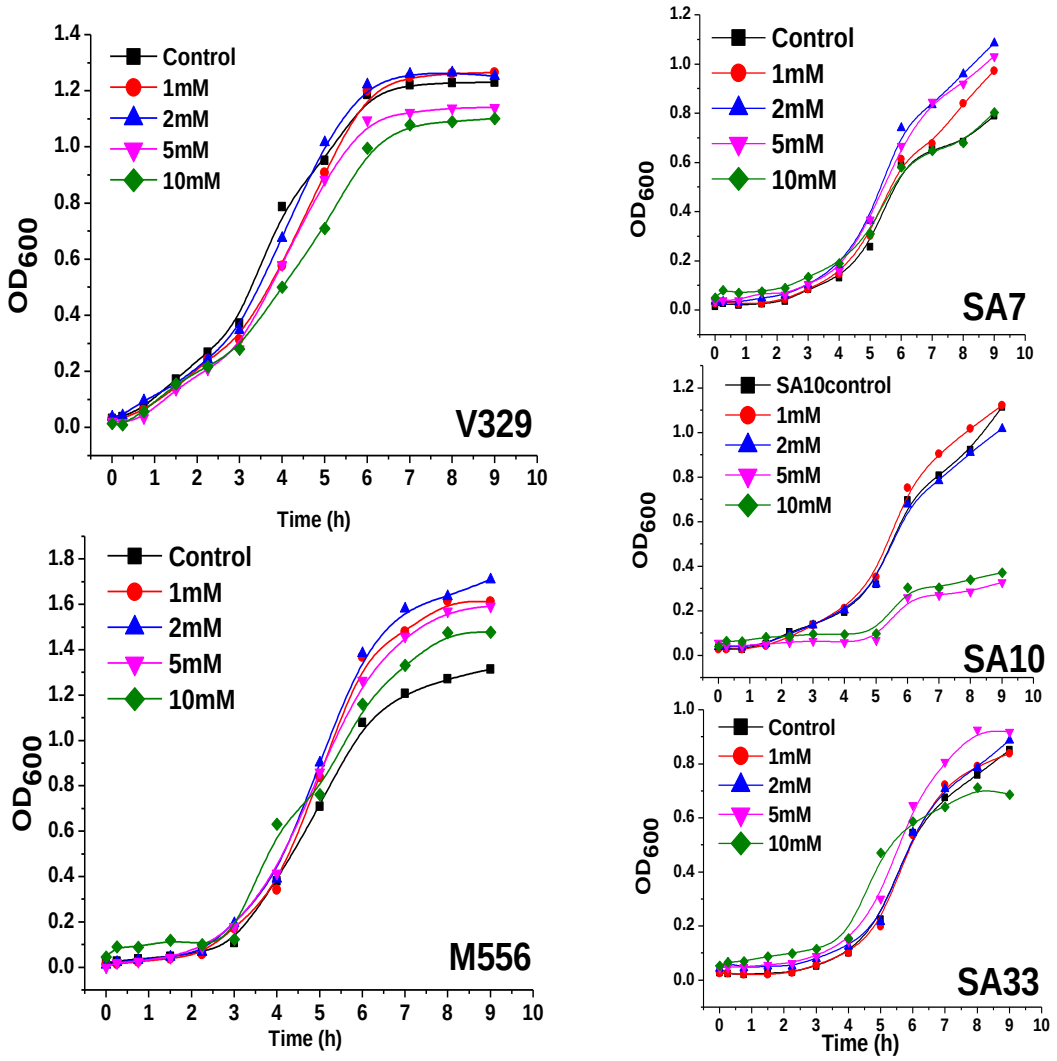


Figure 3.8: Effect of different concentration of Ca^{2+} on planktonic growth of *S. aureus* strains.

3.3.7 Effect of growth medium on *S. aureus* biofilm development in the presence of Ca^{2+}

In the present study ICP-AES analysis showed that the concentration of Ca^{2+} in TSB medium was 7.36 ± 0.85 mM. It was observed that addition of Ca^{2+} to TSB medium enhanced the turbidity. This observation contemplates that the essential nutrients or factors which might be required for biofilm formation could have been complexed with Ca^{2+}. To rule out this possibility minimal media was used to observe the effect of Ca^{2+} on biofilm formation of *bap*-positive and *bap*-negative strains. Biofilm assay using TRIS minimal medium supplemented with

1% glucose (TRISG), showed that *S. aureus* V329 exhibited similar behavior in presence of Ca^{2+} as shown in TSB complex media (Figure 3.9). Thus it can be inferred that biofilm producing capacity was decreased in the presence of Ca^{2+}. Strain M556, a *bap* mutant formed very weak biofilm in TSB as well as TRIS and did not show any response in presence of Ca^{2+}. Similarly SA7 and SA33 strains did not show biofilm inhibition in presence of Ca^{2+}. However, SA10 showed reduced biofilm formation in minimal media TRISG (Figure 3.9). It can be speculated that this particular *S. aureus* strain (SA10) needs an unknown factor present in complex media for growth which also competes with Ca^{2+} and binds to the unknown-ligand. At very high concentration (>12.5 mM) Ca^{2+} showed more affinity to its ligand than unknown factor hence reduced the growth of biofilm. On the other hand in minimal media the unknown factor is absent hence biofilm formation is significantly decreased and we did not see any competing effect at all concentrations of Ca^{2+}.

3.3.8 Effect of divalent ions and increasing salt on concentration on Bap-mediated Ca^{2+} biofilm inhibition in *S. aureus* V329.

In this experiment, it was studied whether the biofilm inhibition in V329 is Ca^{2+} specific or any other divalent ion can manifest similar effect. To test this assumption, similar concentrations of Mg^{2+} ions i.e. 0.78 mM to 50 mM were used in biofilm assay. Figure 3.10 describes that Mg^{2+} did not show any significant change in biofilm forming capacity of V329 even up to 50 mM concentration, while Ca^{2+} showed a dose dependent inhibition. Apart from the above study, it was also evaluated whether Bap and Ca^{2+} interaction is specific or mere electrostatic in nature. To rule out the possibility of Bap and Ca^{2+} interaction is not electrostatic, three inhibitory concentrations of Ca^{2+} were tested; 5 mM, 10 mM and 20 mM.

V329 biofilm was grown in presence of different concentration of salt (NaCl) in TSB medium along with three inhibitory concentrations of Ca^{2+}. Figure 3.11 show that increasing concentration of NaCl did not affect Ca^{2+} inhibition of V329 biofilm development. Similar inhibition pattern was observed at three different inhibitory concentration of Ca^{2+} (5 mM, 10 mM and 20 mM) when salt concentration was increased from 0.1 to 0.5%.

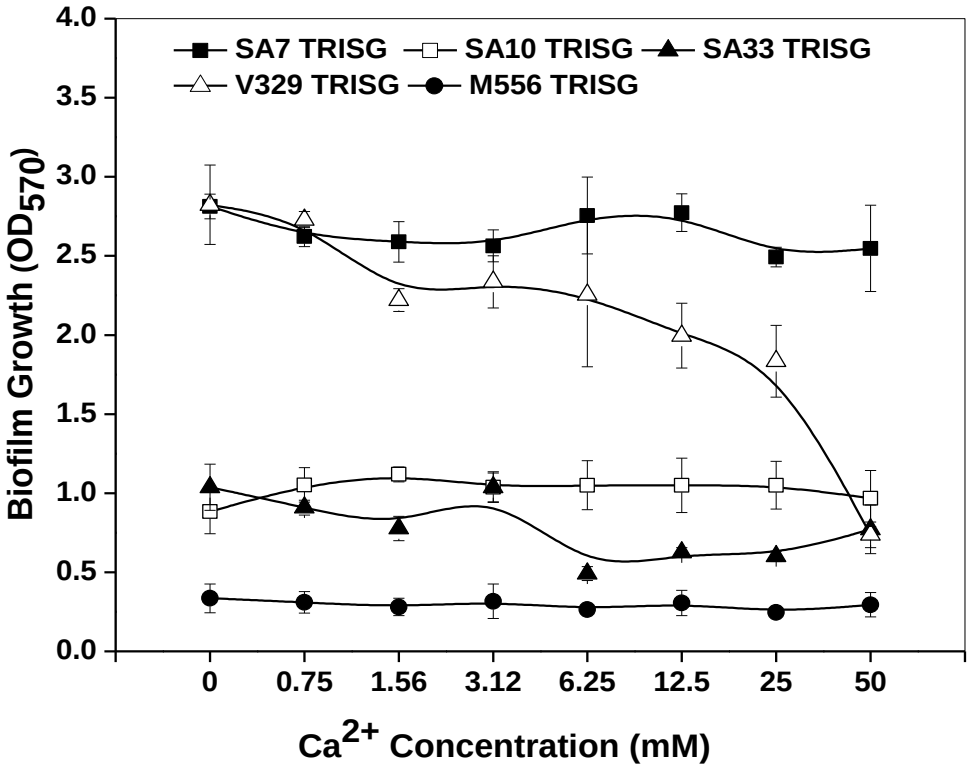


Figure 3.9: Comparative study of biofilm forming capacity among *bap*-positive and *bap*-negative strains of *S. aureus* in the presence of different concentration of Ca^{2+} in TRIS-glucose minimal media. Biofilms were evaluated after 16 h of growth. Error bars at some points are not visible due to very small variation.

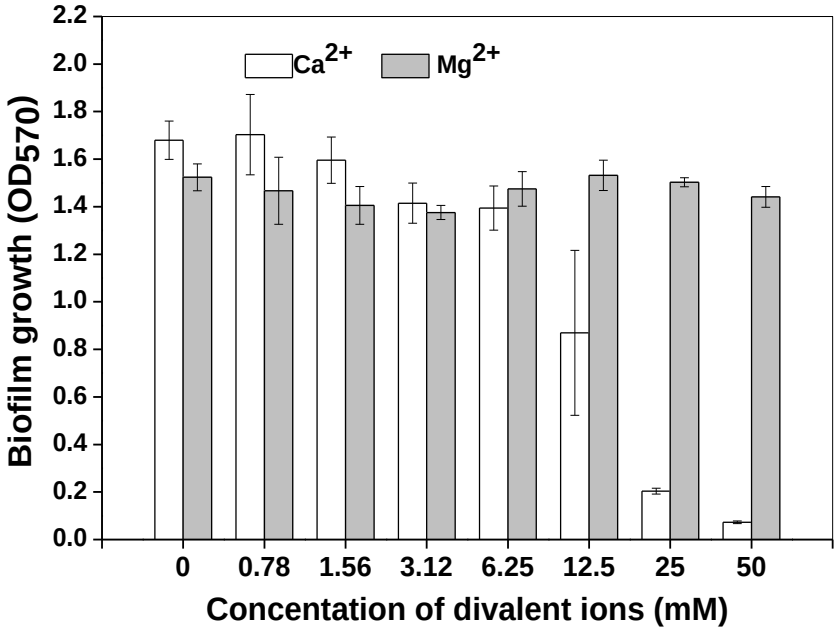


Figure 3.10: Effect of divalent ions (Ca^{2+} and Mg^{2+}) on biofilm forming capacity in *S. aureus* V329. Biofilms were evaluated after 16 h of growth. Error bars are shown as ±1SD.

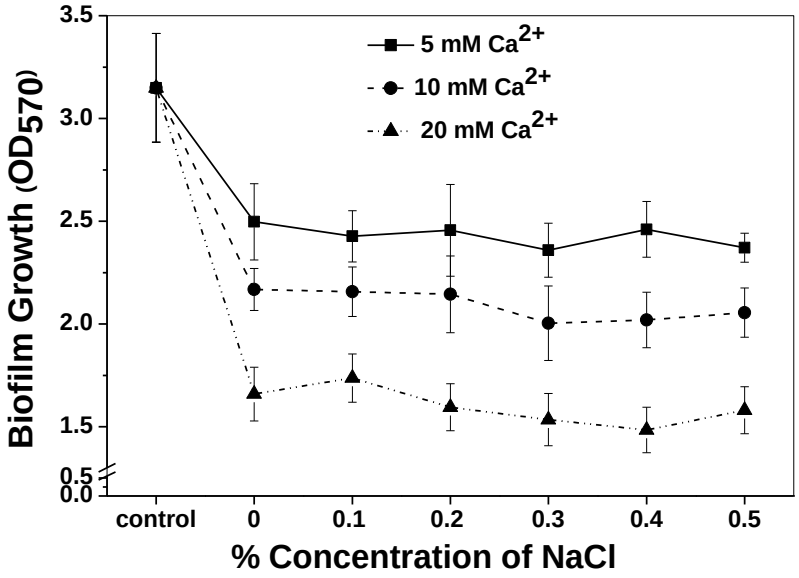


Figure 3.11: Effect of decreased electrostatic interaction by increasing salt concentration on biofilm forming capacity of *S. aureus* V329 in presence of Ca^{2+}. In control neither Ca^{2+} nor NaCl was added. Biofilms were evaluated after 16 h of growth. Error bars are shown as ±1SD.

3.3.9 Effect of Ca^{2+} on *S. aureus* V329 biofilm architecture and topography

CSLM studies were carried out to examine the effect of Ca^{2+} on *S. aureus* biofilm topography and architecture. Here, two *bap*-negative *S. aureus* strains (SA7 and SA10) were studied along with *bap*-positive strain. SA33 and M556 were excluded from the CSLM study since SA33 was found to form weak biofilm on glass surface and M556 did not form biofilm at all. CSLM images manifest the topographical changes in the growth *of S. aureus* V329 biofilm in presence of sub-inhibitory (1 mM and 2 mM) to critical inhibitory concentration (≥5 mM) of Ca^{2+} on glass slides as shown in Figure 3.12. Ca^{2+} influenced the architecture and topography of the *S. aureus* V329 biofilm which varied in response to different concentrations as shown in the CSLM images. Analysis of V329 biofilm parameters using COMSTAT program in presence of different concentration is shown in Table 3.4. Figure 3.13A and Figure 3.13B shows the dose dependent decrease in average thickness and maximum thickness of the *S. aureus V329* biofilm in response to Ca^{2+}. It was found that at 1 mM Ca^{2+} concentration there was slight decrease in biofilm biomass, average thickness and maximum thickness whereas the roughness coefficient and surface to biovolume changed positively. It indicates at 1 mM V329 biofilm was less dense and biofilm was more exposed to bulk liquid.

Figure 3.12A and Figure 3.12B show dense protruding pillars of clumped cells distributed all over and make water channels. From 2 mM to 10 mM Ca^{2+} concentration range; total biomass, average thickness and maximum thickness changed negatively whereas roughness coefficient and surface to biovolume

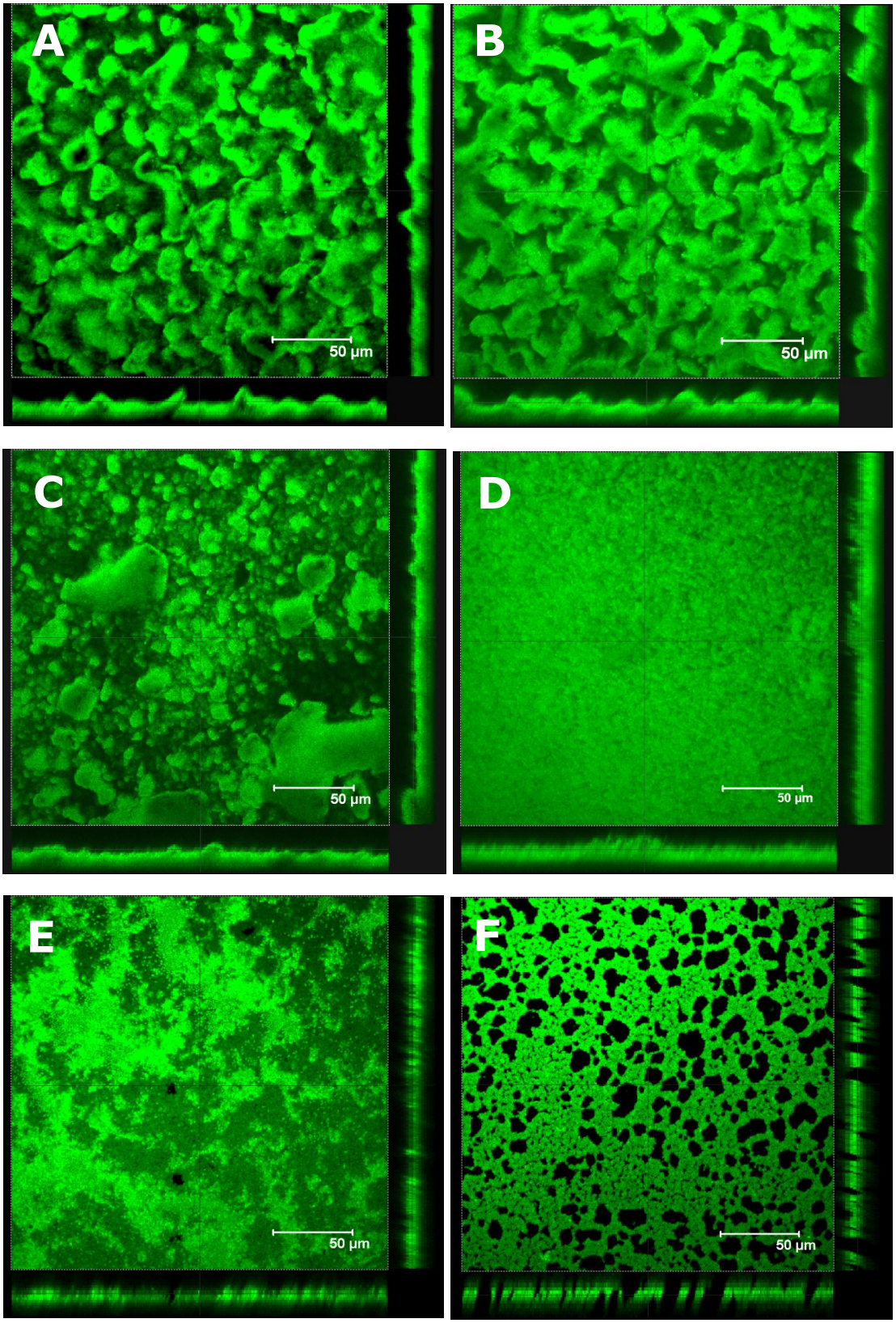
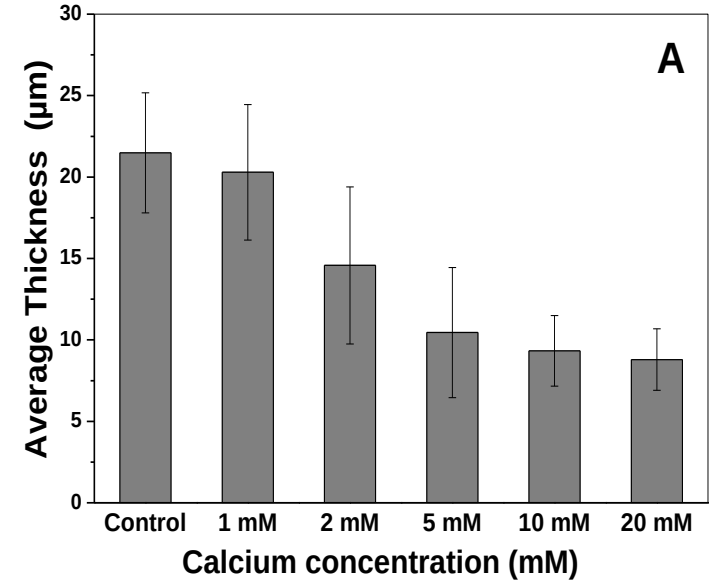
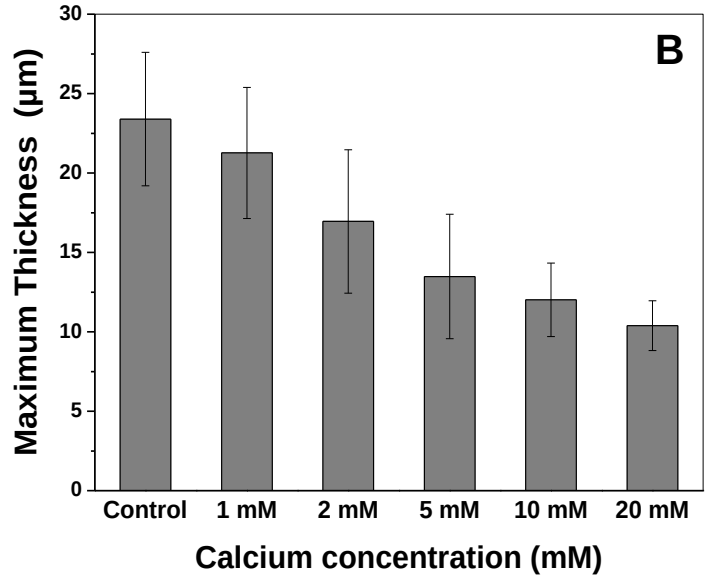
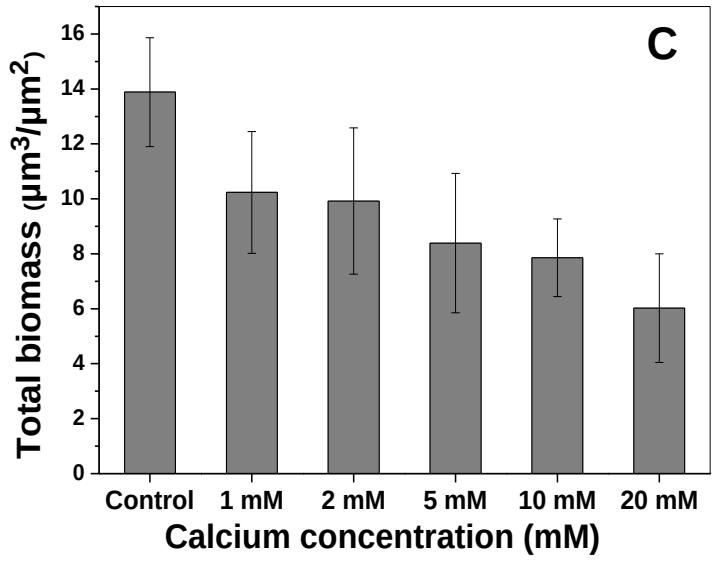


Figure 3.12: CSLM images of *S. aureus* V329 16 h old biofilm in presence of different concentration of calcium. A) Control; B) 1 mM Ca^{2+}; C) 2 mM Ca^{2+}; D) 5 mM Ca^{2+}; E) 10 mM Ca^{2+}; F) 20 mM Ca^{2+} (Scale bar 50µm).


A
Average Thickness (µm)
0
5
10
15
20
25
30
Control
1 mM
2 mM
5 mM
10 mM
20 mM
Calcium concentration (mM)


B
Maximum Thickness (µm)
0
5
10
15
20
25
30
Control
1 mM
2 mM
5 mM
10 mM
20 mM
Calcium concentration (mM)


C
Total biomass (µm3/µm2)
0
2
4
6
8
10
12
14
16
Control
1 mM
2 mM
5 mM
10 mM
20 mM
Calcium concentration (mM)

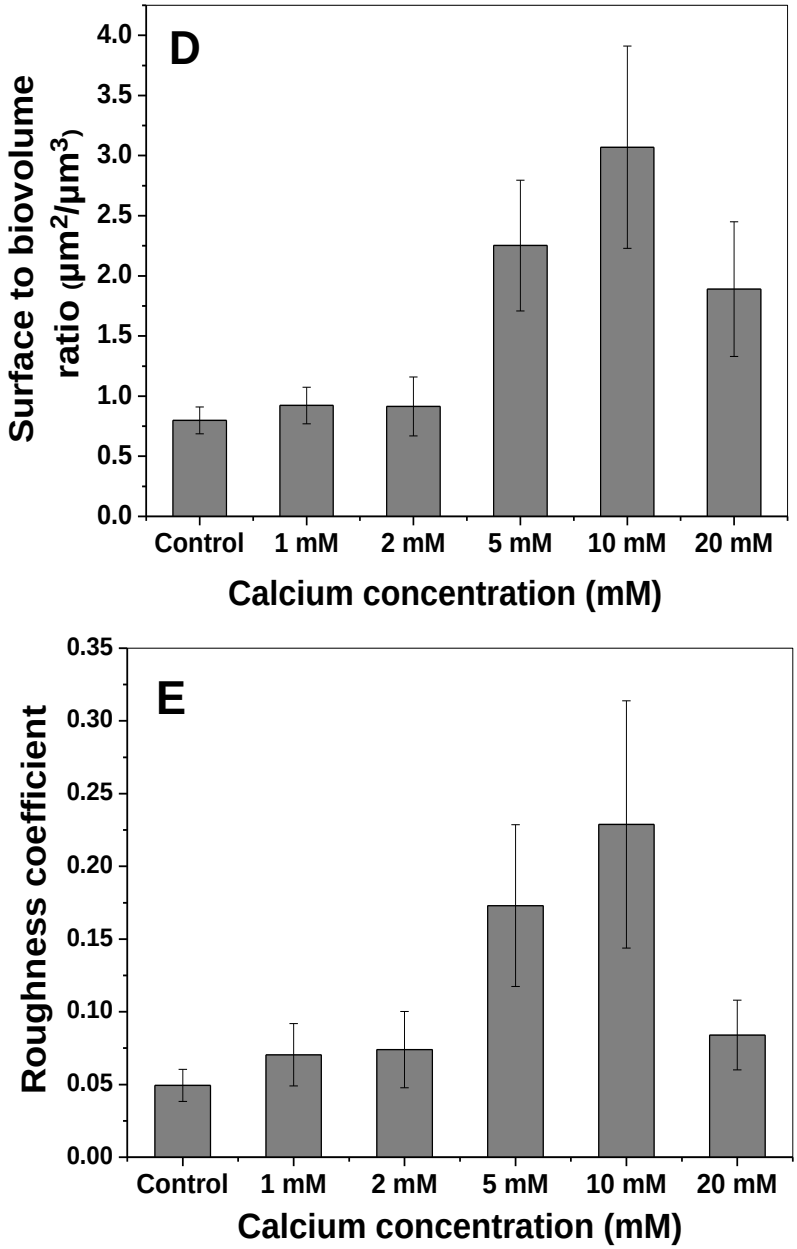


Figure 3.13: Effect of different concentration of Ca^{2+} on *S. aureus* V329 biofilm parameters. Error bars are shown as ±1SD. Asterisk, P value < 0.0001 as compared with *S. aureus* cells wherein no calcium was added (control). (A) Average thickness (B) maximum thickness (C) total biomass (D) surface to biovolume ratio (E) Roughness coefficient.

Table 3.4: Structural parameters of biofilm formed by *S. aureus* V329 in TSBG in the presence of different concentration of Ca^{2+}. Biofilm parameters were quantified using COMSTAT from the CSLM images.

Biofilm Parameters	TSBG	TSBG+ Ca^{2+}				
		1 mM	2 mM	5 mM	10 mM	20 mM
Total biomass (μm^3 μm^{-2})	13.884 ± 1.9781	10.234 ± 2.214	9.921 ± 2.659	8.388 ± 2.536	7.855 ± 1.412	6.02 ± 1.97
Average thickness (μm)	21.479 ± 3.687	20.289 ± 4.16	14.575 ± 5.824	10.45 ± 3.997	9.327 ± 2.168	8.79 ± 1.89
Maximum thickness (μm)	23.394 ± 4.198	21.262 ± 4.123	16.952 ± 5.517	13.48 ± 3.919	12.016 ± 2.319	10.39 ± 1.57
Roughness coefficient	0.0494 ± 0.011	0.0704 ± 0.0214	0.074 ± 0.0262	0.173 ± 0.0557	0.23 ± 0.09	0.08 ± 0.03
Surface to biovolume ratio (μm^2 μm^{-3})	0.799 ± 0.112	0.922 ± 0.152	0.915 ± 0.245	2.252 ± 0.5435	3.069 ± 0.84	1.89 ± 0.56

ratio changed positively. However at ≥ 5 mM Ca^{2+} all biofilm parameters under investigation showed significant change, positively or negatively. Continuous decrease in total biomass, maximum thickness and average thickness and enhanced roughness coefficient and surface to biofilm ratio suggests that increasing Ca^{2+} affect the biofilm architecture and arrangement of the cells. V329 biofilm gradually became thinner and flatter as Ca^{2+} concentration was increased in the TSB medium. At 20 mM Ca^{2+} V329 biofilm showed little change in maximum and average thickness but substantial reduction in total biomass as well as

roughness coefficient and surface to biovolume ratio entailed that biofilm had more porous, and mosaic structured with voids as compared to 10 mM Ca^{2+}.

To confirm whether the observed topographical changes are due to Ca^{2+}, the critical inhibitory concentrations of Ca^{2+} (5 mM) along with non-inhibitory concentration of EDTA (0.15 mM) which chelate the Ca^{2+} ions was tested. Figure 3.14 (A-C) shows CSLM image of vertical section of control V329 biofilms, in presence of Ca^{2+} and Ca^{2+} along with EDTA respectively. Figure3.15A shows that the thickness of the biofilm developed in presence of Ca^{2+} and EDTA did not show significant difference ($P = 0.3264$) as compared to control biofilm. Whereas biofilm formed in presence of only Ca^{2+} (5 mM) showed significant difference from the control biofilm ($P < 0.0001$) i.e. when Ca^{2+} is chelated by EDTA biofilm forming capacity of V329 strain was restored. Figure 15B shows thickness observed in each case. Identical treatment of Ca^{2+} was given to *S. aureus* SA7 and SA10 where no substantial differences were observed (see Figure 3.15A and Figure 3.15B).

3.4 Discussion

Microorganisms acquire diverse mechanisms to adhere and initiate biofilm development in various environments (Johnson et al. 2005). In the present work, the role of a surface protein, Bap in *S. aureus* biofilm formation was investigated and the topographic changes in response to Ca^{2+} variation. Bioinformatic analysis showed the presence of four calcium binding motifs with 80 % homology with EF hand motif; this indicates that the Bap protein has inherent property to chelate Ca^{2+} rather than having normal electrostatic / ionic interaction. In their pioneering study Arrizubieta et al. (2004), gave more emphasis on the effect of Ca^{2+} on multi-

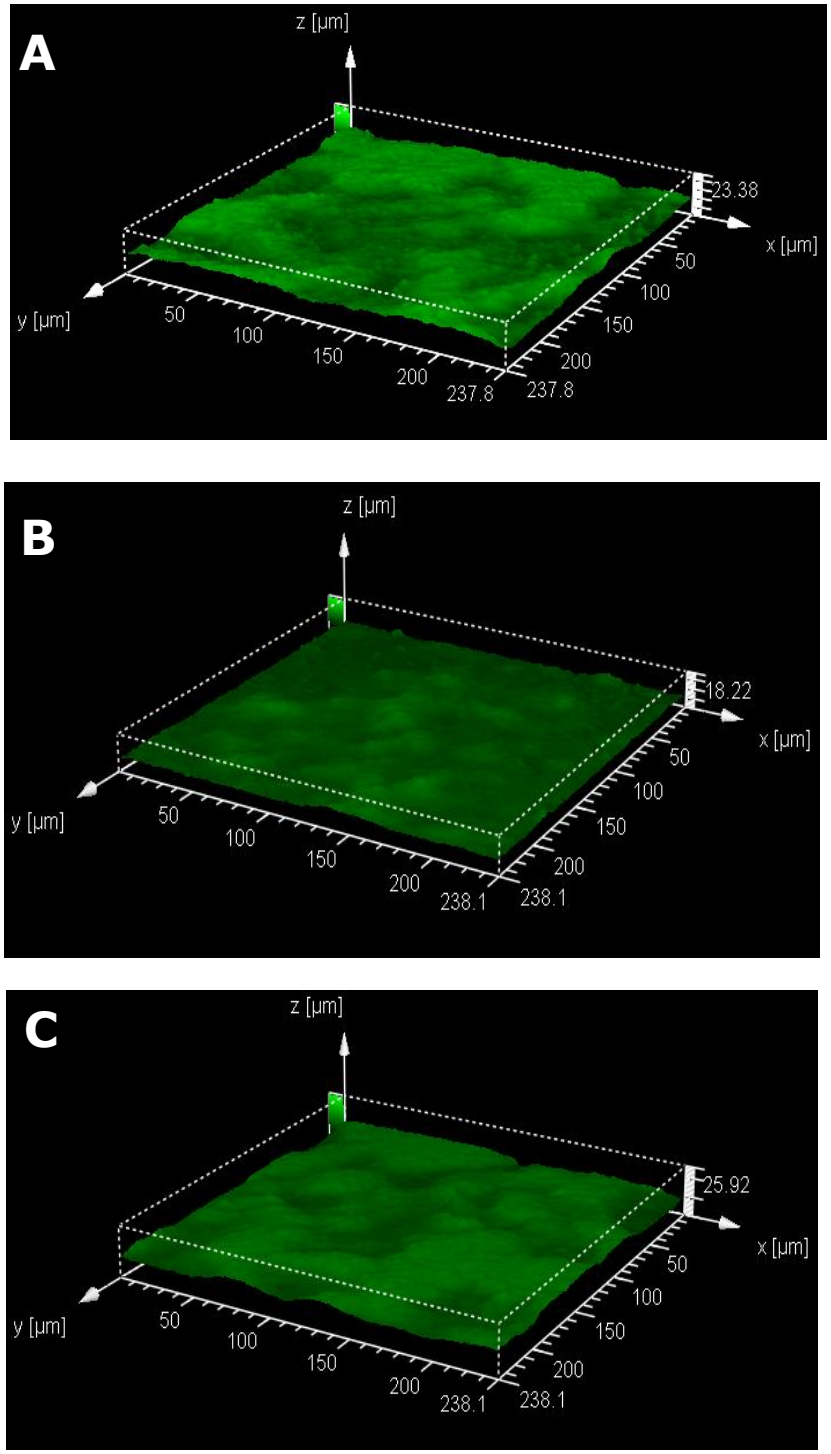


Figure 3.14: CSLM images showing thickness of 16 h old *S. aureus* V329 biofilm in presence of chelating agent A) control V329 biofilm; B) V329 biofilm in presence of 5 mM calcium; C) biofilm in presence of 5 mM calcium and 0.15 mM EDTA.

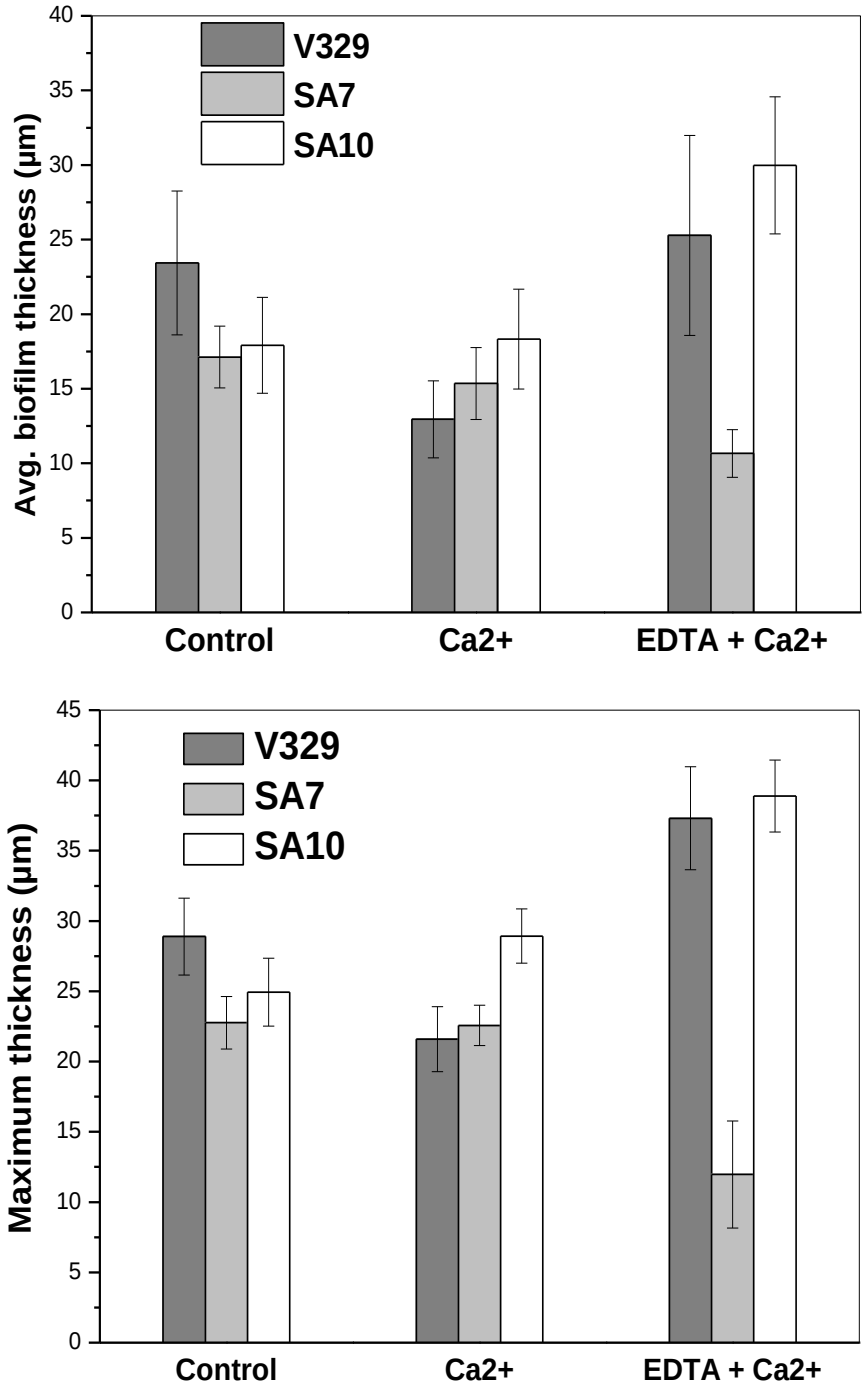


Figure 3.15: Comparative CSLM study of 16 h old biofilm among bap-positive, *bap*-negative strains of *S. aureus* in the presence of critical inhibitory concentration of Ca^{2+} in one set and Ca^{2+} in the presence of non-inhibitory concentration of EDTA in other set. Error bars are shown as ±1SD. (A) Average biofilm thickness (B) Maximum biofilm thickness.

cellular behavior of *S. aureus* cells and experiments were carried out using TSB supplemented with glucose. However, in this study it was observed that addition of Ca^{2+} to TSB media has increased the turbidity and it was difficult to interpret the aggregation behavior in turbid conditions. When PBS was used there was no turbidity hence the buffer was used to observe auto-aggregation in presence of Ca^{2+}. The results showed that auto aggregative behavior is independent of presence of Bap. The biofilm assay results strengthens the hypothesis that putative presence of EF hand motifs for Ca^{2+} in Bap might influence the biofilm development in *bap*-positive *S. aureus.* This effect was found to be true in presence of calcium, since *bap*-isogenic mutant M556 did not manifest similar response. In order to evaluate, whether inhibition of biofilm was due to the hampered growth or due to the life style switch over of the cells to planktonic form, growth studies were carried out with all strains of *S. aureus* in presence of different concentration of Ca^{2+} (Figures 3.8). Interestingly, higher absorbance values and more *S. aureus* V329 planktonic cells was noticed after 6-7 hours at low Ca^{2+} concentration. This could be simply because of the fact that in presence of Ca^{2+}, the *S. aureus* V329 cells prefer to be in planktonic phase rather than changing to biofilm mode. However, strain M556 showed less absorbance values in presence of Ca^{2+}. The growth study results infer that Ca^{2+} does not inhibit the cell division of the bacterial cells. Thus, the possibility of decrease in biofilm forming capacity in *bap*-positive V329 due to the inhibition of cell division was ruled out. Biofilm assay using minimal medium proved that biofilm inhibition by Ca^{2+} in V329 is not due to any complexation of Ca^{2+} with medium component.

According to Michiels (2002), EF hand motifs are known to have very specific interaction with Ca^{2+}. Biofilm assay in presence of Mg^{2+} showed that Bap-

dependent Ca^{2+} inhibition of V329 biofilm development is highly specific. If other surface proteins or Bap had non-specific interactions with the divalent ions then, presence of Mg^{2+} would have resulted in the inhibition of biofilm forming capacity. Increased salt concentration reduces the electrostatic interaction between the two charged moieties. If EF hand and Ca^{2+} had electrostatic interaction increased salt concentration would have reduced electrostatic interaction thereby decreasing Ca^{2+} inhibition. Biofilm assay in presence of high salt concentration showed that Bap-dependent Ca^{2+} inhibition of biofilm development in V329 is highly specific. If other surface proteins or Bap had non-specific interactions with Ca^{2+} ions then, high salt concentration would have resulted in the inhibition of biofilm forming capacity. Therefore, the probability of inhibition of biofilm formation because of nonspecific electrostatic interactions between Ca^{2+} and Bap as well as other surface proteins is ruled out. It can be hypothesized that this specificity of Bap towards Ca^{2+} can confer structural or enzymatic functionality to the protein.

COMSTAT analysis of CSLM images of V329 biofilm development at higher concentration of Ca^{2+} showed a decrease in biofilm formation and presence of Ca^{2+} substantially affected the architecture of V329 biofilm. Among the various biofilm parameters, the surface to biovolume ratio showed positive response with increase in Ca^{2+} concentration up to 10 mM which signifies that a high fraction of cells in the V329 biofilm were exposed to the bulk liquid. Similarly a positive change in roughness coefficient indicated an increase in biofilm heterogeneity up to 10 mM Ca^{2+}. COMSTAT analysis showed that there was gradual increase in maximum thickness, average thickness, total biomass and increase in roughness coefficient and surface to bio volume ratio in range of 1 – 10 mM Ca^{2+} which indicated that Ca^{2+} inhibited the biofilm development and V329 biofilm become

uneven and flat. Further increase in Ca^{2+} concentration resulted in decrease of biomass, roughness coefficient and surface to biovolume ratio which indicates the formation of voids and mosaic structure. Reduction of pillar size, flattening of biofilm, and reduction in the biomass and ultimately the formation of voids with increase in Ca^{2+} concentration suggests that the possible mechanism of biofilm inhibition by Ca^{2+} is by reducing the cell to cell interaction. The role of Ca^{2+} in biofilm inhibition by V329 was further investigated by adding EDTA to the medium. Addition of divalent chelator EDTA confirmed that observed architectural change was due to the presence of Ca^{2+}only.

The present study supports one of the earlier proposed mechanisms by Thomas et al. (1993) which were postulated for the adherence of *S. aureus*, one involving surface proteins that bind Ca^{2+} and the other involving surface proteins that interact with mucin independent of Ca^{2+}. This study highlights Bap as one of the possible surface proteins that binds to Ca^{2+} and affect the adhesion. This study provides possible explanations towards answering the unusual adhesion behavior observed by van Hoogmoed et al. (1997) in *S. thermophillus* where maximum adhesion rate was found at 1 mM Ca^{2+}. Since, Bap and Ca^{2+} were found to specific, it could be hypothesized that homologous surface protein/s might be involved in regulation of Ca^{2+} specific adhesion in *S. thermophillus*. Furthermore this study strengthens the concept of non-involvement of exo-polysaccharides in adhesion to hydrophobic surfaces in *S. epidermidis* as was proposed by Dunne and Burd (1992).

Till date all *bap*-positive *S. aureus* have been isolated from mastitis infection where Ca^{2+} is profoundly present. The presence of EF hand motifs in Bap protein seems to be a contradiction to the fact that Bap helps in the protein mediated

biofilm establishment. However, other data indicate that the role of Bap may not be that straightforward as expected. It may have other regulatory role than in initial adherence. In a distinct and interesting study by Patrauchan et al. (2005), it was observed that extracellular Ca^{2+} can alter the proteomics of the *Pseudoalteromonas sp*. which is dose dependent. Therefore, the possibility of altered proteome of V329 in response to Ca^{2+} might be responsible for the altered topology of the biofilm. The principal finding of this study is that, Ca^{2+} can modulate the biofilm development in *bap*-positive *S. aureus* V329. Based on the present findings and previous reports (Dunne and Burd 1992; Arrizubieta et al. 2004; Patrauchan et al. 2005) it can be inferred that Bap confers *S. aureus* V329 the ability to adapt in different environments, with varying Ca^{2+} concentrations. However, at this stage with limited knowledge on adherence mechanism of bacteria, it cannot be presumed that other *bap*-negative staphylococcal biofilms might also be modulated by Ca^{2+}. According to Davey and O'Toole (2000) and O'Toole et al. (2000) multiple mechanisms are adopted by different staphylococci for biofilm formation.

3.5 Conclusion

This study establishes the following salient points; (1) PCR mediated approach followed by dot blotting identifies SA7, SA10 and SA33 as *bap* negative *S. aureus* strains. (2) Bap-mediated biofilm development in *S. aureus* V329 is inhibited at $\geq$ 5 mM Ca^{2+} (3) *bap*-isogenic mutant M556 produced a very weak biofilm after 16 hrs which is not influenced by Ca^{2+} (4) among other *bap*-negative strains of *S. aureus*, SA7 and SA33 did not show any inhibition in presence of Ca^{2+} even up to 50 mM (5) Bap dependent biofilm inhibition in V329 was found to be Ca^{2+} specific since addition of Mg^{2+} did not inhibit biofilm growth. (6) Interaction

of Bap and Ca^{2+} is not mere electrostatic, since increasing salt concentration did not revert the Ca^{2+} inhibition. (7) CSLM study showed that varying Ca^{2+} concentrations significantly influenced the architecture and topography of the *S. aureus* biofilm**.** It can be inferred that auto-aggregation could also be due to nonspecific bridging of acidic functional groups of cell surface polymers with Ca^{2+}. This observation was found to be contradictory to the previous findings where Bap was claimed to be an essential factor for aggregative behaviour of *S. aureus* V329 strain (Arrizubieta et al. 2004). Biofilm development is widely believed to be initiated by bacteria sensing the surface that trigger the transition from a planktonic state to a biofilm mode of growth (Stoodley et al. 2002). Adhesion is a threshold event in formation of biofilms, studies on molecular and biochemical aspects have highlighted that the protein matrix of the biofilm structure is of interest in developing strategies to combat microbial fouling/chronic biofilm infections (Cucurella et al. 2001). Microbiota acquires diverse mechanisms to adhere and initiate the biofilm development in various environments. Although, the present study was carried out using *S. aureus* as model organism, it establishes the fact that cell surface proteins play important role in biofilm development and the presence of Ca^{2+} might affect its growth. A better understanding of molecular aspects of adhesion and biofilm phenomenon will certainly help in developing control strategies for clinical manifestations as well as aid in designing better industrial cooling water treatment programs for prevention of biofouling.

References

Arrizubieta MJ, Toledo-Arana A, Amorena B, Penades JR, Lasa I. 2004. Calcium inhibits *bap*-dependent multicellular behaviour in *Staphylococcus aureus*. J Bacteriol. 186: 7490–7498.

Beloin C, Michaelis K, Lindner K, Landini P, Hacker J, Ghigo JM, Dobrindt U. 2006. The transcriptional antiterminator RfaH represses biofilm formation in *Escherichia coli*. J Bacteriol. 188:1316-1331

Bramley AJ, Patel AH, O'Reilly M, Foster R, Foster TJ. 1989. Roles of alpha-toxin and beta-toxin in virulence of *Staphylococcus aureus* for the mouse mammary gland. Infect Immun. 57: 2489-2494.

Brown EM, Vassilev PM, Hebert SC. 1995. Calcium ions as extracellular messengers. Cell 83:679-682.

Christensen GD, Simpson W, Younger J, Baddour L, Barrett F, Melton D, 1985. Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. J Clin Microbiol. 22: 996-1006.

Combet C, Blanchet C, Geourjon C, Deleage G. 2000. NPS@: network protein sequence analysis. Trends Biochem Sci. 25**:**147–150.

Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM. 1995. Microbial biofilms. Annu Rev Microbiol 49:711-45.

Cucarella C, Solano C, Valle J, Amorena B, Lasa I, Penades JR. 2001. *Bap*, a *Staphylococcus aureus* surface protein involved in biofilm formation. J Bacteriol. 183:2888–2896.

Davey ME, O'Toole GA. 2000. Microbial biofilms: from ecology to molecular genetics. Microbiol Mol Biol Rev. 64:847-867.

Donlan RM. 2001. Biofilms and device-associated infections. Emerg Infect Dis 7:277–281.

Dunne Jr WM, Burd EM. 1992. The effects of magnesium, calcium, EDTA, and pH on the in vitro adhesion of *Staphylococcus epidermidis* to plastic. Microbiol Immunol. 36:1019-1027.

Eaton TJ, Gasson MJ. 2002. A variant enterococcal surface protein Esp (fm) in *Enterococcus faecium*; distribution among food, commensal, medical, and environmental isolates. FEMS Microbiol Lett. 216:269–275.

Fletcher M. 1988. Attachment of *Pseudomonas fluorescens* to glass and influence of electrolyte on bacterial-substratum separation distance. J Bacteriol. 170:2027-2030.

Heydorn A, Nielsen AT, Hentzer M, Sternberg C, Givskov M, Ersboll BK and Molin S. 2000. Quantification of biofilm structures by the novel computer program COMSTAT. Microbiology146: 2395-2407.

Holland IB, Jones HE, Campbell AK, Jacq A. 1999. An assessment of the role of intracellular free Ca^{2+} in *E. coli*. Biochimie 81:901–907.

Johnson M, Cockayne A, Williams PH, Morrissey JA. 2005. Iron-responsive regulation of biofilm formation in *Staphylococcus aureus* involves fur-dependent and fur-independent mechanisms. J Bacteriol. 187:8211-8155.

Kretsinger RH, Nockolds CE. 1973. Carp muscle calcium-binding protein. II. Structure determination and general description. J Biol Chem. 248:3313–3326.

Lewit-Bentley A, Réty S. 2000. EF-hand calcium-binding proteins. Curr Opinion Structural Biol. 10: 637-643.

Lowy FD. 1998. *Staphylococcus aureus* infections. N Engl J Med. 339:520-532

Marshall KC, Stout R, Mitchell R. 1971. Mechanisms of the initial events in the sorption of marine bacteria to surfaces. J Gen Microbiol. 68:337-348.

Maurer P, Hohenester E, Engel J. 1996. Extracellular calcium-binding proteins. Curr Opin Cell Biol 8:609–617.

McNab R, Holmes AR, Clarke JM, Tannock GW, Jenkinson HF. 1996. Cell surface CshA mediates binding of *Streptococcus gordonii* to other oral bacteria and to immobilized fibronectin. Infect Immun. 64:4204-4210.

McNab R, Jenkinson HF. 1998. Altered adherence properties of a *Streptococcus gordonii* hppA (oligopeptide pennease) mutant result from transcriptional effects on cshA adhesion gene expression. Microbiology 144:127-136.

Michiels J, Xi C, Verhaert J, Vanderleyden J. 2002. The functions of Ca^{2+} in bacteria: a role for EF-hand proteins? Trends Microbiol. 10:87–93.

Norris V, Chen M, Goldberg M, Voskuil J, McGurk G, Holland IB. 1991. Calcium in bacteria: a solution to which problem? Mol Microbiol. 5:775–778.

Norris V, Grant S, Freestone P, Canvin J, Sheikh FN, Toth I, Trinei M, Modha K, Norman RI. 1996. Calcium signalling in bacteria. J Bacteriol. 178:3677–3682.

O'Toole G, Kaplan HB, Kolter R. 2000. Biofilm formation as microbial development. Annu Rev Microbiol. 54:49-79.

O'Toole GA, Kolter R. 1998. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. Mol Microbiol. 28:449–461.

Patrauchan MA, Sarkisova S, Sauer K, Franklin MJ. 2005. Calcium influences cellular and extracellular product formation during biofilm-associated growth of a marine *Pseudoalteromonas sp.* Microbiology 151:2885–2897.

Potter A, Ceotto H, Giambiagi-Demarval M, dos Santos KR, Nes IF, Bastos Mdo C. 2009. The gene *bap*, involved in biofilm production, is present in *Staphylococcus* spp. strains from nosocomial infections. J Microbiol. 47: 319-326.

Saravanan P, Nancharaiah YV, Venugopalan VP, Rao TS, Jayachandran S. 2006. Biofilm formation by *Pseudoalteromonas ruthenica* and its removal by chlorine. Biofouling 22: 371-381.

Shankar N, Baghdayan AS, Gilmore MS. 2002. Modulation of virulence within a pathogenicity island in vancomycin-resistant *Enterococcus faecalis*. Nature 417: 746–750.

Smith RJ. 1995. Calcium and bacteria. Adv Microb Physiol. 37:83–133.

Stanley PM. 1983. Factors affecting the irreversible attachment of *Pseudomonas aeruginosa* to stainless steel. Can J Microbiol. 29:1493-1499

Stepanovic S, Vukovic D, Jezek P, Pavlovic M, Svabic-Vlahovic M. 2001. Influence of dynamic condition on biofilm formation by *Staphylococci.* Eur J Clin Microbiol Infect Dis. 20:502-504.

Stoodley P, Sauer K, Davies DG, Costerton JW. 2002. Biofilms as complex differentiated communities. Annu Rev Microbiol. 56:187-209.

Sutra L, Poutrel B. 1990. Detection of capsular polysaccharide in milk of cows with natural intramammary infection caused by *Staphylococcus aureus.* Am J Vet Res. 51:1857-1859.

Tendolkar PM, Baghdayan AS, Gilmore MS, Shankar N. 2004. Enterococcal surface protein, Esp, enhances biofilm formation by *Enterococcus faecalis*. Infect Immun. 72: 6032–6039.

Thomas VL, Sanford BA, Ramsay MA. 1993. Calcium- and mucin-binding proteins of S*taphylococci.* J Gen Microbiol. 139:623-629.

van Hoogmoed CG, van der Mei HC, Busscher HJ. 1997. The influence of calcium on the initial adhesion of *S. thermophilus* to stainless steel under flow studied by metallurgical microscopy. Biofouling 11:167-176.

Vautor E, Abadie G, Pont A, Thiery R. 2008. Evaluation of the presence of the *bap* gene in *Staphylococcus aureus* isolates recovered from human and animals species. Vet Microbiol. 127: 407-411.

Virji M, Saunders JR, Sims G, Makepeace K, Maskell D, Ferguson DJ. 1993. Pilus-facilitated adherence of *Neisseria meningitidis* to human epithelial and endothelial cells: modulation of adherence phenotype occurs concurrently with changes in primary amino acid sequence and the glycosylation status of pilin. Mol Microbiol. 10:1013-28.

Whittaker CJ, Klier CM, Kolenbrander PE. 1996. Mechanisms of adhesion by oral bacteria. Ann Rev Microbiol. 50: 513-552.

CHAPTER 4 : Dispersal of Bap-like surface protein mediated *S. aureus* biofilm by Proteinase K

4.1 Introduction

Biofilms are complex transient microbial communities attached to surfaces and embedded in an extracellular polymeric matrix (Flemming and Wingender 2010). A mature biofilm is generally composed of polysaccharides, proteins, and extracellular DNA. Generally, the process of biofilm development proceeds through a set of discreet stages; a) adherence of cells to a substratum, b) development of micro-colonies, c) maturation of micro-colonies into biofilms and d) detachment of bacteria and dispersal of motile cells (O'Toole et al. 2000). Among the variety of bacterial species, *Staphylococcus aureus* and *Staphylococcus epidermidis* are the predominant microorganisms causing nosocomial infections, mainly associated with implanted medical devices such as catheters, cardiac valves and orthopaedic prostheses (Gotz 2002). The biofilm mode of *S. aureus* propagation enhances its recalcitrance and enables the bacterium to survive antibiotic action (Parsek and Singh 2003). This also imposes a challenge to host defence mechanisms and antimicrobial therapy (Parsek and Singh 2003; del Pozo and Patel, 2007). Biofilm dispersal is a natural process, which allows bacterial cells to leave biofilm and migrate to a more favourable environment for resettlement. However, during this transition phase, bacteria lose the advantages of the biofilm mode of life and become relatively more susceptible to antimicrobial agents.

A recent study reported that biofilm disassembly/dispersion by signalling pathway plays a very important role in pathogenicity and environmental dispersion of bacteria (Boles and Horswill 2008). In order to promote dispersion, bacterial cells synthesize various EPS degrading enzymes (Boyd and Chakrabarty, 1994; Kaplan et al. 2003). Dispersal mechanisms vary in different bacteria and this event

is considered as a novel approach to treat drug resistant *S. aureus* infections in body implants and catheters (Kiedrowski and Horswill 2011). In spite of its high potential, there is not much information on biofilm dispersion products because of the ratio of higher investment cost to commercial outcome (Romero and Kolter, 2011).

Quorum sensing, among other things, is involved in biofilm dispersion. In the context of *S. aureus*, it was reported that biofilm dispersal is controlled by *Agr* - quorum-sensing system (Boles and Horswill 2008). There are several reports on surface protein dependent biofilm formation by *S. aureus,* among which protein A and biofilm associated protein (Bap) were studied in detail (Cucarella et al. 2001; Merino et al. 2011). Bap is a large, multi-domain, cell surface anchored protein, which has been reported to play crucial role in the early stages of *S. aureus* biofilm development (Cucarella et al. 2001). Importance of the Bap in *S. aureus* biofilm was so significant that *bap*- positive *S. aureus* can form biofilm even though, its *ica*- operon was disrupted (Cucarella et al. 2001; Tormo et al. 2005). The *ica*-operon synthesizes polymeric intercellular adhesin (PIA), a major constituent in *S. aureus* biofilm. The role of PIA component in the *S. aureus* biofilm formation was studied in detail (O'Gara, 2007). Earlier studies showed that the presence of *bap* gene and its homologues was restricted to bovine mastitis (Cucarella et al. 2001; Tormo et al. 2005). Of late, the presence of *bap* gene was also reported in human nosocomial infections (Potter et al. 2009). Furthermore the increase in number of occurrences of *bap* also indicates the presence of alternate PIA-independent mechanism of biofilm formation (Tormo et al. 2005; Hennig et al., 2007).

Biofilm disassembly/dispersion is believed to play very important role in pathogenicity, environmental distribution and also in phase transition (Flemming and Wingender 2010, Mangwani et al. 2014). The dispersal phenomenon can also be triggered by several environmental signals or due to unfavourable condition (Karatan and Watnick 2009). *S. aureus* secretes four major extracellular proteases such as metallo-protease aureolysin (*aur*), two cysteine proteases (*scpA* and *sspB*) and seven serine proteases (Dubin, 2002). Theoretically, these extracellular proteases contribute to biofilm detachment; however, their role in staphylococci is still unknown (Dubin, 2002, Boles and Horswill, 2004; Shaw, 2004). An earlier study showed that among the above four classes of extracellular proteases, serine proteases are dominant and believed to play major role in biofilm detachment (Boles, and Horswill, 2004). Proteinase K is a highly reactive serine protease, stable in a broad range of conditions: pH, buffer salts, detergents (SDS), and temperature (Kristjansson et al., 1999). This makes Proteinase K an ideal choice for disassembly among the various proteases. According to a previous report, biofilms of *S. epidermidis, S. lugdunensis* and *S. aureus* showed susceptibility to enzymatic treatments, which was dependent on chemical composition of their biofilm matrices (Chaignon et al., 2007). Thus, mimicking the extracellular degrading enzymes is a possible approach that could be used to disintegrate the biofilms. Concurrent use of antibiotics can kill the detached cells and help in eliminating the infection. It was reported that Proteinase K was more effective in dispersing *S. aureus* biofilm when PIA content was very less and biofilm was probably dominated by the presence of proteins (Chaignon et al., 2007). In the above study, the authors used Proteinase K at a high concentration (1 mg/ml), which may increase the cost of the treatment. Thus, there is a need for

generating data on the use of lesser concentrations of Proteinase K for biofilm dispersal. It is already discussed that in Bap-expressing *S. aureus* strains, the protein plays a major role in establishing cell to cell interaction. Thus, targeting Bap with a protease may be a very useful approach to control staphylococci infections.

Antibiotics are used to eradicate biofilm infections and excessive use of antibiotics led to selection pressure and resulted in development of resistant strains among many pathogenic bacteria (Alanis, 2005; Soderblom, et al. 2010). This has motivated researchers to look for alternative therapeutic approaches, which reduce the use of antibiotics or completely eliminate the need of antibiotics for biofilm removal (Soderblom, et al. 2010; Hryniewicz, 2011). In this study, the possibility of imitating naturally produced proteases with Proteinase K was investigated and whether it can be used to enhance the biofilm dispersal through cleavage of surface proteins i.e. Bap- dependent *S. aureus* biofilm establishment. In this study, it was investigated whether this approach would be useful in general and has wider applicability by using six *S. aureus mastitis* isolates. Apart from the above study, the effect of calcium binding to Bap on its stability against proteolytic activity of Proteinase K was also investigated. To address the problem, five *S. aureus* bovine mastitis isolates were included in the study along with *bap*–positive *S. aureus* V329 and a *bap*-isogenic mutant M556. It was also tested whether Proteinase K could be used in combination with antibiotics to enhance the dispersal/control of *S. aureus* biofilms.

4.2 Materials and Methods

4.2.1 Microorganisms and culture conditions

A *bap*–positive *S. aureus* V329 and its isogenic mutant *S. aureus* M556 were used in this study along with five other clinical isolates of *S. aureus* viz., SA7, SA10 SA33, SA252 and SA392. M556 was generated by transposon insertion in the downstream part of bap gene of *S. aureus V329* in such a way that Bap protein is synthesized but remains non-functional as cell wall anchoring region is truncated (Curcarella et al. 2001). For each experiment, single colonies were picked from Tryptic Soy Agar (TSA) culture plates and inoculated in Tryptic Soy Broth (TSB) medium supplemented with 0.25% glucose and incubated at 37^{o}C at 150 rpm. Overnight grown cultures were used for all experiments.

Table 4.1: Details of *S. aureus* strains used in this study

Strain	Details	Source
S. aureus V329	V329, *bap*-positive mastitis isolates	Prof. I. Lasa, Spain
***S. aureus*M556**	M556, *bap*-isogenic mutant of V329	Prof. J.R. Penades, Spain
***S. aureus*7**	SA7, mastitis isolate	Department of Microbiology, Karnataka veterinary college, Bengaluru, India
***S. aureus*10**	SA10, mastitis isolate	-Do-
***S. aureus*33**	SA33, mastitis isolate	-Do-
***S. aureus*352**	SA352, mastitis isolate	Madras Veterinary college, Chennai, India
***S. aureus*392**	SA392, mastitis isolate	-Do-

4.2.2 Proteinase K preparation

The enzyme Proteinase K was purchased from Sigma-Aldrich, USA, which had specific activity of 30 units/mg, where one unit is defined as the amount of

enzyme needed to hydrolyse urea-denatured haemoglobin to produce colour equivalent to 1.0 µmole of tyrosine per min at pH 7.5 at 37°C (colour reaction by Folin-Ciocalteu reagent). The working concentration of Proteinase K was chosen as 2 µg/ml in most of the experiments. To inactivate the Proteinase K after the prescribed experimental time, 2 mM of freshly prepared phenyl methyl sulphonyl fluoride (PMSF) was added.

4.2.3 Quantitative biofilm assay

Biofilm assay was performed in micro-titre plates to estimate the inhibitory/dispersion action of Proteinase K. The working concentration of Proteinase K was chosen as 2 µg/ml for all the experiments. The overnight grown cultures of the *S. aureus* cells in TSB-G were diluted 1:40 in sterile TSB-G medium and added to the pre-sterilized 96 well flat bottom polystyrene micro-titre plates. To estimate the inhibitory action *S. aureus* biofilms were grown in the presence of 2 µg/ml of Proteinase K. To study dispersion, biofilms were grown on micro-titre plates, washed after prescribed time and 200 µl of fresh TSB-G amended with 2 µg/ml of Proteinase K was added to the wells and the plates were incubated at 37°C for 24h. To study the effect of Ca^{2+} on proteolytic cleavage of Bap in terms of biofilm formation, V329 biofilms were grown at 37°C for 24h in the presence of 2 µg/ml of Proteinase K along with increasing concentration of Ca^{2+} in the range of 1.56 mM to 50 mM. V329 biofilm grown in the presence of only Ca^{2+} in the similar concentration range acted as control for Ca^{2+}. After 24 h of incubation, biofilm growth was quantified. Biofilm quantification was done by classical crystal violet assay as described previously (Mangwani et al. 2014). For dissolution of bound crystal violet, 33% acetic acid was used

(Stepanovic et al. 2001). Biofilm growth was monitored in terms of absorbance at 570 nm using a multimode micro-plate reader (BioTek, USA).

4.2.4 Early adhesion assay

Overnight grown cultures in TSB supplemented with 0.25% glucose were diluted 1:40 in sterile TSB medium and Proteinase K treatment (2 µg/ml) was given to the cells in order to cleave and remove all surface proteins. Thereafter the Proteinase K treated cells were added to 96 well micro-titre plates. Untreated *S. aureus* cells were used as control. In order to evaluate the role of surface protein in initial adhesion, post Proteinase K treated cells (Proteinase K was inactivated by addition of PMSF), were allowed to adhere for different time intervals, i.e. 0 h, 1 h, 2 h and 3 h at 37^{o}C to aid in surface protein-mediated adherence. Adherence of cells was measured by crystal violet staining assay as described earlier (see sub topic 3.2.7)

4.2.5 Planktonic growth studies

Cell from overnight grown bacterial cultures were harvested and washed twice with phosphate buffered saline (PBS) and the OD_{600} of each culture was set to 0.1. absorbance, 100 µl volume of each resuspended culture was inoculated in 1900 µl of TSB-G having different concentrations of Proteinase K. Cultures were incubated at 37^{o}C and 150 rpm. Absorbance of each culture was recorded at different time intervals after vortexing for 5 sec, to re-suspend the settled cells.

4.2.6 EPS extraction and quantification of biofilm matrix components

Staphylococcus aureus biofilm was grown for 48 h on glass slides immersed in 20 ml of TSB supplemented with 0.25% glucose (TSB-G). After 48 h, planktonic cells were aspirated and biofilm was gently washed twice with PBS. *S. aureus* biofilms were treated with 2 µg/ml Proteinase K in TBS-G for 4 h at 37 ^{o}C. After 4

h biofilm was gently washed with PBS and then remaining biofilm was scrapped and collected in 5 ml of PBS. Biofilm was disintegrated by gentle vortexing using glass beads. A 5 ml of the biofilm sample was centrifuged at 8000 rpm and 4^{o}C for 30 min. Supernatant was collected and mixed with double volume of 90% chilled ethanol and kept at 4^{o}C overnight. EPS was collected by centrifugation at 10000 rpm and 4^{o}C for 10 min. The supernatant was discarded and pellet was collected and dried at 60^{o}C to remove ethanol. Pellet was resuspended in 100 µl PBS buffer. The protein and eDNA content in the resuspension was quantified using Qubit Fluorometer (Invitrogen, Carlsbad, CA, USA), the quantification protocol was followed as described by the vendor (Beaudet et al. 2007; Bajrami et al. 2009). Glucose concentration as a measure of polysaccharide content was quantified by the Dubois method as described elsewhere (Paraneeiswaran et al. 2015).

4.2.7 Viable cell counts in biofilm after antibiotic-Proteinase K treatment

To investigate the anti-biofilm activity of Proteinase K in combination with antibiotics, streptomycin (STR) gentamycin (GEN) and ampicillin (AMP) were used *against S. aureus* V329 biofilms. The antibiotic concentrations were chosen as 10 and 50 times of the MIC (indicated as X and 5X respectively) against *S. aureus* planktonic cells. Proteinase K treatment was given in combination with X concentration of antibiotics. Against other *S. aureus* biofilm forming strains *viz;* SA7, SA10, SA33 and SA352 Proteinase K treatment was given with combination of gentamycin*. S. aureus* biofilms were grown in 96 well micro-titre plates at 37ºC and 150 rpm. After 24 h, planktonic cells were aspirated by pipette and the biofilms were gently rinsed twice with sterile phosphate buffered saline (PBS). After

rinsing, the biofilms were treated with antibiotics alone (GEN 150 µg/ml and 750 µg/ml; STR 100 µg/ml and 500 µg/ml; AMP 100 µg/ml and 500 µg/ml) and antibiotics-Proteinase K combinations, as per the experimental plan. After 24h of treatment planktonic cells were aspirated using a pipette and biofilms were gently rinsed twice with PBS. Then 200 µl of PBS was added to each well and the biofilm cells were dislodged by ultra-sonication for 5 min. Cells released from the biofilms were harvested and the viable cell count was obtained by plating on TSA media and incubated at 37^{o}C overnight.

4.2.8 Fluorescein di-Acetate (FDA) assay

Post-treatment to antibiotics and Proteinase K the dispersion of left over biofilms was done by sonication. Micro-titre plates were subjected to Fluorescein di-acetate (FDA) assay to check the efficiency of sonication. FDA stock solution (5 mg/ml in acetone) was diluted 1:50 times in PBS to get the working solution. A 200 µl of working solution of FDA was added to micro-titre wells. Plate was incubated in dark condition at 37^{o}C for 4h. After 4h fluorescence was measured at 490 nm using multimode plate reader (BioTek, USA).

4.2.9 Confocal Laser Scanning Microscopy (CSLM) study

Confocal laser scanning microscopy analysis was done with a Leica TCS-SP2 AOBS microscope. V329 biofilms grown on glass cover slips in six-well cell culture plates. Biofilms (24 h old) were treated with Proteinase K for a given time period, gently rinsed with PBS and stained with 0.2 % acridine orange for 5 min. A thin cover slip was placed over the biofilms and the slide was mounted upside down over the objective lens of CSLM, equipped with DM IRE 2-inverted microscope (Leica Microsystems). Image stacks were collected from 20 different random points of the biofilm. In order to get statistically significant data, each

experiment was repeated 3 times. Image stacks were analyzed by COMSTAT (Heydorn et al. 2000) and Imaris software (version 6.2).

4.2.10 Statistical Analysis

A Two-tailed Student's t test was used to determine the differences in biofilm formation between the groups. Differences were considered statistically significant when P value was < 0.005.

4.3 Results

4.3.1 Proteinase K interferes with early adhesion of *S. aureus* cells and inhibits biofilm development

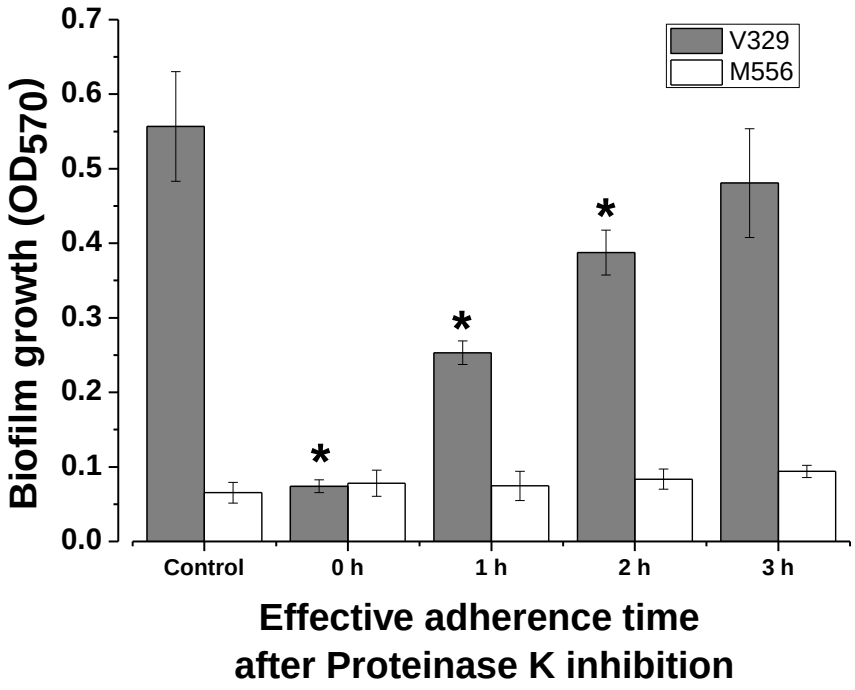


Figure 4.1: Effect of Proteinase K on Bap- mediated early adhesion. Results are average of 5 replicates ±1 S.D. and are representative of three independent experiments. Asterisk, P value < 0.0001 as compared with *S. aureus* cells exposed to no Proteinase K (control). Zero hour point shows that Proteinase K was not inhibited.

Figure 4.1 illustrates the results of early adhesion assay which shows that proteinase K treatment hampered the primary attachment of V329 cells to 96 well microtitre plate. The adherence property of the V329 cells was restored when proteinase K was inactivated by the addition of PMSF. Figure 4.1 also shows the number of adhered cells increased with time. On the contrary, strain M556 did not show any change in adhesion or biofilm formation in the presence of proteinase K. This observation signifies the Bap surface protein is important in early phase of biofilm formation by V329.

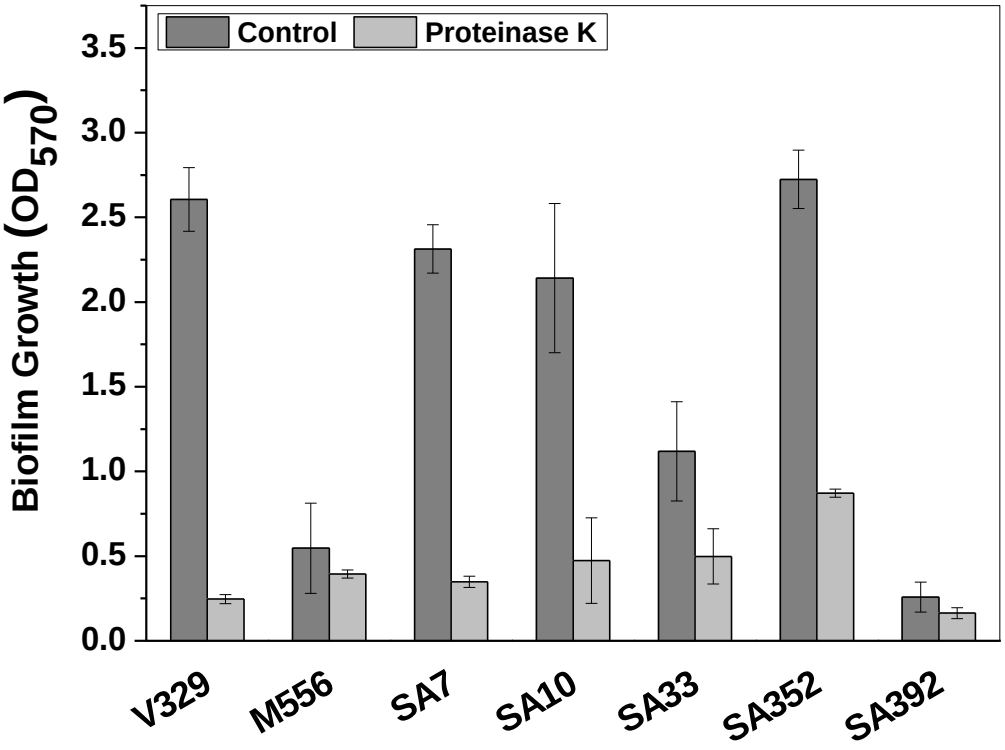


Figure 4.2: Effect of Proteinase K on the growth of *S. aureus* biofilms.

Figure 4.2 illustrates the results of biofilm inhibition assay, which shows that Proteinase K treatment hampered the biofilm development of most *S. aureus* strains viz. SA7, SA10, SA33, SA352and *bap*- positive V329. All *S. aureus* strains except SA392 (weak biofilm producing strain) showed significant inhibition in biofilm growth when treated with 2 µg/ml.

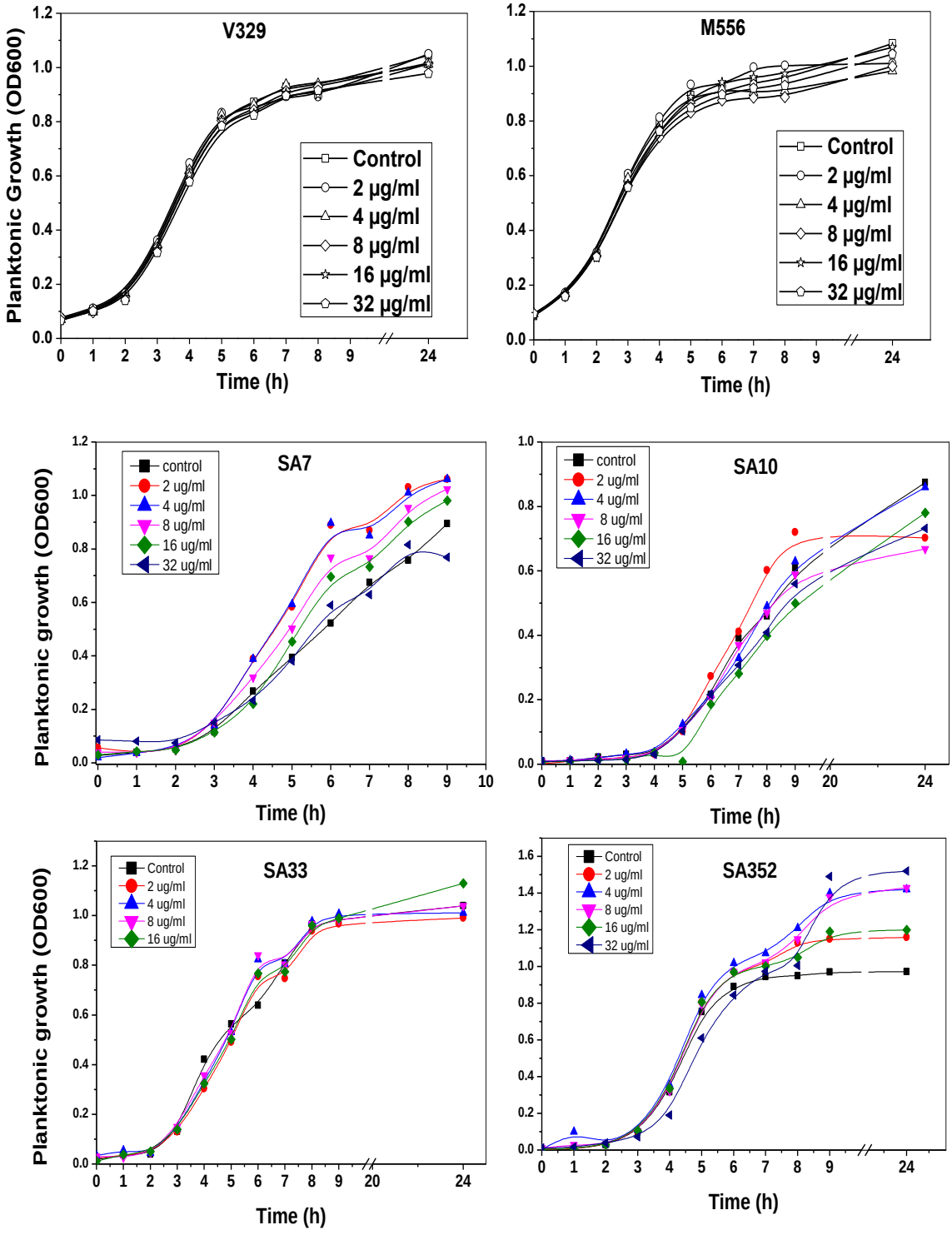


Figure 4.3: Effect of different concentrations of Proteinase K on planktonic growth of *S. aureus* V329 and M556 and other *S. aureus* strains SA7, SA10, SA33 and SA352. Results are shown as mean ±1 S.D.

SA7, SA10, SA33, SA352 biofilms showed 84, 71, 83 and 68% reduction in biofilm growth in the presence of 2 µg/ml Proteinase K. On the contrary, strains M556 and SA392 were found to be weak biofilm producers and there was no significant inhibition of biofilm formation in the presence of Proteinase K. Planktonic growth studies of *bap*- positive V329 and *bap*-mutant M556 and other *S. aureus* strains was carried out in the presence of different concentrations of proteinase K. Figure 4.3 shows that there was no effect of Proteinase K on the planktonic growth of either *S. aureus* strains when tested up to 32 µg/ml.

4.3.2 Effect of different concentrations of Ca^{2+} on proteolytic degradation of Bap and biofilm development of V329

Biofilm assay in the presence of Proteinase K with increasing concentrations of Ca^{2+} was carried out. Since Bap contains Ca^{2+} binding EF hand motif, it was investigated whether binding of Ca^{2+} to Bap would confer resistance against Proteinase K mediated degradation. Biofilm assay showed that in the presence of Proteinase K, biofilm formation of V329 was inhibited. Addition of Ca^{2+} (3 mM) did not influence Proteinase K mediated inhibition of early adherence of cells and biofilm development (Figure 4.4). Addition of 3 mM Ca^{2+} had no significant effect on V329 biofilm formation. This observation demonstrates that binding of Ca^{2+} to Bap does not confer any immunity against proteolytic degradation. Figure 4.4 shows that increasing concentrations of Ca^{2+} had no effect on Proteinase K mediated inhibition of biofilm development. In other words, Ca^{2+} did not affect the proteolytic degradation of surface protein Bap by Proteinase K. On the other hand, lower concentration of Ca^{2+} (up to 6.25 mM) had no significant effect on V329 biofilm formation but higher concentrations showed an inhibitory effect.

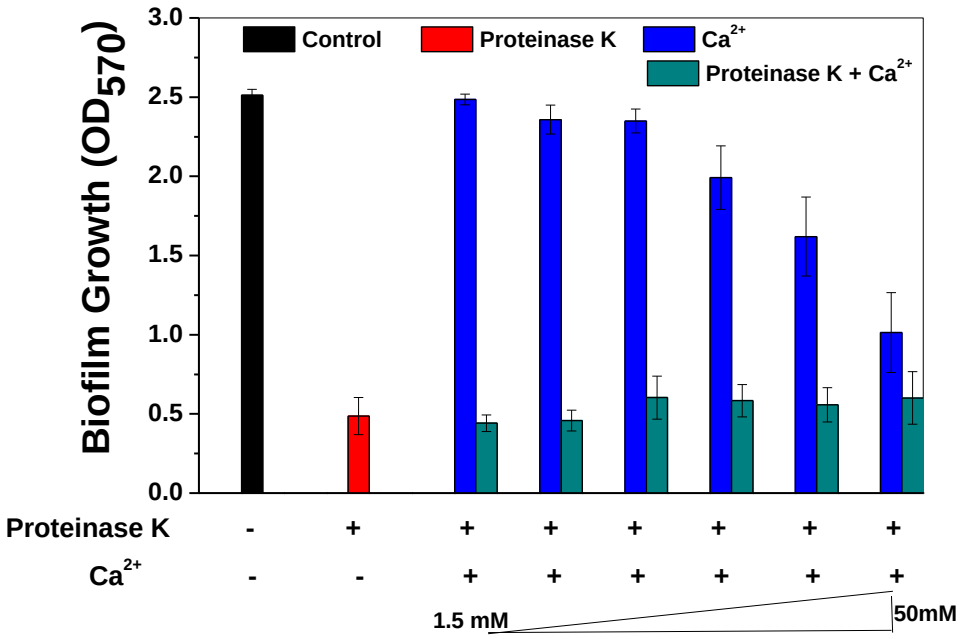


Figure 4.4: Effect of Ca^{2+} on Proteinase K mediated biofilm inhibition in bap-positive *S. aureus* biofilm. Results are average of 5 replicates ±1 S.D.

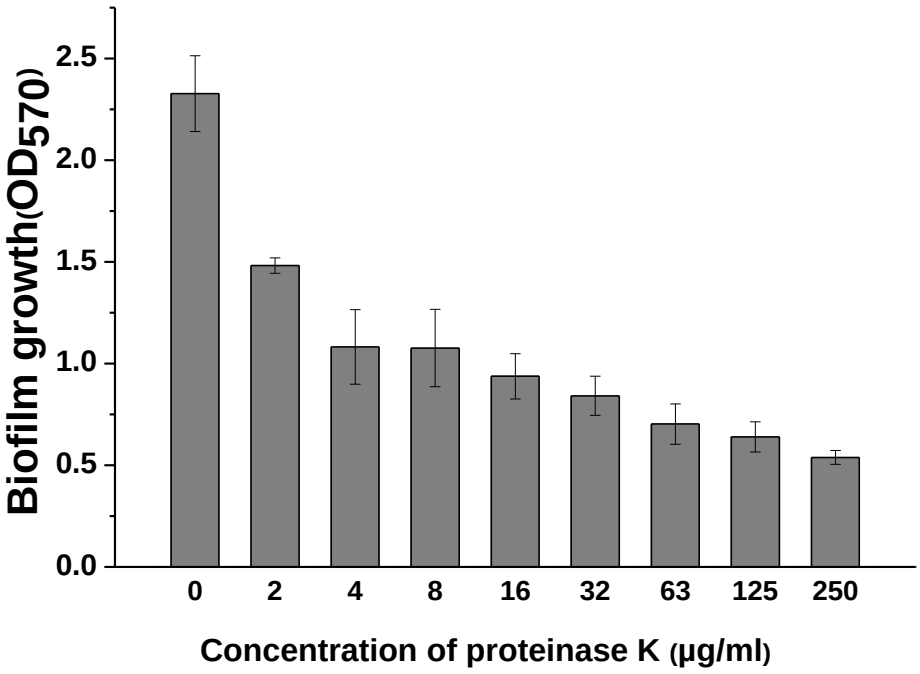


Figure 4.5: Effect of different concentration of Proteinase K (in TSB) on 24 h old *bap*- positive *S. aureus* V329 biofilm. Results are average of 5 replicates ±1 S.D.

4.3.3 Proteinase K enhances dispersal in *bap*-positive *S. aureus*

In order to investigate the biofilm dispersal activity, a range of Proteinase K concentrations were tested against mature *bap*-positive *S. aureus* V329 biofilms. Figure 4.5 shows dose dependent dispersal of V329 biofilm. Roughly, 36% biofilm dispersal was observed with 2 µg/ml Proteinase K treatment, whereas the highest dose of 250 µg/ml showed ~76% biofilm dispersal. It is clearly evident from this observation that increasing concentration of Proteinase K did not result in enhanced biofilm dispersal hence 100% biofilm dispersal could not be achieved. In order to keep this approach economical, 2 µg/ml of Proteinase K was chosen for further experiments.

Figure 4.6 illustrates that Proteinase K treatment of 24 and 48 h old V329 and M556 biofilms. Proteinase K treatment enhanced the dispersion of *bap*-positive V329 biofilm, while the *bap*-isogenic mutant M556 biofilm was not at all dispersed. Figure 4.6 also shows that M556 forms a weak biofilm as compared to V329 at 24 h hence estimating M556 biofilm dispersal was difficult and inappropriate. However, after 48h it also produced significant biofilm and it was clearly evident that Proteinase K treatment did not disperse M556 biofilm. Based on the crystal violet assay data, percentage removal of V329 biofilm by Proteinase K was estimated to be 15%, 24% and 30% after 2, 4 and 6 h of contact time, respectively.

In order to investigate the biofilm dispersal activity of Proteinase K against other *S. aureus* biofilms, Proteinase K treatment was given to 24 h old *S. aureus* biofilms. Figure 4.7 show that Proteinase K treatment of *S. aureus* biofilms caused a significant disruption of all *S. aureus* biofilms except M556 and SA392.

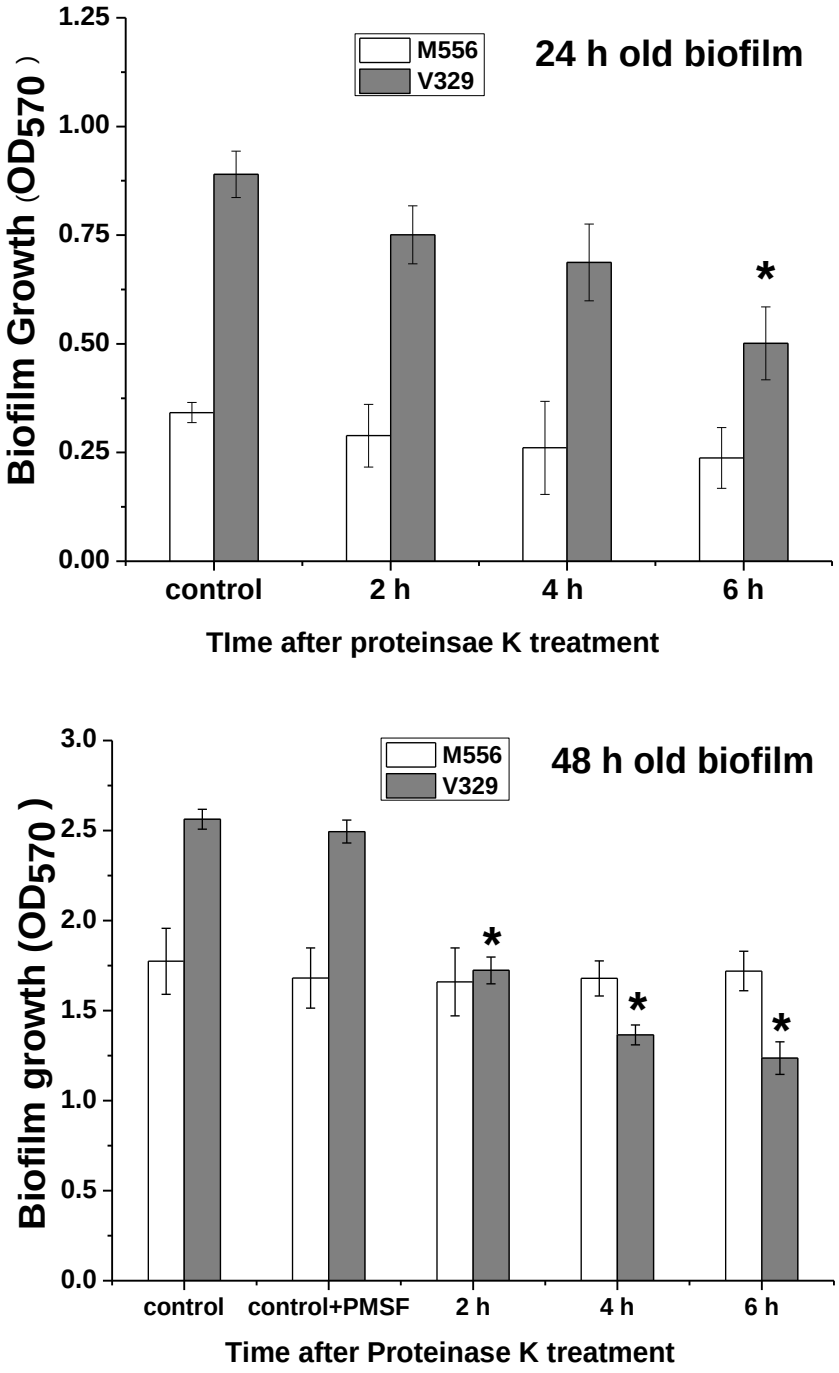


Figure 4.6: Effect of Proteinase K on 24 h and 48 h old *S. aureus V329* and M556 biofilm. Results are average of 5 replicates ±1 S.D. and are representative of three independent experiments. Asterisk, $P < 0.005$ as compared with control *S. aureus* biofilm, which was not exposed to Proteinase K.

Upon 2 µg/ml Proteinase K treatment for 24 h, approximately 92.5 %, 23.4%, 90.1%, 95.8%, 81.9% and 60% biofilm dispersal was observed in V329, M556, SA7, SA10, SA33 and SA352 respectively. Though, Proteinase K enhanced the biofilm dispersal, 100% biofilm removal could not be achieved in any case. Moreover, SA392 formed a weak biofilm as compared to V329 after 24 h, hence estimating SA392 biofilm dispersal was difficult and inappropriate.

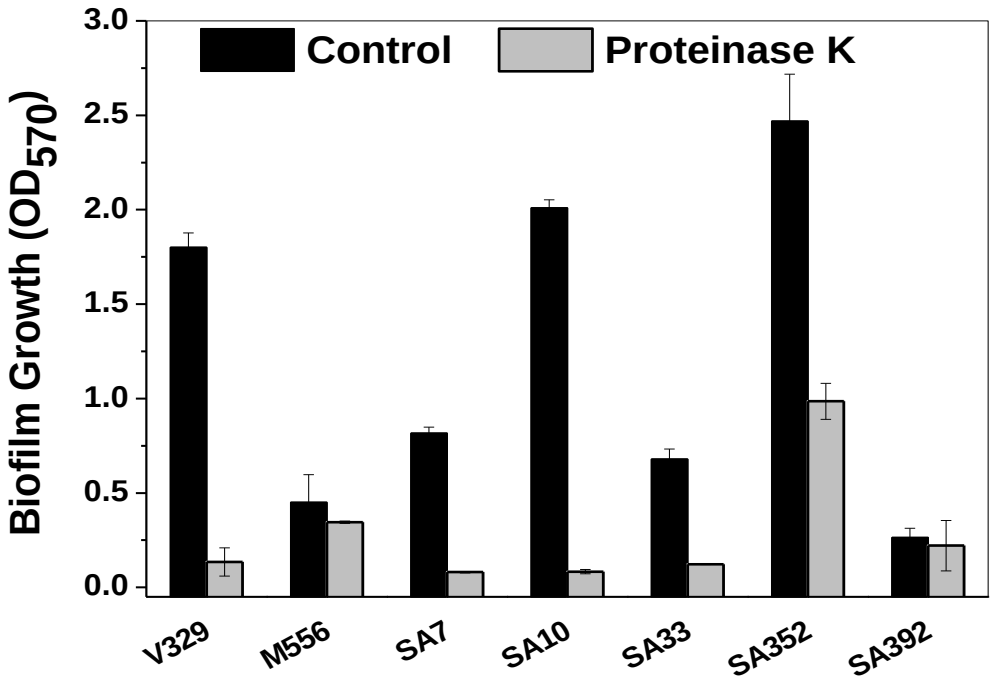


Figure 4.7: Dispersal of pre-grown 24 h old *S. aureus* biofilms by Proteinase K. Results are shown as mean ± 1 S.D.

Figure 4.8 and Table4.2 show the constituents of *S. aureus* biofilms before and after the Proteinase K treatment. *S. aureus* V329 biofilm had significantly higher amount of carbohydrate as well as eDNA ($P < 0.05$, $n = 3$) as compared to Bap mutant M556 biofilm. On the other hand M556 biofilm was comprised of significantly higher amount of biofilm matrix proteins ($P < 0.05$, $n = 3$). Figure 4.8 and Table 4.2 show that there was significant decrease in the protein as well as

eDNA content in V329 and M556 biofilms after Proteinase K treatment, however there was no significant decrease in the carbohydrate content of biofilm matrix in either case.

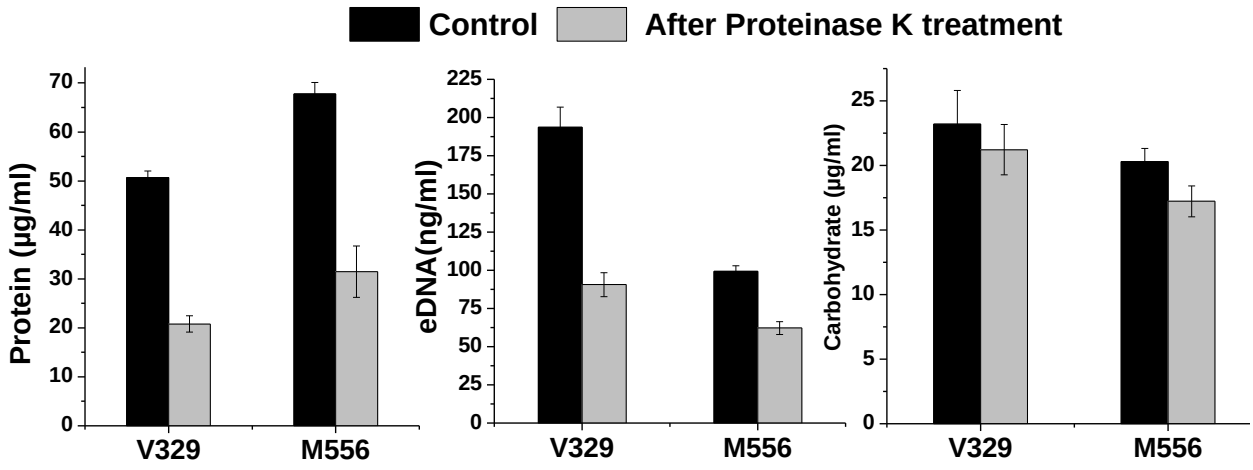


Figure 4.8: Constituents of *S. aureus* EPS before and after treatment of Proteinase K. Results are shown as mean ± 1 S.D.

Table 4.2: Constituents of *S. aureus* EPS before and after treatment of Proteinase K. Results are shown as mean ± 1 S.D.

Biofilm Constituents	V329	After Proteinase K	M556	After Proteinase K
Protein (µg/ml)	50.7 ± 1.34	20.8 ± 0.654	67.8 ± 2.34	31.467 ± 5.26
eDNA (ng/ml)	193.6 ± 13.15	90.6 ± 7.82	99.4 ± 3.61	62.16 ± 4.14
Carbohydrate (µg/ml)	23.20 ± 2.60	21.22 ± 1.94	20.30 ± 1.017	17.22 ± 1.19

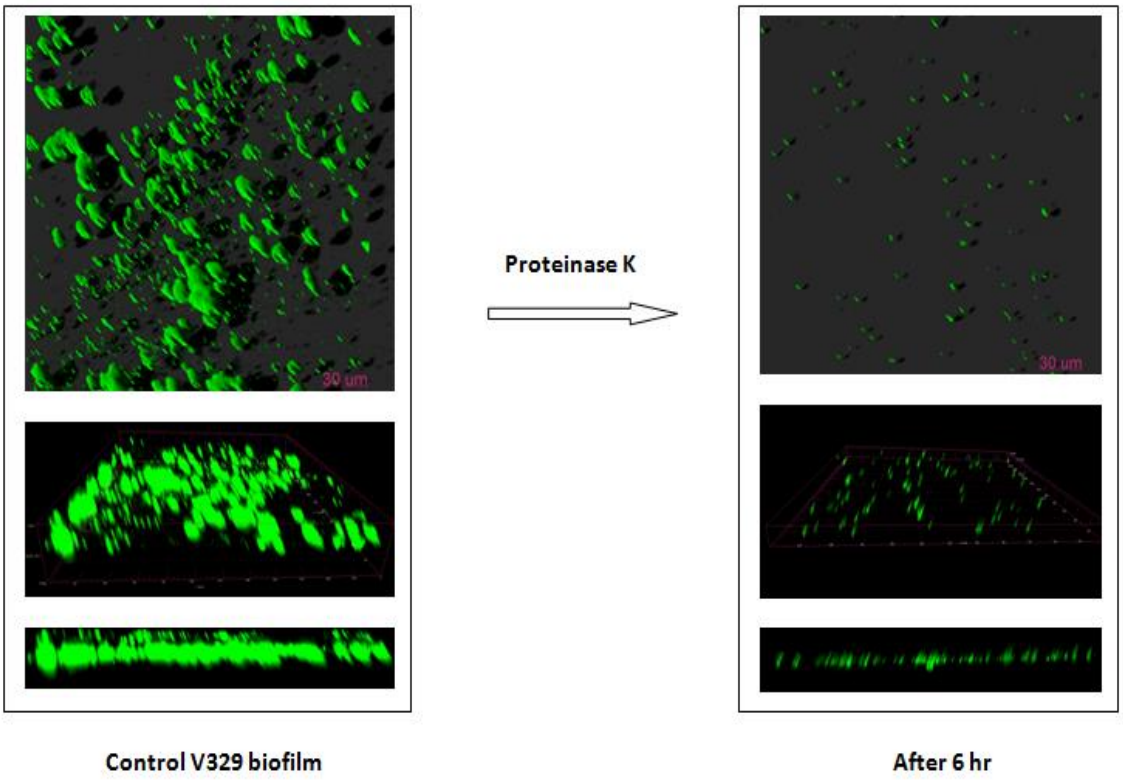


Figure 4.9: CSLM study of Proteinase K mediated dispersal of V329 biofilm**.** A) Control V329 biofilm; B) After 6 h of Proteinase K treatment.

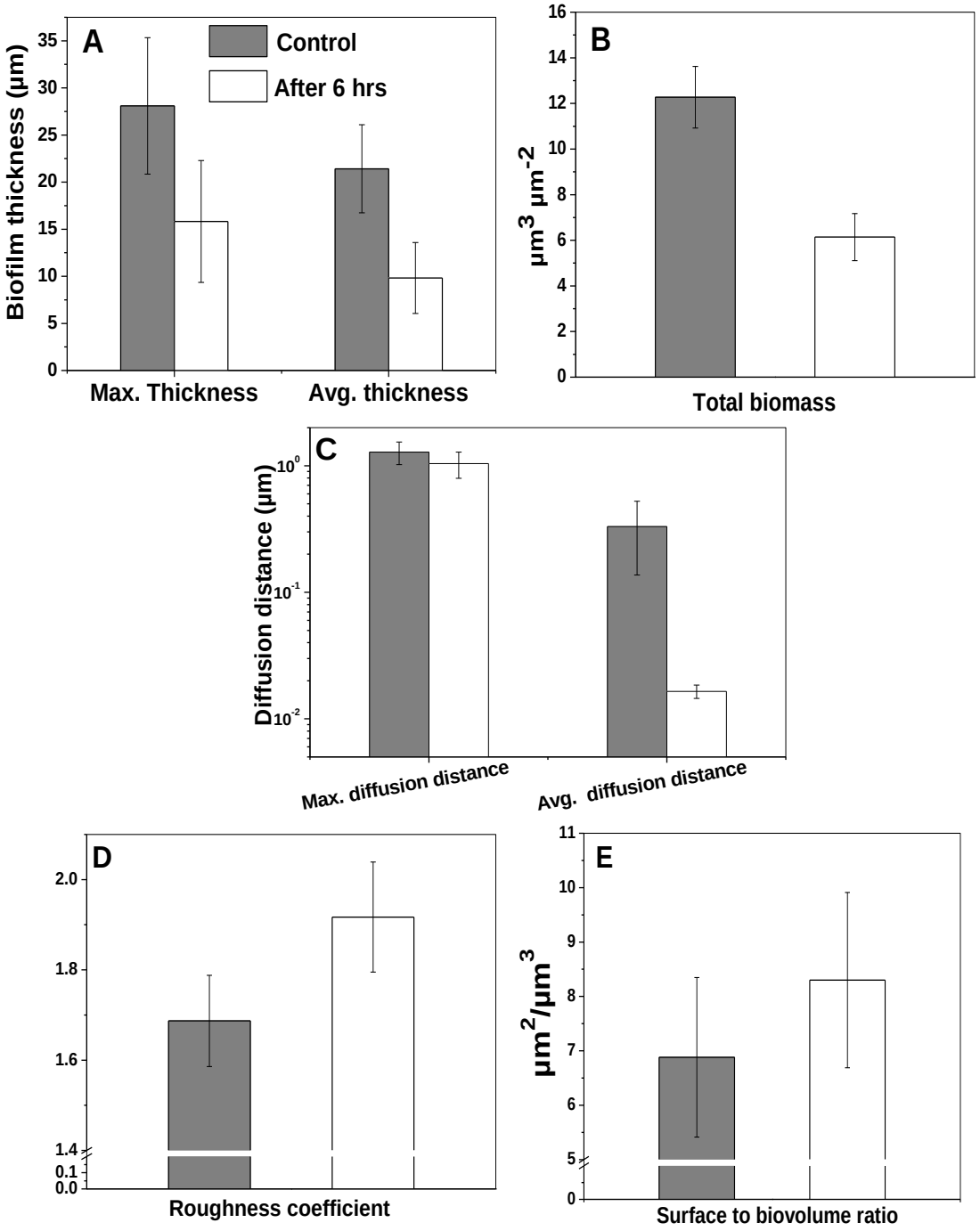


Figure 4.10: Biofilm parameters in control *S. aureus* V329 biofilm and after 6 h treatment of Proteinase K (A) Maximum thickness and average thickness (B) Total biomass (C) Maximum and average diffusion distances (D) Roughness coefficient (E) surface to biovolume ratio.

Table 4.3: COMSTAT analysis of biofilm parameters in control *S. aureus* V329 biofilm and after 6 h treatment of Proteinase K. Proteinase K treatment was given on 24 hours old *S. aureus V329* biofilm.

Biofilm Parameters	**Control V329 biofilm**	**After 6 h of Proteinase K treatment**
Total biomass ($\mu m^3/\mu m^2$)	12.270 ± 1.350	6.140 ± 1.033
Maximum thickness (µm)	28.094 ± 7.239	15.82 ± 6.473
Avg. thickness (µm)	21.412± 4.68	9.818 ± 3.769
Roughness coefficient	1.887 ±0.101	1.917 ± 0.122
Max. diffusion distance (µm)	1.282 ± 0.260	1.039 ± 0.243
Avg. diffusion distance (µm)	0.3310 ± 0.194	0.0165 ± 0.002
Surface to biovolume Ratio ($\mu m^2/\mu m^3$)	6.881 ± 1.466	8.30 ± 4.61

CSLM study further strengthened the observations of the biofilm assay. The V329 biofilm after 2, 4 and 6 h of contact with Proteinase K showed considerable dispersion (Figure 4.6). COMSTAT analysis of the CSLM images showed that the maximum biofilm thickness was reduced from 28.09 ± 7.24 µm to 15.82 ± 6.47 µm (P value=0. 0008, n=10), while average thickness was reduced from 21.41 ± 4.68 µm to 9.82 ± 3.77 µm (P value < 0.0001, n=10) (Figure 4.10A). Total biofilm biomass ($\mu m^3/\mu m^2$) was reduced from 34 to 62% after 6 h of Proteinase K treatment (Figure 4.10B). A substantial change was noticed in the maximum diffusion distance that reduced from 1.28 ± 0.26 µm to 1.03 ± 0.24 (P value = 0.0446, n=10) (Figure 4.10C). Considerable reduction was observed in the average diffusion distance which was reduced from 0.331 ± 0.194 µm to 0.0165 ± 0.002 µm (P value < 0.0001) (Figure 4.10C). Another important change in biofilm

parameter that determines the interface for exchange of the metabolites, flow of nutrient/antimicrobial agents through biofilm, was roughness coefficient and surface to biovolume ratio (Figure 4.10D &10E; Table 4.3). Enhanced roughness coefficient and surface to biovolume ratio indicates more accessibility of nutrients as well as penetration of antimicrobial agents into the biofilm.

4.3.4 Proteinase K enhances antibiotic efficacy against *bap*-positive *S. aureus* biofilm

Figure 4.11 shows that the efficacy of the antibiotics (GEN, STR AMP and KAN) increased when the biofilm was treated with Proteinase K. The addition of Proteinase K in combination with antibiotics had more impact against *S. aureus* biofilm cells when compared to antibiotics alone addition. When the antibiotic concentration was increased by five times, one log reduction in cfu count was observed. On the other hand, Proteinase K in combination with antibiotics resulted in 3 logs reduction in the case of GEN and STR and 1.3 logs reduction in the case of AMP.

4.3.5 Proteinase K enhances antibiotic efficacy against *S. aureus* biofilms in general

Figure 4.12 shows the reduction in cfu count in *S. aureus* biofilms (SA7, SA10, SA33 and SA352) upon treatment of gentamycin in various combinations. It was observed that addition of Proteinase K in combination with gentamycin had more impact against *S. aureus* biofilm cells when compared to gentamycin alone. When the antibiotic concentration was increased by five times, there was no significant increase in log reduction of cfu count in any case. On the other hand, the addition of 2 µg/ml Proteinase K in combination with antibiotics resulted in significant reductions in bacterial cells. A significant reduction in cfu in biofilms by

3.85, 5.0, 3.03 and 3.76 log units was observed in the case of SA7, SA10, SA33 and SA352 respectively.

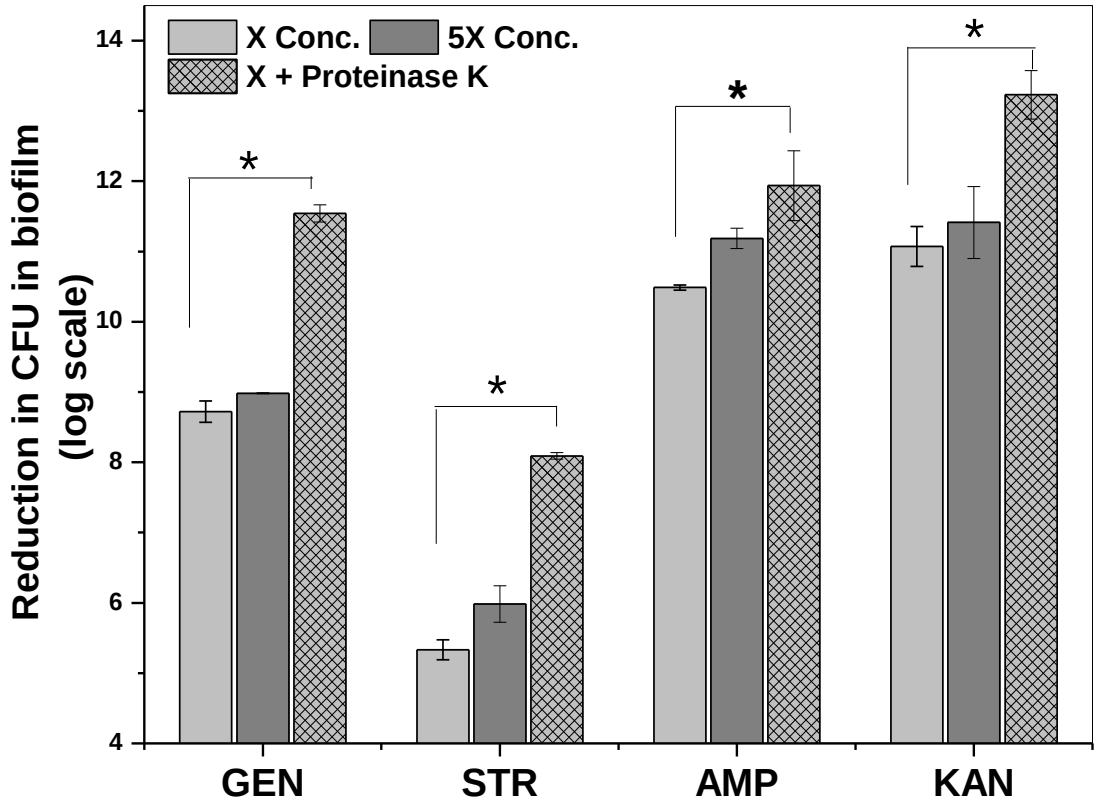


Figure 4.11: Antimicrobial efficacy of antibiotics in combination of Proteinase K against 48 h old *S. aureus V329* biofilm. Results are average of 5 replicates ±1 S.D. and are representative of three independent experiments. Proteinase K was used at 2 µg /ml. Asterisk, $P < 0.05$ as compared with corresponding *S. aureus* biofilm exposed to no Proteinase K.

On the other hand, only 0.45, 1.27, 0.88 and 0.79 log units enhancement was noticed when gentamycin concentration was increased by 5 times. Figure 4.13 show that sonication effectively removed the cells from leftover biofilms after Proteinase K and antibiotic (Gentamycin) treatments. SA352 had a very significant amount of biofilm at the end of the experiment.

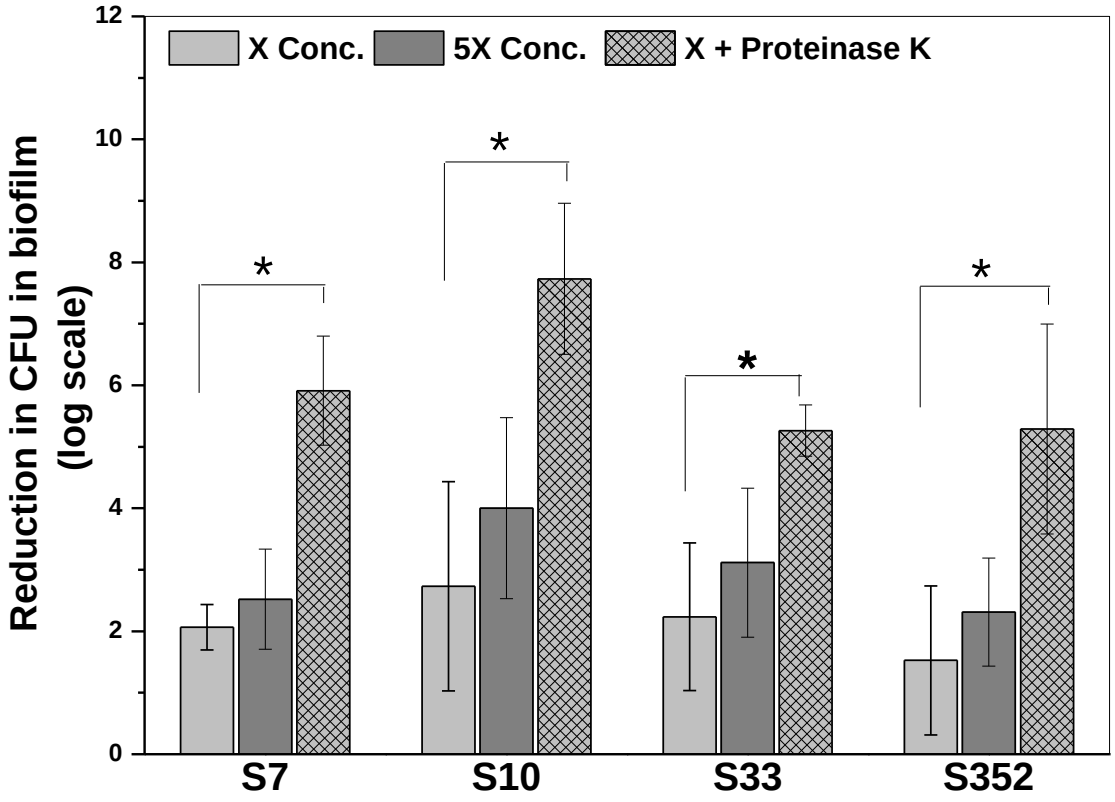


Figure 4.12: Antimicrobial efficacy of antibiotics in combination with Proteinase K against 24 h old *S. aureus* biofilms. Proteinase K was used at 2 µg/ml. Two concentrations of gentamycin were used; X = 150 µg/mL and 5X = 750 µg/ml. Asterisk shows significant difference when $P < 0.05$.

4.4 Discussion

Staphylococcus aureus and *S. epidermidis* are the predominant microorganisms among HAIs such as catheters, cardiac valves and orthopedic prostheses (Gotz 2002). In clinical settings, *S. aureus* biofilms impose resistance to host immune/defense mechanisms and antimicrobial therapy thus enabling the bacterium to persist (Parsek and Singh 2003; del Pozo and Patel 2007). During the last decade many reports have come up highlighting the important role played by surface proteins in early adhesion and establishment of biofilm.Among the

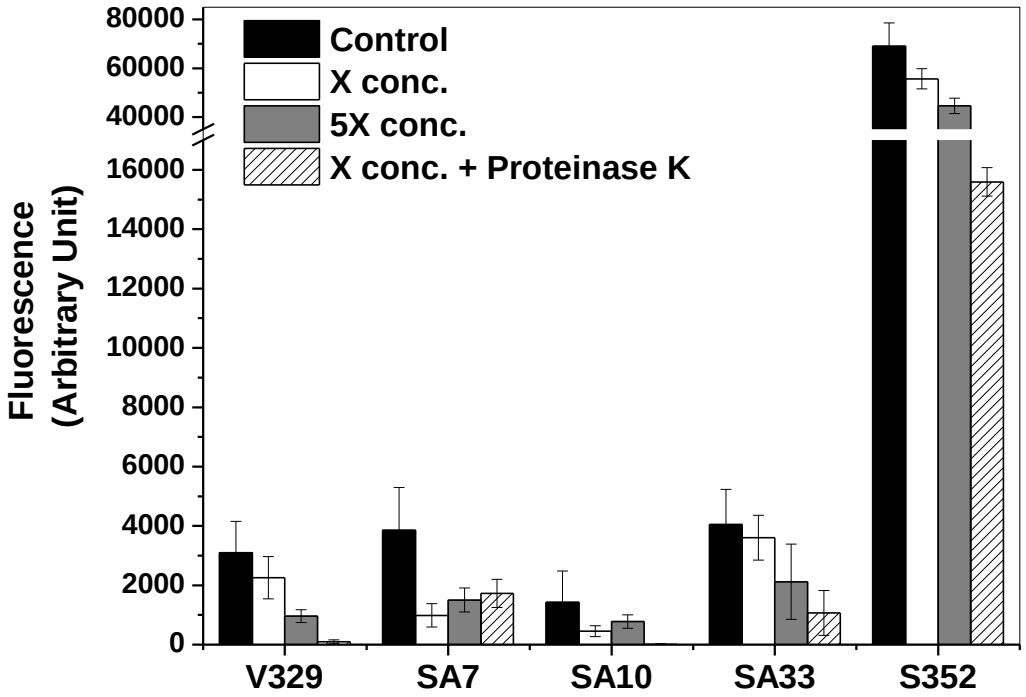


Figure 4.13: Efficacy of sonication used to disrupt remaining S. aureus biofilm after antibiotics-Proteinase K treatment. Two concentrations of gentamycin were used; X = 150 µg/mL and 5X = 750 µg/ml. Asterisk shows significant difference when $P < 0.05$.

various proteins, Bap has been reported to play a major role in early adhesion as well as in biofilm development (Cucarella et al. 2001). It was anticipated that proteolytic cleavage of these biofilms would hamper the initial adhesion process and in turn progression of biofilm. In this study, it was noticed that when mild treatment of Proteinase K was given to *S. aureus* V329 cells to remove surface proteins, a reduction in early cell adhesion was observed. The observations emphasize the fact that Bap plays an important role in early adhesion, as similar effect was not observed in *bap*-negative M556. The observations recorded in Figure 4.2 showed that Proteinase K treatment had significantly impacted the biofilm development of most *S. aureus* isolates. In order to evaluate, whether

inhibition of biofilm was due to the hampered growth or due to the life style switch over of the cells to planktonic form and effect of different concentration of Proteinase k on planktonic cells were monitored. Figure 4.3 shows that there was no impact of Proteinase K on cell viability, hence biofilm inhibition was due to proteolytic cleavage of surface proteins. These observations also reemphasize the important role played by Bap like surface proteins in biofilm development, as similar effect was not observed in *bap*- mutant M556.

In general, metal binding proteins are structurally more stable when they are bound to a specific metal and can show higher resistance towards proteolytic degradation (Villalonga *et al.* 2004). As Bap contains four putative Ca^{2+} binding EF hand motifs, it was evaluated whether binding of Ca^{2+} to Bap would confer any resistance against Proteinase K mediated degradation and in turn biofilm dispersion. The observations shown in Figure 4.4 indicates that there was no apparent difference between biofilms grown in the presence of proteinase K alone and Proteinase K along with increasing concentrations of Ca^{2+}. Whereas in another set of increasing concentrations of Ca^{2+} alone showed inhibition of V329 biofilm at higher concentrations as shown in an earlier report. This result demonstrates that binding of Ca^{2+} ions to Bap had no effect on Proteinase K mediated inhibition of V329 biofilm. In other words, biofilm assay using Ca^{2+} alone and Ca^{2+} with Proteinase K showed that Ca^{2+} did not confer any immunity against proteolytic degradation of Bap.

As surface proteins also play an important role in stability of the *S. aureus* biofilms, preformed biofilms of various *S. aureus* strains (V329, M556, SA7, SA10, SA33, SA352 and SA392) were also tested with Proteinase K. Treatment of 24 h old *S. aureus* biofilm *with* Proteinase K showed that the enzyme enhanced the

dispersion of *bap*- positive V329 in a time dependent manner (2 – 6 h) but did not affect the M556 biofilm, since up to 24 hrs M556 did not produce significant biofilm. Figure 4.7 show that Proteinase K treatment also caused a significant dispersal of all pre-grown (24 h) *S. aureus* biofilms except M556 and SA392. 48h old biofilms of V329 and M556 were also investigated for the treatment of Proteinase K. Since in the case of M556, Bap protein does not remain attached to the cell wall, it remains non-functional and does not contribute in biofilm stability. M556 harbours functional *ica*-operon and hence could produce significant amount of biofilm after 48 h (Cucarella et al. 2001). Therefore, it can also be speculated that in weak biofilms formed by M556 and SA392, PIA might play a predominant role as their biofilms were not affected by Proteinase K treatment.

The effect of Proteinase K treatment on the composition of *bap*-positive and *bap*-mutant *S. aureus* biofilms was also investigated. After Proteinase K treatment, a significant decrease in the protein and eDNA but not in the carbohydrate content in EPS was observed. eDNA is also known to play very important role in *S. aureus* biofilm stability(Mann et al. 2009). As there was a significant decrease in eDNA content along with the biofilm matrix protein content, it is speculated that matrix proteins might also be involved in eDNA retention in the biofilm. Since Ca^{2+} binds with Bap (Arrizubieta et al. 2004) as well as eDNA (Das et al. 2014), it is speculated that Ca^{2+} might act as a cross-linking agent between Bap and eDNA thereby the presence of Bap can assist in retention of eDNA. Therefore, upon Proteinase K treatment that degraded Bap, a significant amount of eDNA was also lost along with the proteins. As shown in Figure 4.8 and Table 4.2 M556 biofilm was comprised of significant amount of eDNA and carbohydrate, which suggest that in the absence of functional Bap protein, eDNA

and carbohydrate i.e. polysaccharide polymers in matrix might play a crucial role in *S. aureus* biofilms. In M556 biofilm, matrix proteins do not contribute to biofilm stability despite having higher amount of protein content. The results obtained also indicate that matrix proteins were neither protected by sugars and DNA nor resistant to Proteinase K and hence degraded by protease. It is speculated that carbohydrate polymers retains the biofilm structure and do not allow the M556 biofilm to get dispersed upon Proteinase K treatment.

The potential application of this study denotes that, Proteinase K can be used in biofilm dispersion as well as for anti-biofilm activity. However Proteinase K treatment showed significant removal of biofilm (Figure 4.5) but 100% removal could not be accomplished even after 24 h of treatment. It is an established fact that biofilm cells are extremely (1000 times or more) resistant to antibiotics as compared to planktonic cells due to physical as well as the genetic reasons (Hoyle and Costerton 1991; Mah et al. 2003). The Proteinase K mediated dispersal of *S. aureus* biofilms suggested its potential use in enhancing the susceptibility of bacterial cells towards antibiotic treatment. The results indicated that Proteinase K treatment increased the susceptibility of V329 bacterial cells in biofilms towards the antibiotic treatment. CSLM data analysis by COMSTAT (Figure 4.10 and Table 4.3) showed that Proteinase K treatment increased the surface to volume ratio and roughness coefficient of the V329 biofilm. If the surface to biovolume ratio as well as roughness coefficient is more, it indicates that a high fraction of cells in the biofilm are exposed to the bulk liquid and consequently to dissolved antimicrobial agents (Heydorn et al. 2000). Thus, the enhanced values of surface to biovolume ratio and roughness coefficient after Proteinase K treatments signify that more biofilm surface becomes available for antibiotics action. Generally,

biofilm forms a physical barrier, limiting diffusion of antibiotics and thereby reducing its efficacy (Hoyle & Costerton, 1991; Hoyle et al., 1992; Singh et al. 2010). COMSTAT analysis showed that Proteinase K treatment significantly decreased the average diffusion distance as well as maximum diffusion distance. This enables the antibiotics to penetrate deep inside the biofilm and kill the cells. Thus it can be inferred that Proteinase K shows synergistic effect when associated with antibiotics for biofilm removal. On similar lines gentamycin efficacy in combination with Proteinase K was investigated against other biofilm forming *S. aureus* strains in this study. Result showed that Proteinase K treatment significantly enhanced the efficacy of gentamycin against all *S. aureus* biofilm tested i.e., SA7, SA10 SA33 and SA352. In addition, FDA assay was performed to ensure that sonication did not contribute to any experimental error. The trend as seen in Figure 4.12, the respective well fluorescence and cfu count showed that sonication treatment was uniformly applied to all wells and did not contribute to any experimental error.

In conclusion, the potential application of this study denotes that, Proteinase K mediated biofilm removal has a wider application. Proteinase K can be used in biofilm dispersion as well as for anti-biofilm activity as most of the biofilms do comprise of surface proteins as a major constituent of biofilm matrix. The present study show a wider applicability of Proteinase K treatment against *S. aureus* biofilms and it also establishes the fact that antibiotics in combination with Proteinase K can be more effective in controlling biofilm mediated infections particularly *S. aureus*. Apart from clinical biofilms, Proteinase K has also been shown to impair *Listeria monocytogenes* biofilm formation and induce dispersal of pre-existing biofilms in food industry (Nguyen and Burrows, 2014). This study

shows that targeting biofilm matrix components by specific enzymes, e.g. surface proteins could be a potential approach towards controlling biofilms related problems.This study concludes that proteolytic degradation of the Bap- mediated biofilm matrix could be a promising therapeutic approach in controlling infections involving *S. aureus*. However, in other *S. aureus* strains where PIA, rather than surface protein, plays a major role in adhesion, this approach will not be useful.

References

Alanis AJ. 2005. Resistance to antibiotics: are we in the post-antibiotic era? Arch Med Res. 36:697-705.

Arrizubieta MJ, Toledo-Arana A, Amorena B, Penadés JR, Lasa I. 2004. Calcium inhibits bap-dependent multicellular behavior in *Staphylococcus aureus*. J Bacteriol. 186:7490-7498.

Bajrami B, Shi Y, Lapierre P, Yao X. 2009. Shifting unoccupied spectral space in mass spectrum of peptide fragment ions. J Am Soc Mass Spectrom. 20:2124-2134.

Beaudet MP, Ahnert N, Dallwig JA, Goodman T, Thomas JA. 2007. A fast, easy, accurate method for protein quantitation. Faseb J. 21:A1006-A1007

Boles BR, Horswill AR. 2008. Agr-mediated dispersal of *Staphylococcus aureus* biofilms. PLoS Pathog. 4:e1000052.

Boyd A, Chakrabarty AM. 1994. Role of alginate lyase in cell detachment of *Pseudomonas aeruginosa*. Appl Environ Microbiol. 60:2355-2359.

Chaignon P, Sadovskaya I, Ragunah C, Ramasubbu N, Kaplan JB, Jabbouri S. 2007. Susceptibility of staphylococcal biofilms to enzymatic treatments depends on their chemical composition. Appl Microbiol Biotechnol. 75:125-132.

Cucarella C, Solano C, Valle J, Amorena B, Lasa I, Penades JR. 2001. Bap, a *Staphylococcus aureus* surface protein involved in biofilm formation. J Bacteriol. 183:2888–2896.

Das T, Sehar S, Koop L, Wong YK, Ahmed S, Siddiqui KS, Manefield M. 2014. Influence of calcium in extracellular DNA mediated bacterial aggregation and biofilm formation. PLoS One.9:e91935.

del Pozo JL, Patel R. 2007. The challenge of treating biofilm-associated bacterial infections. Clin Pharmacol Ther. 82:204-209.

Dubin G. 2002. Extracellular proteases of Staphylococcus spp. Biol Chem. 383:1075-86.

Flemming HC, Wingender J. 2010. The biofilm matrix. Nat Rev Microbiol. 8:623-633.

Gotz, F., 2002. Staphylococcus and biofilms. Mol Microbiol 43, 1367-78.

Hennig S, Wai SN, Ziebuhr W. 2007. Spontaneous switch to PIA-independent biofilm formation in an ica-positive *Staphylococcus epidermidis* isolate. Int J Med Microbiol. 297:117-122.

Heydorn A, Nielsen AT, Hentzer M, Sternberg C, Givskov M, Ersboll BK, Molin S. 2000. Quantification of biofilm structures by the novel computer program COMSTAT. Microbiology 146:2395-40.

Hoyle BD, Alcantara J, Costerton JW. 1992. *Pseudomonas aeruginosa* biofilm as a diffusion barrier to piperacillin. Antimicrob Agents Chemother. 36:2054-2056.

Hoyle BD, Costerton JW. 1991. Bacterial resistance to antibiotics: the role of biofilms. Prog Drug Res. 37:91-105.

Hryniewicz W. 2011. [Antibiotic resistance--what we have to do now?]. Pol Merkur Lekarski. 179:305-9.

Kaplan JB, Ragunath C, Ramasubbu N, Fine DH. 2003. Detachment of *Actinobacillus actinomycetemcomitans* biofilm cells by an endogenous beta-hexosaminidase activity. J Bacteriol 185:4693-8.

Karatan E, Watnick P. 2009. Signals, regulatory networks, and materials that build and break bacterial biofilms. Microbiol Mol Biol Rev. 73:310-347.

Kiedrowski MR, Horswill AR. 2011. New approaches for treating staphylococcal biofilm infections. In: Annals of the New York Academy of Sciences. 104-121.

Kristjansson MM, Magnusson OT, Gudmundsson HM, Alfredsson GA, Matsuzawa H. 1999. Properties of a subtilisin-like proteinase from a psychrotrophic Vibrio species comparison with proteinase K and aqualysin I. Eur J Biochem 260:752-60.

Mah TF, Pitts B, Pellock B, Walker GC, Stewart PS, O'Toole GA. 2003. A genetic basis for *Pseudomonas aeruginosa* biofilm antibiotic resistance. Nature. 426:306-310.

Mangwani N, Kumari S, Shukla SK, Rao TS, Das S. 2014. Phenotypic switching in biofilm-forming marine bacterium *Paenibacillus lautus* NE3B01. Curr Microbiol. 68:648-656.

Mann E, Rice K, Boles B, Endres J, Ranjit D, Chandramohan L, Tsang L, Smeltzer M, Horswill A, Bayles K. 2009. Modulation of eDNA release and degradation affects *Staphylococcus aureus* biofilm maturation. PLoS ONE. 4:e5822.

Merino N, Toledo-Arana A, Vergara-Irigaray M, Valle J, Solano C, Calvo E, Lopez JA, Foster TJ, Penades JR, Lasa I. 2009. Protein A-mediated multicellular behavior in *Staphylococcus aureus*. J Bacteriol. 191:832-843.

Nguyen UT, Burrows LL. 2014. DNase I and proteinase K impair *Listeria monocytogenes* biofilm formation and induce dispersal of pre-existing biofilms. Int J Food Microbiol. 187:26-32.

O'Gara JP. 2007. ica and beyond: biofilm mechanisms and regulation in *Staphylococcus epidermidis* and *Staphylococcus aureus*. FEMS Microbiol Lett. 270:179-88.

O'Toole GA, Kolter R. 1998. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. Mol Microbiol. 28:449-61.

O'Toole G, Kaplan HB, Kolter R. 2000. Biofilm formation as microbial development. Annu Rev Microbiol. 54:49-79.

Paraneeiswaran A, Shukla SK, Prashanth K, Rao TS. 2015. Microbial reduction of [Co(III)–EDTA]− by *Bacillus licheniformis* SPB-2 strain isolated from a solar salt pan. J Hazard Mat. 283:582-590.

Parsek MR, Singh PK. 2003. Bacterial biofilms: an emerging link to disease pathogenesis. Annu Rev Microbiol. 57:677-701.

Potter A, Ceotto H, Giambiagi-Demarval M, dos Santos KR, Nes IF, Bastos Mdo C. 2009. The gene *bap*, involved in biofilm production, is present in *Staphylococcus* spp. strains from nosocomial infections. J Microbiol.47:319-326.

Romero D, Kolter R. 2011. Will biofilm disassembly agents make it to market? Trends Microbiol. 19:304-306.

Shaw L. 2004. The role and regulation of the extracellular proteases of *Staphylococcus aureus*. Microbiology. 150:217-228.

Singh R, Ray P, Das A, Sharma M. 2010. Penetration of antibiotics through *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms. J Antimicrob Chemother.65:1955-1958.

Soderblom T, Aspevall O, Erntell M, Hedin G, Heimer D, Hokeberg I, Kidd-Ljunggren K, Melhus A, Olsson-Liljequist B, Sjogren I, et al. 2010. Alarming spread of vancomycin resistant enterococci in Sweden since 2007. Euro Surveill.15:19620.

Stepanovic S, Vukovic D, Jezek P, Pavlovic M, Svabic-Vlahovic M. 2001. Influence of dynamic conditions on biofilm formation by staphylococci. Eur J Clin Microbiol Infect Dis 20:502-504.

Stoodley P, Sauer K, Davies DG, Costerton JW. 2002. Biofilms as complex differentiated communities. Annu Rev Microbiol 56:187-209.

Tormo MA, Knecht E, Gotz F, Lasa I, Penades JR. 2005. Bap-dependent biofilm formation by pathogenic species of *Staphylococcus*: evidence of horizontal gene transfer? Microbiology. 151:2465-2475.

Vautor E, Abadie G, Pont A, Thiery R. 2008. Evaluation of the presence of the bap gene in *Staphylococcus aureus* isolates recovered from human and animals species. Vet Microbiol. 18:127:407

Villalonga ML, Reyes G, Villalonga R. 2004. Metal-induced stabilization of trypsin modified with alpha-oxoglutaric acid. Biotechnol Lett. 26:209-212.

CHAPTER 5 : Effect of c-di-GMP on *S. aureus* biofilm and cell morphology

5.1 Introduction

Staphylococcus aureus is the predominant pathogen however the molecular mechanisms of its pathogenesis remain largely unclear. Biofilm development has been identified as main cause of persistent infections by this pathogen (Gotz 2002). Prevention and treatment of *S. aureus* infection is becoming increasingly cumbersome since it is associated with biofilm development which per se confers tolerance to antimicrobial agents (Donlan and Costerton 2002, Lowy 1998). The poor success rate in the treatment of *staphylococci* is also due to the emergence of multidrug resistance (Alanis 2005). Once upon a time, methicillin was the antibiotic of choice as last line of defence (Muto et al. 2003). However, excessive use of this antibiotic resulted in the development of many methicillin resistant *S. aureus* strains (Sieradzki et al. 1999, Soderblom et al. 2010). As per a recent report, approximately 60% of *S. aureus* isolates only remain sensitive to this drug (Roghmann et al. 2001).The increasing emergence of antimicrobial resistance in bacterial pathogens and the importance of biofilms in the infection process has directed researchers to look for alternate antimicrobial strategies (Kiedrowski and Horswill 2011). Therefore, there is an urgent need to develop novel treatment regimes to prevent biofilm formation and minimize the antibiotics use.

Among many factors, nucleotide-based secondary messenger such as cCMP, cAMP, c-dinucleotide GMP etc. are involved in the regulation of almost all aspects of microbial life (Pesavento and Hengge 2009). In prokaryotic systems, cyclic di-GMP (cyclic-diguanylate-[3,5-cyclic diguanylic acid) has emerged as an extremely important and ubiquitous intracellular secondary messenger molecule involved in bacterial life-style transitions i.e., biofilm formation, virulence, and planktonic life style (Ross et al. 1991); D'Argenio and Miller 2004, Jenal 2004,

Römling 2002, Römling and Amikam 2006). c-di-GMP consists of two cGMP molecules joined by a 3,5-phosphodiester bond (Jenal 2004). c-di-GMP was first identified in *Gluconacetobacter xylinus* in the context of regulation of cellulose production. Of late, it was found to be a critical factor in biofilm formation by many bacteria such as *Vibrio cholerae*, *Yersinia pestis*, *Salmonella enteritidis* serovar Typhimurium, and *Pseudomonas aeruginosa* (García et al. 2004, Hengge 2009, Tischler and Camilli 2004, Waters et al. 2008). In general, up-regulation of c-di-GMP enhances the biofilm formation, decreases the motility and virulence in gram-negative bacteria (Hengge 2009). However, their role in the regulation of gram positive bacterial biofilm formation, such as *S. aureus* biofilm is not very clear. c-di-GMP is associated with proteins containing GGDEF protein domains (Ausmees et al. 2001, Jenal 2004, Paul et al. 2004, Römling 2002, Ross, Mayer and Benziman 1991, Simm et al. 2004). As per the existing literature, *S. aureus* has only a few proteins that contains a GGDEF domain (Corrigan et al. 2011, Tatusov et al. 2001). Karaolis et al. reported that c-di-GMP can inhibit Bap mediated cell-cell interactions in *S. aureus* and in turn its biofilm formation (Karaolis et al. 2005). The chapter's objective was to carry out a detail study of the effect of c-di-GMP on Bap-mediated biofilm inhibition and to investigate the possibility of c-di-GMP treatment as a novel approach to control *S. aureus* biofilm formation.

5.2 Materials and Methods

5.2.1 Bacterial strains and growth conditions

All *S. aureus* strains used in this study were stored at -80°C in 50% glycerol. A *bap*–positive *S. aureus* V329 and its isogenic mutant *S. aureus* M556 were used in this study along with five other clinical isolates of *S. aureus* viz., SA7, SA10

SA33, SA252 and SA392. M556 was generated by transposon insertion in the downstream part of bap gene of *S. aureus* V329 in such a way that Bap protein is synthesized but remains non-functional as cell wall anchoring region is truncated (Cucarella et al. 2001). For each experiment, single colonies were picked from Tryptic Soy Agar culture plates and inoculated in Tryptic Soy Broth (TSB) medium supplemented with 0.25% glucose (TSB-G) and incubated at 37^{o}C at 150 rpm. Overnight grown cultures were used for all experiments.

5.2.2 c-di-GMP preparation

The c-di-GMP used in these studies was procured from as pure and high-yield preparation of c-di-GMP di sodium salt (Biolog, India). Before use, lyophilized c-di-GMP was resuspended in 0.9% NaCl with final stock concentration of 2 mM and stored at 4°C until needed.

5.2.3 Biofilm formation inhibition assay

Staphylococcus aureus strains were sub-cultured from glycerol stocks onto TSA agar plates and incubated at 37°C for 18 h. A single colony was inoculated into 5 ml of TSB using a sterilized loop and incubated at 37°C for 18 h with shaking at 150 rpm. The overnight grown cultures of the *S. aureus* cells in TSB-G were diluted 1:100 in fresh sterile TSB-G medium and added to the pre-sterilized 96 well flat bottom polystyrene micro-titre plates. To estimate the inhibitory action of c-di-GMP, *S. aureus* biofilms were grown in the presence of c-di-GMP (final concentration 4 µM and 20 µM) at 37°C for 24 h. After 24 h of incubation, biofilm growth was quantified. Biofilm quantification was done by classical crystal violet assay as described previously (Mangwani et al. 2014). For dissolution of bound crystal violet, 33% acetic acid was used (Stepanovic et al. 2001). Biofilm growth

was monitored in terms of OD_{570} using a multimode micro-plate reader (BioTek, USA).

5.2.4 Planktonic growth study

The overnight grown bacterial cultures were harvested and washed twice with phosphate buffered saline (PBS) and OD_{600} of each culture was set to 0.1. A 10 µl of each resuspended culture was diluted in sterile TSB-G medium having final concentration of c-di-GMP at 4 µM to the final volume of 200 µl and transferred to 96 well micro-titre plate. Planktonic growth of cultures was monitored at 37^{o}C in terms of absorbance at 600 nm using a multimode micro-plate reader (BioTek, USA).

5.2.5 Confocal Scanning Laser Microscopy

The overnight grown *S. aureus* V329 culture was harvested and washed twice with phosphate buffered saline (PBS) and OD_{600} of each culture was set at 0.1. A 10 µl of each resuspended culture was diluted in sterile TSB-G medium having final concentration of c-di-GMP at 4 µM to the final volume of 2 ml and incubated at 37^{o}C for 4 h. After incubation, 50 µl of the culture was taken on to a glass slide, heat fixed and stained with 0.2% acridine orange. Planktonic cells were observed under the CSLM (Leica, Germany) as described earlier (sub topic 3.2.9).

5.3 Results

5.3.1 Effect of c-di-GMP on *S. aureus* biofilm formation

Figure 5.1 shows the effect of c-di-GMP on biofilm formation of different *S. aureus* strains at different concentrations of c-diGMP. Figure 1a shows that there was no substantial effect of c-di-GMP on *S. aureus* biofilm formation in any strains when tested at 4 µM and 20 µM. Figure 1b shows the effect of c-di-GMP on biofilm

formation of *bap*-positive V329 and isogenic *bap*-mutant M556 at very high concentration i.e. 200 µM to see if there was any effect on their biofilm formation capability. Figure 5.1b clearly depicts that there was no effect on biofilm formation capability of either strains.

5.3.2 Effect of c-di-GMP on planktonic growth of *S. aureus* strains

Figure 5.2 shows the results of planktonic growth study in the presence of 4 µM c-di-GMP with all the *S. aureus* strains. Results showed there was no substantial effect on planktonic growth of any of the *S. aureus* strains as well.

5.3.3 Effect of c-di-GMP on intercellular adhesion of *bap*-positive *S. aureus* V329

Effect of c-di-GMP on intercellular adhesion of *bap*-positive *S. aureus* V329 was investigated using confocal laser scanning microscopy. Figure 5.3 shows the effect of 200 µM c-di-GMP on intercellular adhesion of *bap*-positive *S. aureus* V329 which is hypothesized to be mediated by Bap surface protein. It was observed that there was a marginal reduction in intercellular adhesion. However, in terms of biofilm formation after 24 h there was no significant effect as shown in Figure 5.1.

5.4 Discussion

Few reports have shown that the extracellular c-di-GMP interfere with Bap protein in *S. aureus* and inhibit the biofilm formation (Brouillette et al. 2005, Karaolis et al. 2005). As stated earlier, the purpose of the work carried out in this chapter was to investigate inhibition of Bap-mediated biofilm formation in *S. aureus* by c-di-GMP.

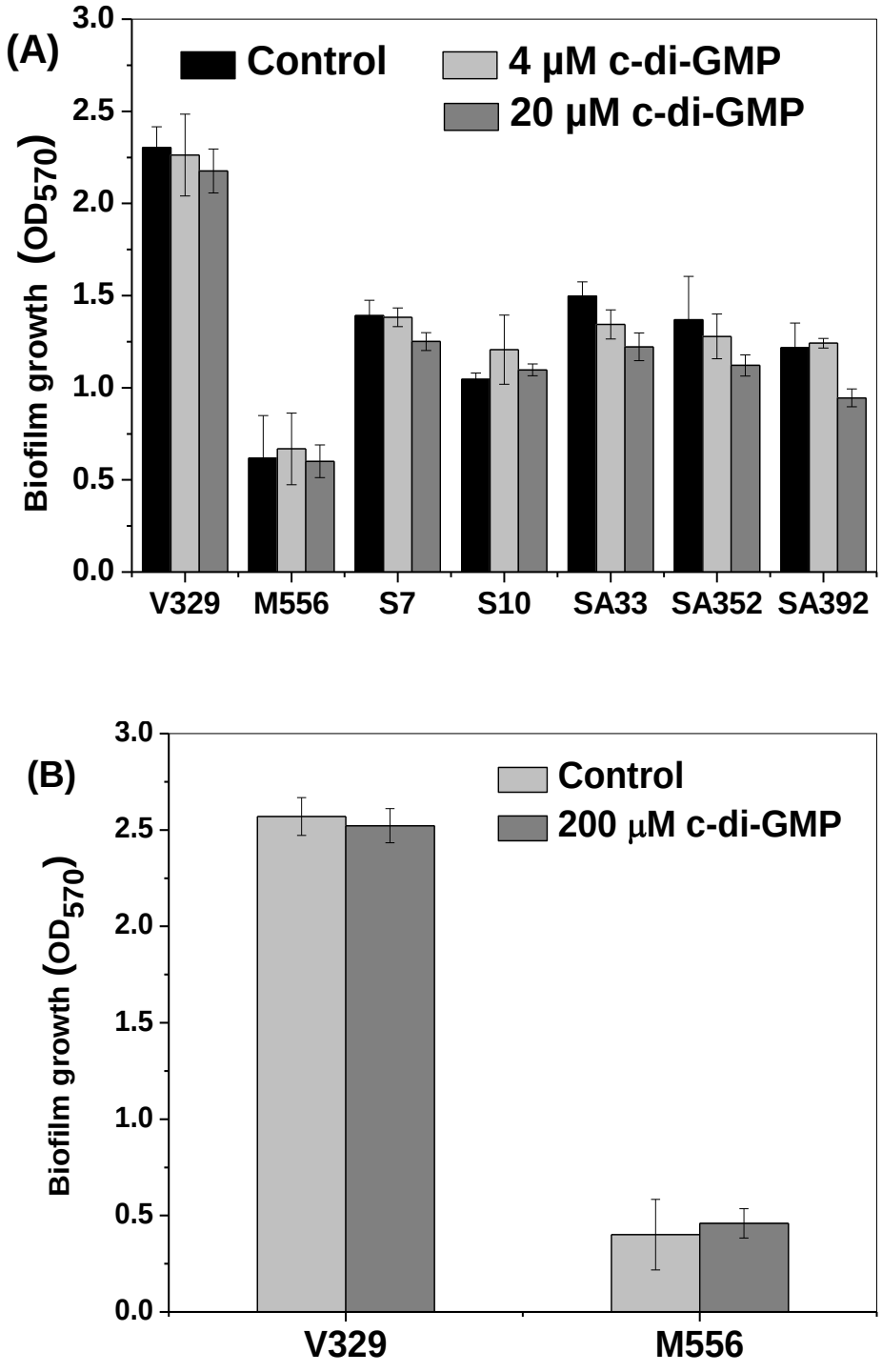


Figure 5.1: Effect of c-di-GMP on *S. aureus* biofilm formation.

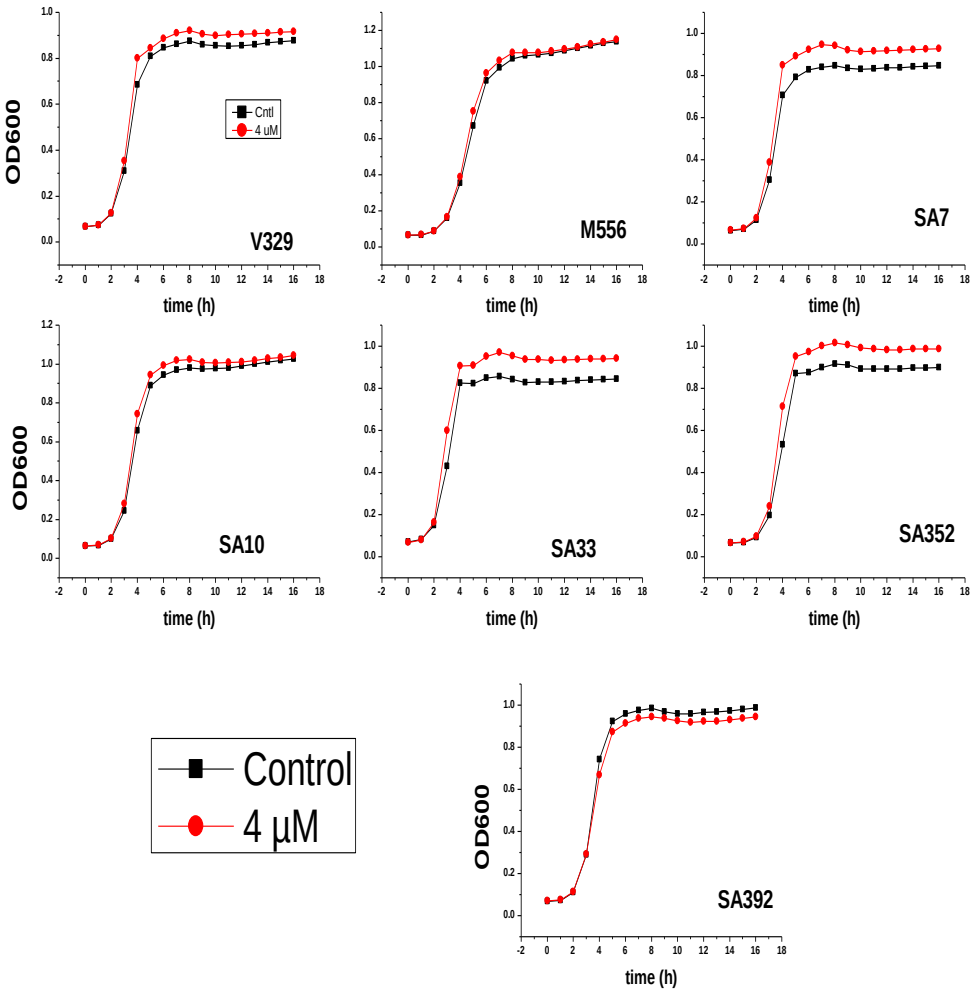
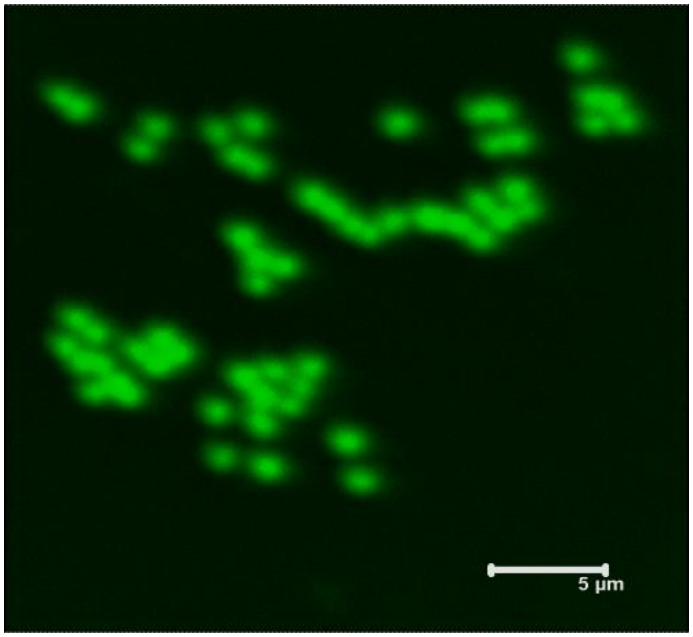


Figure 5.2: Effect of c-di-GMP on *S. aureus* planktonic growth.

Control V329



200 µM c-di-GMP

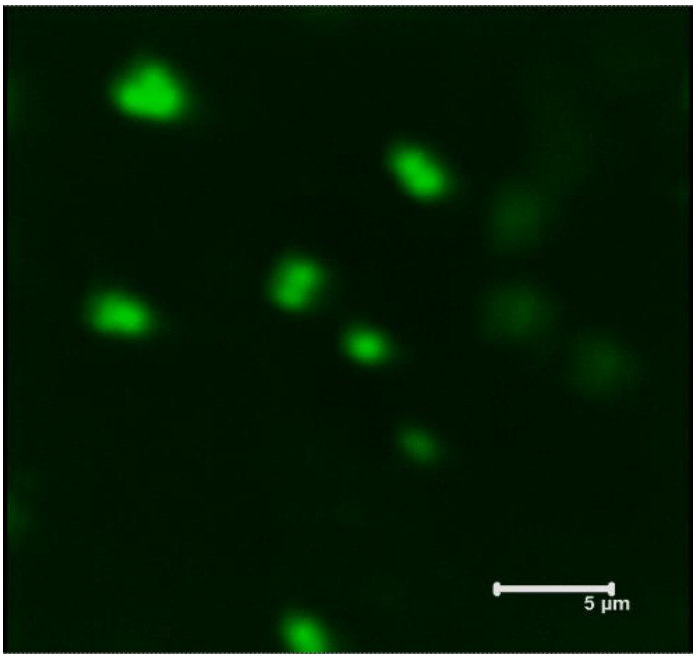


Figure 5.3: Effect of c-di-GMP on *S. aureus* V329 cell –cell interaction.

However, the outcome of the primary experiments suggested that there was no effect on biofilm formation or planktonic growth of *S. aureus*. Results did not corroborate with earlier studies by Karaolis et al., (2005) where they had claimed that the treatment of *S. aureus* with extracellular c-di-GMP inhibited cell-to-cell (intercellular) adhesive interaction in liquid medium and reduced the biofilm formation by >50% in human and bovine isolates compared to untreated controls.

Quoc et al. (2007) identified *a* GGDEF Domain Protein (GdpS) gene important for early steps in *S. aureus* biofilm formation. The role of the GGDEF-containing protein and its molecular mechanism underlying *S. aureus* biofilm formation are unclear. *S. aureus* GdpS lacks an EAL domain which is required for the hydrolysis of c-di-GMP (Lasa 2006). Later, another recent study indicated that GGDEF domain protein may regulate staphylococcal biofilm formation independently of c-di-GMP (Holland et al. 2008). Shang et al., (2009) showed that GdpS regulates the protein A expression in *S. aureus* but it was found to be independent of c-di-GMP. Recently, Corrigan et al. (2011) showed that c-di-GMP was not produced by *S. aureus* strain RN4220. Rather *GdpS* was involved in the synthesis of c-di-AMP which acts as secondary messenger in *S. aureus.* The ground breaking study by Corrigan et al., helped in understanding the limitations of earlier studies by Holland et al. (2008), Quoc et al. (2007), Shang et al. (2009). Later it was also reported to have an important role in *Bacillus subtilis* biofilm regulation (Gundlach et al. 2015).

Results of this study corroborated with studies by Shang et al., (2009) where biofilm formation was found not to be affected by the presence of extracellular c-di-GMP. This observation can now easily be explained in light of important role of a newly emerged secondary messenger c-di-AMP rather than c-di-GMP in *S. aureus* biofilm formation. However, it cannot be denied that use of a wide range of concentrations of c-di-GMP could have shown some positive and negative effects. The discovery of c-di-AMP rather than c-di GMP as secondary messenger in *S. aureus* biofilm regulation is also apparent from the present study. Since high concentration (200 µM) of c-di GMP had no effect on *S. aureus* biofilms the further

experiments were stopped on *S. aureus* biofilm formation and cell-to-cell interaction.

5.5 Conclusion

The study suggested that there was no substantial effect of c-di-GMP on *S. aureus* biofilm formation, planktonic cell growth or cell-cell interaction.

References

Alanis AJ. 2005. Resistance to antibiotics: are we in the post-antibiotic era? Arch Med Res. 36:697-705.

Ausmees N, Mayer R, Weinhouse H, Volman G, Amikam D, Benziman M, Lindberg M. 2001. Genetic data indicate that proteins containing the GGDEF domain possess diguanylate cyclase activity. FEMS Microbiol Lett. 204:163-167.

Brouillette E, Hyodo M, Hayakawa Y, Karaolis DK, Malouin F. 2005. 3′, 5′-Cyclic diguanylic acid reduces the virulence of biofilm-forming *Staphylococcus aureus* strains in a mouse model of mastitis infection. Antimicrob Agents Chemther. 49:3109-3113.

Corrigan RM, Abbott JC, Burhenne H, Kaever V, Gründling A. 2011. c-di-AMP is a new second messenger in *Staphylococcus aureus* with a role in controlling cell size and envelope stress. PLoS pathogens.7:e1002217-e1002217.

Cucarella C, Solano C, Valle J, Amorena B, Lasa I, Penades JR. 2001. Bap, a *Staphylococcus aureus* surface protein involved in biofilm formation. J Bacteriol. 183:2888-2896.

D'Argenio DA, Miller SI. 2004. Cyclic di-GMP as a bacterial second messenger. Microbiology.150:2497-2502.

Donlan RM, Costerton JW. 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev. Apr;15:167-193.

García B, Latasa C, Solano C, Portillo FGd, Gamazo C, Lasa I. 2004. Role of the GGDEF protein family in *Salmonella* cellulose biosynthesis and biofilm formation. Mol Microbiol. 54:264-277.

Gotz F. 2002. Staphylococcus and biofilms. Mol Microbiol. 43:1367-1378.

Gundlach J, Mehne FM, Herzberg C, Kampf J, Valerius O, Kaever V, Stülke J. 2015. An essential poison: synthesis and degradation of cyclic di-AMP in *Bacillus subtilis*. J Bacteriol. 197:3265-3274.

Hengge R. 2009. Principles of c-di-GMP signalling in bacteria. Nat Rev Microbiol. 7:263-273.

Holland LM, O'donnell ST, Ryjenkov DA, Gomelsky L, Slater SR, Fey PD, Gomelsky M, O'Gara JP. 2008. A staphylococcal GGDEF domain protein regulates biofilm formation independently of cyclic dimeric GMP. J Bacteriol. 90:5178-5189.

Jenal U. 2004. Cyclic di-guanosine-monophosphate comes of age: a novel secondary messenger involved in modulating cell surface structures in bacteria? Curr Opin Microbiol. 7:185-191.

Karaolis DK, Rashid MH, Chythanya R, Luo W, Hyodo M, Hayakawa Y. 2005. c-di-GMP (3′-5′-cyclic diguanylic acid) inhibits *Staphylococcus aureus* cell-cell interactions and biofilm formation. Antimicrob Agents Chemther. 49:1029-1038.

Kiedrowski MR, Horswill AR. 2011. New approaches for treating staphylococcal biofilm infections. In: Annals of the New York Academy of Sciences. 104-121.

Lasa I. 2006. Towards the identification of the common features of bacterial biofilm development. Int Microbiol. 9:21.

Lowy FD. 1998. *Staphylococcus aureus* infections. N Engl J Med. 339:520-532.

Mangwani N, Shukla SK, Kumari S, Rao T, Das S. 2014. Characterization of *Stenotrophomonas acidaminiphila* NCW-702 biofilm for implication in the degradation of polycyclic aromatic hydrocarbons. J Appl Microbiol. 117:1012-1024.

Muto CA, Jernigan JA, Ostrowsky BE, Richet HM, Jarvis WR, Boyce JM, Farr BM. 2003. SHEA guideline for preventing nosocomial transmission of multidrug-resistant strains of *Staphylococcus aureus* and enterococcus. Infection Control. 24:362-386.

Paul R, Weiser S, Amiot NC, Chan C, Schirmer T, Giese B, Jenal U. 2004. Cell cycle-dependent dynamic localization of a bacterial response regulator with a novel di-guanylate cyclase output domain. Genes Dev. 18:715-727.

Pesavento C, Hengge R. 2009. Bacterial nucleotide-based second messengers. Curr Opin Microbiol. 12:170-176.

Quoc PHT, Genevaux P, Pajunen M, Savilahti H, Georgopoulos C, Schrenzel J, Kelley WL. 2007. Isolation and characterization of biofilm formation-defective mutants of *Staphylococcus aureus*. Infect Immun. 75:1079-1088.

Roghmann M-C, Siddiqui A, Plaisance K, Standiford H. 2001. MRSA colonization and the risk of MRSA bacteraemia in hospitalized patients with chronic ulcers. J Hosp Infect. 47:98-103.

Römling U. 2002. Molecular biology of cellulose production in bacteria. Res Microbiol. 153:205-212.

Römling U, Amikam D. 2006. Cyclic di-GMP as a second messenger. Curr Opin Microbiol. 9:218-228.

Ross P, Mayer R, Benziman M. 1991. Cellulose biosynthesis and function in bacteria. Microbiol Rev. 55:35-58.

Shang F, Xue T, Sun H, Xing L, Zhang S, Yang Z, Zhang L, Sun B. 2009. The *Staphylococcus aureus* GGDEF domain-containing protein, GdpS, influences protein A gene expression in a cyclic diguanylic acid-independent manner. Infect Immun. 77:2849-2856.

Sieradzki K, Roberts RB, Haber SW, Tomasz A. 1999. The development of vancomycin resistance in a patient with methicillin-resistant *Staphylococcus aureus* infection. N. Engl. J. Med. 340:517-523.

Simm R, Morr M, Kader A, Nimtz M, Römling U. 2004. GGDEF and EAL domains inversely regulate cyclic di-GMP levels and transition from sessility to motility. Mol Microbiol. 53:1123-1134.

Soderblom T, Aspevall O, Erntell M, Hedin G, Heimer D, Hokeberg I, Kidd-Ljunggren K, Melhus A, Olsson-Liljequist B, Sjogren I, et al. 2010. Alarming spread of vancomycin resistant enterococci in Sweden since 2007. Euro Surveill.15:19620.

Stepanovic S, Vukovic D, Jezek P, Pavlovic M, Svabic-Vlahovic M. 2001. Influence of dynamic conditions on biofilm formation by staphylococci. Eur J Clin Microbiol Infect Dis. Jul;20:502-504.

Tatusov RL, Natale DA, Garkavtsev IV, Tatusova TA, Shankavaram UT, Rao BS, Kiryutin B, Galperin MY, Fedorova ND, Koonin EV. 2001. The COG database: new developments in phylogenetic classification of proteins from complete genomes. Nucleic acids Res. 29:22-28.

Tischler AD, Camilli A. 2004. Cyclic diguanylate (c-di-GMP) regulates Vibrio cholerae biofilm formation. Mol Microbiol.53:857-869.

Waters CM, Lu W, Rabinowitz JD, Bassler BL. 2008. Quorum sensing controls biofilm formation in *Vibrio cholerae* through modulation of cyclic di-GMP levels and repression of vpsT. J Bacteriol. 190:2527-2536.

CHAPTER 6 : Cloning, over-expression and purification of biofilm-associated protein

6.1 Introduction

Staphylococcus aureus is one of the most common root causes of nosocomial infections because of its dominant biofilm forming property. Biofilms have been implicated in almost 60% of all bacterial infections (Cha and Thilly 1993). The bacterial biofilm formation is governed by several factors that are under the control of diverse genetic elements (CLONEAMP). One of the factors is the expression of Biofilm-associated protein, which confers the capacity to form the biofilm. It also plays a crucial role in bacterial infection process even in the absence of *ica* operon, which is responsible for polysaccharide intercellular adhesion (PIA)/poly-β-1,6-N-acetylglucosamine (PNAG) synthesis (Cucarella et al. 2001). Bap was the first protein among the family of large surface proteins that is reported to be involved in initial attachment to surfaces and assist in cell–cell interactions (Latasa et al. 2006). The gene *bap* has been reported to be widespread among natural isolates of coagulase-negative *Staphylococcus* species, like *S. epidermidis, S. chromogenes, S. xylosus, S. simulans* and *S. hycus* (Tormo et al. 2005). Of late, a number of surface proteins are reported to have structural homology with *bap* and constitute Biofilm-associated proteins family (BAP family). Such proteins have been identified in different organisms viz., Bap in *Staphylococcus aureus*, Esp in *Enterococcus faecalis* (Toledo-Arana et al. 2001) LapA in *Pseudomonas putida* (Hinsa et al. 2003) and BapA in *Salmonella* (Latasa et al. 2005). Common features of member proteins of this family are an extracellular signal sequence, very high molecular weight, repetitive structure and a typical cell wall anchoring domain (Latasa et al. 2006).

In the pioneering work by Cucarella et al. (2001), there is not much information available on Bap protein. Later the studies by (Shukla and Rao

2013a, Shukla and Rao 2013b, Shukla and Rao 2014) showed that how Bap is important for *S. aureus* biofilm stability and how non-antibiotic factors such as calcium ion concentration and Proteinase K can be used to modulate the *S. aureus* biofilms. To understand the molecular mechanism, the functional as well as the evolutionary significance of *bap* gene need to be discerned. The aim of this study was to clone full-length *bap* (~6.8 Kb) gene in *E. coli* and heterologous expression of the Bap protein under the extremely controlled condition to facilitate *in vitro* study of the Bap protein and its functional characterization. Apart from large size of the gene, the presence of the 13 tandem repeats in C- region of the gene, which confers instability due to homologous recombination, were the main challenges in molecular cloning of *bap* gene. To overcome these challenges, a combination of different hosts and incubation temperatures were tried. In this study, an expression vector named pET21b-*bap* was constructed to express the recombinant protein. The high-purity of the recombinant protein was obtained with Ni–NTA affinity chromatography.

6.2 Materials and Methods

6.2.1 Bacterial strains, expression vector and culture conditions

Staphylococcus aureus V329 containing full length *bap* gene was obtained from Dr. I lasa, Spain and was used in the study. Cloning host *E. coli* stbl2 (Invitrogen) and expression host *E. coli* BL21(DE3)-pLysS (Promega) were used as a cloning host and expression host respectively (Table 6.1). Expression vector pET21b (+) was procured from Novagen (Madison, WI) and was used for protein expression in *E. coli*. *S. aureus* was cultured in tryptic soy broth (TSB) supplemented with 0.25% glucose. *E. coli* strains stbl2 and BL21 (DE3) were grown in Luria-Bertani (LB) medium.

Table 6.1: Genotyic details of E. coli strains used in the study. Stbl2 was used as a cloning host whereas BL21 (DE3) pLysS was used as an expression host.

E. coli strain	Genotype
STBL2 (invitrogen)	F- endA1 glnV44 thi-1 recA1 gyrA96 relA1 Δ(lac-proAB) mcrA Δ(mcrBC-hsdRMS-mrr) λ^-
BL21 (DE3) pLysS	F^- ompT gal dcm lon $hsdS_B(r_B^- m_B^-)$ λ(DE3) $pLysS(cm^R)$

Table 6.2: List of primers used in the study.

Primers	Sequence (5'-3')
sab F	CCCTATATCGAAGGTGTAGAATTGCAC
sab R	GCTGTTGAAGTTAATACTGTACCTGC
BFNd1	GAGGTGAGTA**CATATG**GGAAATAAACAAGGTTTTTTACC
BRXh1	CAATAATTTAAA**CTCGAG**TTTTTTATCATTTTCTTTTCT ACGACG

6.2.2 Genomic DNA isolation and plasmid extraction

The genomic DNA was isolated from log phase culture of *S. aureus* V329 and *E. coli* strains using a QiaAmp DNA mini kit according to the manufacturer's protocol. The staphylococcal cells were lysed by lysostaphin (5 mg/ml; Sigma) at 37°C for 2 h before DNA extraction. Plasmid DNA was isolated from *E. coli* strain using a Qiagen plasmid miniprep kit according to the manufacturer's protocol.

6.2.3 Cloning of *bap* gene into a cloning host (*E. coli* Stbl2)

A primer pair, BFNd1 and BRXh1 with the NdeI-XhoI sites (see Table 6.1) was used to amplify full *bap* gene from the published sequence of *bap* of *S. aureus* V329 (GenBank accession no. AY220730.1) and cloned upstream from the His

tag sequence in the pET-21b vector (Novagen). Amplification was carried out on a Master cycler (Eppendorf, Hamburg, Germany) using Qiagen Long Range PCR Kit with a reaction mixture of 50 µl. The PCR program included an initial denaturation at 93^{o}C for 5 min followed by 35 cycles of denaturation (93^{o}C for 15 sec), annealing (56^{o}C for 30 s) and extension (68^{o}C for 8 min). Final extension was carried out at 68^{o}C for 15 min. The PCR product was characterized by gel electrophoresis on 1 % agarose gel with pre-stained ethidium bromide (10 mg/ml) in 0.5 M Tris–EDTA electrophoresis buffer.

The DNA fragment of interest was excised from the gel and extracted from the gel using QIA quick gel extraction kit (Qiagen, Germany). Purified product as well as pET21b(+) (Novagen, Madison, WI) was kept for overnight digestion with restriction enzymes NdeI and XhoI (New England Biolabs, India) to generate sticky ends. Digested DNA fragment and pET21b(+) were mixed (using 3:1 molar ratio) in the ligation reaction mix (2 µl T4 DNA ligase buffer, 1 µl T4 DNA Ligase; 30 ng of pET21b(+) and 100 ng of PCR product in 20 µl reaction mixture) and incubated at room temperature for overnight. Next day, this ligated product was used for transformation using competent *E. coli* (Stbl2, Invitrogen) cells by the classical heat shock method.

6.2.4 Confirmation of cloning of *bap* gene by colony PCR and PCR using *bap* internal primers

Recombinant colonies were randomly picked up from the plate, resuspended in 200 µl of 50 mM NaOH and boiled for 5 min, then quickly placed in ice for 2 min and 32 µl of 1 M Tris-Cl was added. Sample was centrifuged at 10000 rpm for 5 min. 2 µl of supernatant was used for PCR reaction. To carry out PCR with *bap* internal primers, a single colony was picked up from the plate and

inoculated in LB broth containing ampicillin (50 µg /ml) and incubated overnight at 30^{o}C and 150 rpm. The plasmid was extracted using Qiagen Plasmid Miniprep kit following manufacturer's protocol. Extracted plasmid was used as template to carry out PCR reactions.

A primer pair, sabF and sabR (Table 6.2) were used to detect presence of gene from the published sequence of *bap* of *S. aureus* V329 (Vautor et al. 2008). Amplification was carried out with a reaction mixture of 50 µl which contained 1 µl of template DNA, 25 µl of 2x Dynazyme PCR master mix (Finnzymes) and 50 pmol of each of forward and reverse primer. The final volume was made up with nuclease-free water. The PCR programme included initial denaturation at 94^{o}C for 5 min followed by 35 cycles of denaturation (94^{o}C for 1 min), annealing (52^{o}C for 30 s) and extension (72^{o}C for 1 min 30 sec). Final extension was carried out at 72^{o}C for 5 min. The PCR product was characterized by gel electrophoresis on 1% agarose gel with pre-stained ethidium bromide (5 mg/ml) in 0.5 M TAE electrophoresis buffer. A GeneRuler^{TM} DNA ladder mix (Fermentas, Germany) was used as molecular weight marker.

6.2.5 Confirmation of *bap* gene release from vector by double digestion assay

The gene was released from the cloned plasmid vector by double digestion using restriction enzymes (XhoI and NdeI). The digested samples were analyzed by 1% agarose gel electrophoresis.

6.2.6 Nucleotide sequencing of the gene and double digestion with restriction enzymes

The entire open reading frame of cloned *bap* gene in pET21b(+) vector was fully sequenced to exclude polymerase errors and inadvertent mutations. A total

of 11 primers were designed by using an online programme by Genscript, each targeting 600 bp of the *bap* gene (Table 6.3).

Table 6.3: Primers used for full *bap* gene sequencing (by primer walking).

Primers	Sequence (5'-3')	Sequencing Range	Start Site
Bap1F	GGAAATAAACAAGGTTTTTTACC	1-600 bp	1
Bap2F	TGATGATAACATTAAAGAGGACTCAAA	601-1200	552
Bap3F	AGATTCTGATGACGCATTAAATAG	1201-1800	1146
Bap4F	ACAAAAGCTAACCAATGGGC	1801-2400	1756
Bap5F	TCGTTTCTGGTTCTGTTCCTC	2401-3000	2360
Bap6F	GGTACTGCAGATGATCAAGGG	3001-3600	2950
Bap7F	ACGGCAACTGACAAAGACG	3601-4200	3547
Bap8F	GTGACGCTACTCAAGTTACAGGTC	4201-4800	4160
Bap9F	TGCTACGGGTACTGCAGATG	4801-5400	4749
Bap10F	ACGGCAACTGACAAAGACG	5401-6000	5353
Bap11F	CAACGGTGAATGATGTGACT	6001-6600	5945
Bap12F	TGATGACAACTCTGATAACGGAA	6601-6831	6552

6.2.7 Over-expression and purification the 6X-Histidine (His)-Bap fusion protein in *Escherichia coli* BL21(DE3)

Escherichia coli BL21(DE3) harbouring pET21b-*bap* was cultured in LB broth containing 1% glucose and carbenicillin (50 µg/ml) at 30^{o}C. Overnight grown cultures were diluted 1:100 into fresh 500 ml LB broth supplemented with carbenicillin (50 µl/ml) and incubated at 30^{o}C with shaking at 150 rpm to optical density (OD_{600}) 0.8. Culture of this stage was optimized for the soluble expression of Bap. After attaining 0.8 OD, 1 mM IPTG was added and the culture was incubated at 30^{o}C for 5 h at 200 rpm.

Induced cells were harvested and lysed in lysis buffer [50 mM Tris (pH 7.9), 500 mM NaCl, 1 mM protease inhibitor PMSF, and 1 mg/ml lysozyme] followed by sonication for 5 min at 4 °C. The lysate was cleared by centrifugation at 12000 rpm for 20 min at 4 °C, and the supernatant was loaded onto a Ni-NTA column pre-equilibrated with equilibration buffer [50 mM Tris-HCl (pH 7.9) and 500 mM NaCl]. The column was washed with 50 column volumes of wash buffer [50 mM Tris-HCl (pH 7.9), 500 mM NaCl, and 10 mM imidazole], and the protein was eluted out with elution buffer (50 mM Tris (pH 7.9), 500 mM NaCl, and 500 mM imidazole).

6.2.8 SDS PAGE and Western blotting

Purification and molecular weight of expressed His-tagged Bap protein was confirmed by carrying out SDS-PAGE with broad range pre-stained protein ladder having 10 – 230 kDa markers (New England Biolabs, India). The acrylamide concentration was 10% in the resolving gel and 5% in the stacking gel. Expression and purification of Bap was monitored by SDS –PAGE followed by Coomassie blue staining.

Western blotting, semi-dry electro-blotting transfer method were carried out as per following procedure. Proteins bands were transferred to polyvinylidene fluoride (PVDF) membranes using iBLOT gel transfer stacks (Life Technologies) by electro-blotting. The Non-fat-dried milk bovine blocking solution (Sigma) was used at working concentration of 3%. Membranes were washed with PBS-Tween and then incubated for 1 h at room temperature with a 1:10,000 dilution of Anti-His_6 (C-term) monoclonal mouse antibody (Life Technologies). The bound antibodies were detected by using BCIP and NBT.

6.3 Results and Discussion

Staphylococcus aureus and other coagulase-negative staphylococci are the most dominant bacteria among human implant-associated infections. In general, pathogenicity and persistence of these infections are associated with biofilm forming capability. Many surface proteins are reported to be involved in biofilm formation such as SasG (Corrigan et al. 2007), FnBPA and FnBPB (O'Neill et al. 2008), and the biofilm-associated protein, Bap (Cucarella et al. 2001). The Bap protein is encoded by a 6831 bp long *bap* gene. It is speculated that Bap family proteins could be a novel antigen for protection studies. However, very little is understood about its secondary and tertiary structural features. Over-expression and purification of Bap is a prerequisite to carry out its biophysical characterization.

This study appears to be the first report where full Bap protein was expressed with His-tag to aid in purification procedure. Long range DNA polymerase was able to amplify full 6.8 kb *bap* gene with restriction enzymes site at the ends. After double digestion with restriction enzymes it was cloned into the expression vector pTE21b upstream to 6X-His tag sequence (Figure 6.1). Recombinant plasmid (pET21b-*bap*) was transformed in to cloning host *E. coli* stbl2. Earlier, the commonly used cloning hosts were tried such as *E. coli* DH5-α and XL1-blue, however, when transformed colonies were investigated for the presence of *bap* gene, it was found that the fragment size was less than that of expected size i.e. 6.8 kb. Later it was found that such an observation was due to homologous recombination events with 13-direct tandem repeats in C-region of *bap* gene. Therefore, *E. coli* stbl 2 was used for the transformation as these cells

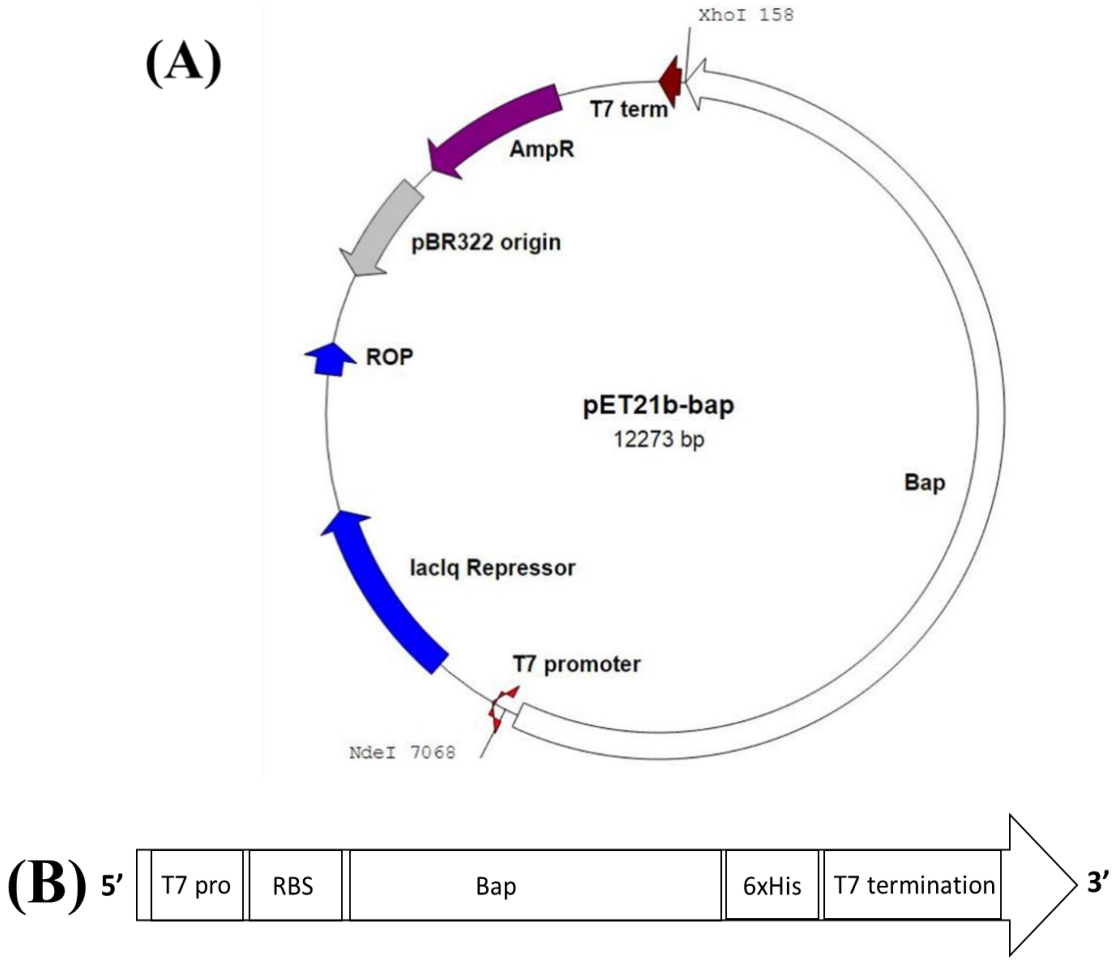


Figure 6.1: Expression plasmid pET21b(+) used for biofilm associate protein (Bap) expression. (A) Schematic representation of recombinant pET21b-*bap* expression vector. (B) Diagram shows the essential elements of the construct for *E. coli* expression. T7 promoter; ribosome binding site, *bap* gene, 6x His Tag and T7 termination element.

are suitable for the cloning of unstable inserts such as retroviral sequences or direct repeats (Singh and Singh 1995). To avoid any anomaly, cloning of full *bap* gene was confirmed in various ways. First of all, transformed colonies were checked for the presence of *bap* gene using internal primers sabF and sabR (Table 6.2) in colony PCR (Figure 6.2). Few-false positive colonies were also

overserved (lane 6 and 9), which happens to be due the self-ligation of vector plasmid. Positive colonies were used to isolate recombinant plasmid and PCR was performed with internal primers (Figure 6.3b). Insert release assay was performed to confirm the size of inserted *bap* gene (Figure 6.3c). Figure 6.3c shows that released DNA fragment was ~6.8 kb equivalent to full *bap* gene whereas undigested plasmid had a size of 12.2 kb (5.4 kb plasmid + 6.8 kb *bap* gene).

Since PCR can introduce some errors during DNA polymerization (Cha and Thilly 1993), it was necessary to investigate whether there was any unintended mutations in 6.8 kb long amplified DNA fragment. To confirm the identity of amplified *bap* gene with the *bap* gene *in situ* (*S. aureus V329*), DNA sequencing was performed with a battery of primers, each targeting 600 bp of gene (Table 6.3). Primers were designed using online programme by Genscript. DNA sequence of the cloned gene was found to be 100% identical to *bap in situ.*

For the expression of the Bap in *E. coli* BL21(DE3)-pLysS, the recombinant plasmid pET21b-*bap* was transformed into the expression host *E. coli* BL21(DE3)-pLysS. In pET expression system, cloned gene is transcribed by T7 promoter that binds specifically to T7 RNA polymerase and is not recognized by the *E. coli* RNA polymerase thereby suppresses the leaky expression. On the other hand T7 RNA polymerase gene remains under the control of the IPTG inducible *lacUV5* promoter. Background expression from pET expression plasmids is further minimized by the co-expression of T7 lysozyme (by either plasmid pLysS or pLysE), which is a natural inhibitor of T7 RNA polymerase. T7 RNA polymerase transcribes five times faster than *E. coli* RNA polymerase hence is suitable for

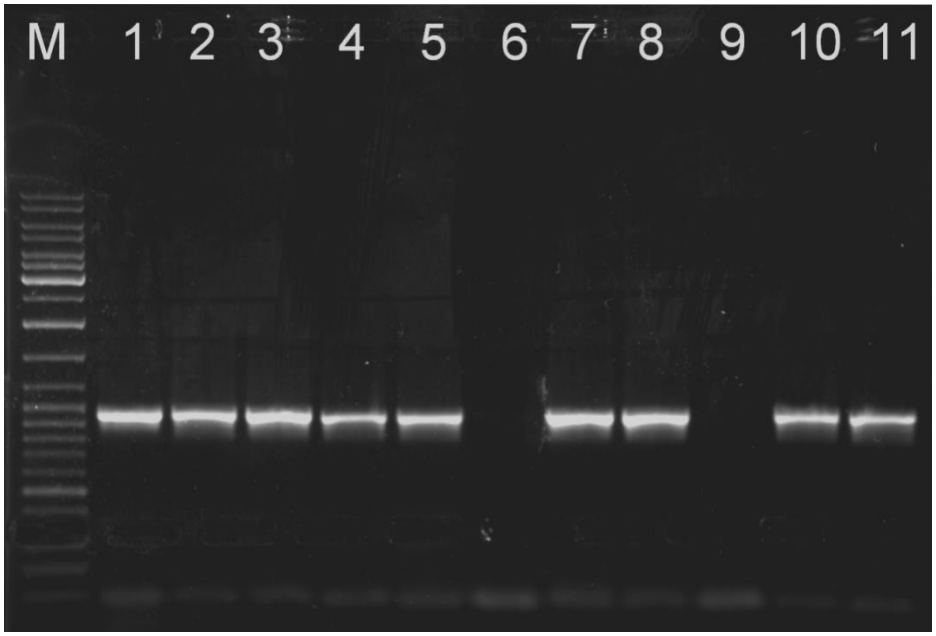


Figure 6.2: Confirmation of positive selection of transformed cells by Colony PCR. Lane M: DNA marker; Lane 1 to 11: 971 bp long PCR products amplified from cell lysate. Lane 6 and 9 were identified as false-positive colonies.

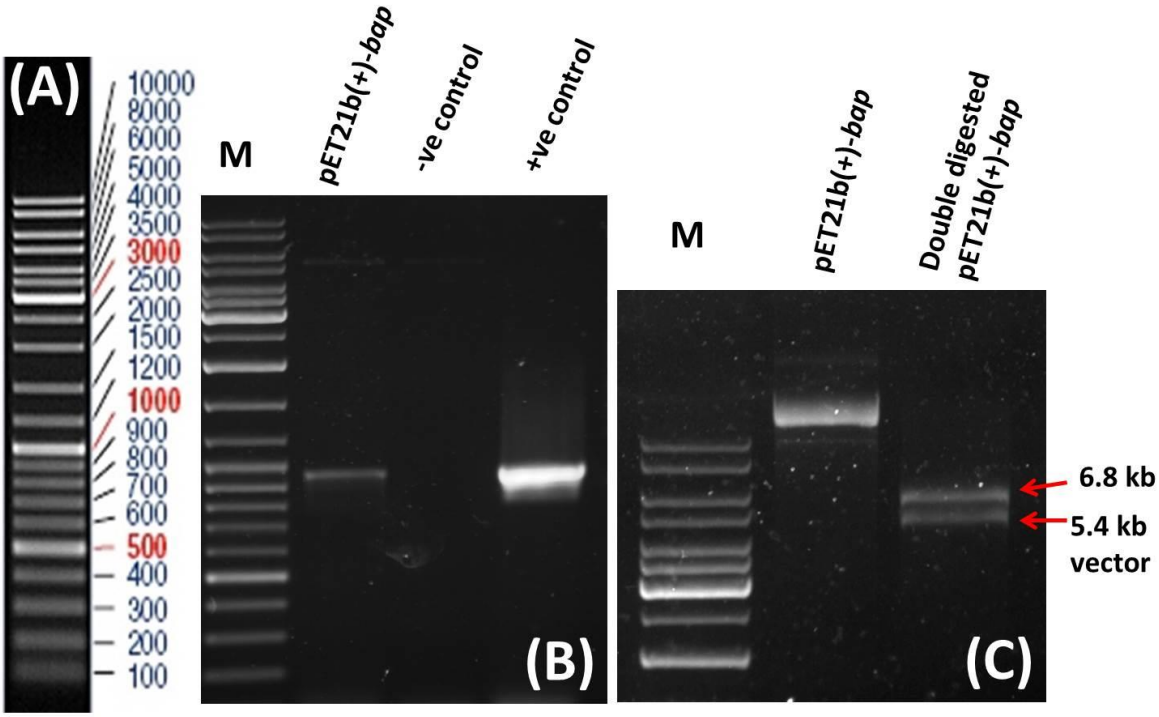


Figure 6.3:(A) DNA ladder showing size of respective bands **(B)** Detection 971 bp bap fragment: lane 1- DNA ladder; lane 2- pET-bap; lane 3- negative control i.e. empty vector pET21b(+); lane 4-+ve control i.e. genomic DNA of *S. aureus V329*. **(C)** Confirmation of cloning of *bap* by release of *bap* gene from recombinant pET21b(+)-bap expression vector by RE digestion with NdeI and XhoI. Lane M- DNA marker; Lane 2- pET21b(+)-*bap*; Lane empty expression vector pET21b(+) (5.4 kb fragment) releasing *bap* gene insert (6.8 kb fragment).

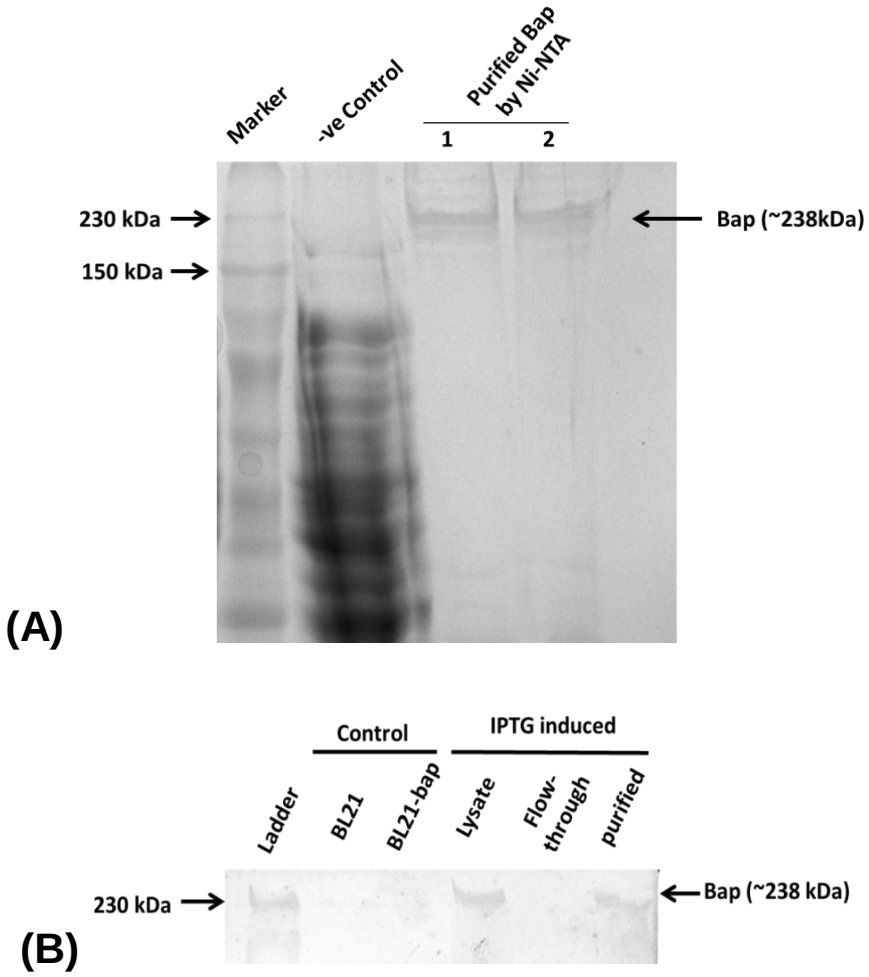


Figure 6.4: Analysis of expression, purification and identification of the Bap protein with C-terminal fused His-tag by 10% SDS–PAGE and Western blotting. (A) The SDS–PAGE analysis of pET21b-*bap* expression in BL21(DE3) induced by 1 mM IPTG. Lane 1, molecular weight marker; lane 2, uninduced *E. coli* BL21 (DE3)/pET21b-*bap*; lane 3, induced *E. coli* BL21(DE3)/pET21b-*bap*; lane 3 & 4, purified fused Bap protein with His_6 tag with Ni-NTA (B) Western blotting analysis of the Bap. Over-expressed Bap with c-terminal His_6-tag was detected in *E. coli* BL21(DE3)/pET21b-*bap* lysate and purified sample by Ni-NTA column with anti-His_6-Tag antibodies.

expressing long genes without falling off. Successful expression of full-length of Bap protein in *E. coli* BL21(DE3)-pLysS was accomplished at 30^{o}C with 1 M IPTG induction. While doing induction and expression of Bap, lower temperature (30^{o}C) was chosen to enhance the stability of *bap* gene, which was unstable due the presence of direct repeats via homologous recombination at higher temperature. Subsequently, the Bap-His_6 fusion protein was purified from *E. coli* BL21(DE3)-pLysS via its His-tag using Ni-NTA affinity chromatography under native conditions. SDS-PAGE of the affinity-purified fusion proteins revealed an approximately 238-kDa protein (Figure 6.4A), which was again confirmed by western blotting experiments using Anti-His tag antibody (Figure 6.4B). Using C-terminal His-tag has its own advantages as detection of C-terminal His-tag by anti-His_6 tag antibody in western blotting confirms that the *bap* gene was fully expressed i.e. 238-kDa as shown in Figure 6.4A and 6.4B.

Interestingly, *bap* gene is reported to be present only in some isolates of *S. aureus* from bovine mastitis (Cucarella et al. 2001, Snel et al. 2015). Of late, some *bap* harbouring *S. aureus* isolates were also reported which suggests that a slow transfer of *bap* gene among *S. aureus* strains is very much possible (Potter et al. 2009, Vautor et al. 2008). Horizontal gene transfer might spread the *bap* gene among other pathogenic *S. aureus* strains because the *bap* gene is present in Pathogenicity Island SaPIbov2, which is a mobile genetic element in *S. aureus V329* (Lasa and Penadés 2006, Latasa et al. 2006). Apart from this, studying functioning and biophysical characterization of Bap is warranted as various other surface proteins exhibit similar structural similarity, such as SasG (Corrigan et al. 2007), SasC (Schroeder et al. 2009) and accumulation-associated protein (Aap) (Hussain et al. 1997, Rohde et al. 2005). SasG is homologous to the

accumulation-associated protein Aap, which mediates biofilm accumulation in *S. epidermidis* (Rohde et al. 2005). Spreading of Bap among staphylococci and the presence of homologous proteins among other pathogens suggest the urgent need of complete characterization of such proteins. This study enables us to do a biophysical characterization study of Bap, which will further pave way to understand and develop a suitable anti-biofilm strategies against *S. aureus*.

6.4 Conclusions

Successfully cloned, over-expressed the 6.83 kb long *bap* gene in *E. coli*. A large amount of soluble Bap after NI-NTA purification was obtained. The procedures of expression and purification of Bap in this study could be used to produce large amount of protein for further biophysical characterization.

References

Cha RS, Thilly WG. 1993. Specificity, efficiency, and fidelity of PCR. Genome Res.3 :18-29.

Cloneamp B. Propagation of plasmid containing an unstable insert of potato virus yo using stbl2™ competent cells. Focus.1:7.

Corrigan RM, Rigby D, Handley P, Foster TJ. 2007. The role of *Staphylococcus aureus* surface protein SasG in adherence and biofilm formation. Microbiology.153:2435-2446.

Cucarella C, Solano C, Valle J, Amorena B, Lasa I, Penades JR. 2001. Bap, a *Staphylococcus aureus* surface protein involved in biofilm formation. J Bacteriol. 183:2888-2896.

Hinsa SM, Espinosa-Urgel M, Ramos JL, O'Toole GA. 2003. Transition from reversible to irreversible attachment during biofilm formation by

Pseudomonas fluorescens WCS365 requires an ABC transporter and a large secreted protein. Mol Microbiol. 49:905-918.

Hussain M, Herrmann M, von Eiff C, Perdreau-Remington F, Peters G. 1997. A 140-kilodalton extracellular protein is essential for the accumulation of *Staphylococcus epidermidis* strains on surfaces. Infect Immun. 65:519-524.

Lasa I, Penadés JR. 2006. Bap: a family of surface proteins involved in biofilm formation. Res Microbiol. 157:99-107.

Latasa C, Roux A, Toledo-Arana A, Ghigo JM, Gamazo C, Penadés JR, Lasa I. 2005. BapA, a large secreted protein required for biofilm formation and host colonization of *Salmonella enterica* serovar Enteritidis. Mol Microbiol. 58:1322-1339.

Latasa C, Solano C, Penadés JR, Lasa I. 2006. Biofilm-associated proteins. C R Biol. 329:849-857.

O'Neill E, Pozzi C, Houston P, Humphreys H, Robinson DA, Loughman A, Foster TJ, O'Gara JP. 2008. A novel *Staphylococcus aureus* biofilm phenotype mediated by the fibronectin-binding proteins, FnBPA and FnBPB. J Bacteriol. 190:3835-3850.

Potter A, Ceotto H, Giambiagi-Demarval M, dos Santos KR, Nes IF, Bastos Mdo C. 2009. The gene bap, involved in biofilm production, is present in *Staphylococcus* spp. strains from nosocomial infections. J Microbiol. 47:319-326.

Rohde H, Burdelski C, Bartscht K, Hussain M, Buck F, Horstkotte MA, Knobloch JKM, Heilmann C, Herrmann M, Mack D. 2005. Induction of *Staphylococcus epidermidis* biofilm formation via proteolytic processing of the accumulation-

associated protein by staphylococcal and host proteases. Mol Microbiol. 55:1883-1895.

Schroeder K, Jularic M, Horsburgh SM, Hirschhausen N, Neumann C, Bertling A, Schulte A, Foster S, Kehrel BE, Peters G. 2009. Molecular characterization of a novel *Staphylococcus aureus* surface protein (SasC) involved in cell aggregation and biofilm accumulation. PloS one.4:e7567.

Shukla SK, Rao TS. 2013a. Dispersal of Bap-mediated *Staphylococcus aureus* biofilm by proteinase K. J Antibiot. 66:55-60.

Shukla SK, Rao TS. 2013b. Effect of calcium on *Staphylococcus aureus* biofilm architecture: A confocal laser scanning microscopic study. Colloids Surf B. 103:448-454.

Shukla SK, Rao TS. 2014. Calcium-mediated modulation of Staphylococcal bacterial biofilms. Ind J Mar Sci. 43.

Singh M, Singh R. 1995. Propagation of plasmid containing an unstable insert of potato virus YO using Stbl-2 competent cells. Focus.17:72-73.

Snel GG, Monecke S, Ehricht R, Piccinini R. 2015. Molecular characteristics of *bap*-positive *Staphylococcus aureus* strains from dairy cow mastitis. J Dairy Res.1-5.

Solano C, García B, Valle J, Berasain C, Ghigo JM, Gamazo C, Lasa I. 2002. Genetic analysis of *Salmonella enteritidis* biofilm formation: critical role of cellulose. Mol Microbiol. 43:793-808.

Spoering AL, Lewis K. 2001. Biofilms and planktonic cells of *Pseudomonas aeruginosa* have similar resistance to killing by antimicrobials. J Bacteriol. 183:6746-6751.

Toledo-Arana A, Valle J, Solano C, Arrizubieta MaJ, Cucarella C, Lamata M, Amorena B, Leiva J, Penadés JR, Lasa I. 2001. The enterococcal surface protein, *Esp*, is involved in *Enterococcus faecalis* biofilm formation. Appl Environ Microbiol. 67:4538-4545.

Tormo MA, Knecht E, Gotz F, Lasa I, Penades JR. 2005. Bap-dependent biofilm formation by pathogenic species of *Staphylococcus*: evidence of horizontal gene transfer? Microbiology. 151:2465-2475.

Vautor E, Abadie G, Pont A, Thiery R. 2008. Evaluation of the presence of the bap gene in *Staphylococcus aureus* isolates recovered from human and animals species. Vet Microbiol. 18:127:407

CHAPTER 7 : Conclusions, Summary and Future Work

7.1 Conclusions

The work described in this thesis has touched various aspects of *S. aureus* biofilm as far as biofilm mediated proteins are concerned. *Staphylococcus aureus* is a universal pathogen which causes mild to severely life threatening diseases and constitutes one of the leading causes of hospital-acquired/healthcare-associated infections. With time there is growing knowledge about *ica*-independent *S. aureus* biofilm development which is predominantly mediated by biofilm-associated surface proteins (Bap, Aap, FnBPs, etc.,). Recent studies showed the higher frequencies of *bap* gene presence in coagulase-negative *Staphylococcus* strains isolated from the nosocomial infections. This has increased the urgency to develop fundamental understanding upon protein based *S. aureus* biofilm development. Studies in this thesis establish the fact that cell surface proteins play an important role in biofilm development. Few molecular aspects of understanding of adhesion and biofilm phenomenon helped in developing anti-biofilm control strategies *in vitro*. Major conclusion of the thesis are summarized as below:

- Bioinformatics analysis showed a conserved hepta-peptide in many BAP family proteins and identified a putative antigenic region in Bap proteins.
- Non antibiotic factors such as high concentration of Ca^{2+} and proteases can be of use in controlling Bap-mediated biofilms development.
- Dominant role of surface proteins in biofilm establishment suggested that Proteinase K can be used in biofilm dispersion as well as for anti-biofilm activity, this can have implications for food industry.
- Moreover treatment of Proteinase K also enhanced the antimicrobials efficiency against *S. aureus* biofilms.

- Cloning and expression of full *bap* gene in *E. coli* will enable to carry out further biophysical characterization of the Bap protein.

7.2 Summary of the thesis

In Chapter 1, a brief introduction of cell surface proteins and biofilm associated proteins in *S. aureus* was described. CWA associated proteins plays a critical role in initial phase and later phase of biofilm development. Attachment of staphylococci to an abiotic surface is dependent on microbial surface components such as the biofilm associated proteins, accumulation-associated protein, autolysins, wall teichoic and lipoteichoic acids. Role of many CWA proțeins including clumping factors A and B and the fibrinogen-binding proteins FnBPA and FnBPB in *S. aureus* in primary attachment to a biotic surface and synthetic surfaces coated with plasma proteins, such as fibronectin, fibrinogen etc., was discussed. A new classification of CWA proteins in *S. aureus* was also described. A brief introduction and review of literature on current understanding on biofilm-associated protein in *S. aureus* was also presented.

In Chapter 2 the outcome of *in silico* analysis of biofilm associated protein that was performed to identify functional and conserved regions in BAP proteins as well as to identify potential antibody-triggering regions in Bap protein was presented. Bioinformatics studies, as describe in this chapter, established that Bap like proteins in staphylococci are highly acidic, large and cell-wall anchored proteins with tandem repeats. Structurally they have two distinct parts; N-terminal part, which contains at least 2-3 calcium-binding EF-hand domains, might play a regulatory role in Bap functioning. Whereas C-terminal part which predominantly consist of tandem repeats plays functional as well as the structural role. An

amyloidogenic heptapeptide (STVTVTF) was found to be conserved in tandem repeats and predicted to participate in domain-domain interaction via hydrophobic forces. Homology modelling studies shows that TR domains show the highest homology with giant Ca^{2+} binding adhesin SiiE in *Salmonella enteric* followed by an immunoglobulin-like structure in ice-binding adhesins of *Marinomonas primoryensis.* Amyloidogenic heptapeptide was found to be present on the β strand. An amino acid sequence (DVTSDATQVTGQAEPNST) in tandem repeats that forms a random coil in immunoglobulin-like structure, was predicted to be potential antigenic region.

The significant occurrence of Bap in bovine mastitis *S. aureus* isolates and the putative presence of Ca^{2+} binding EF-hand motifs in Bap indicated that Ca^{2+} might have a role in *bap*- mediated regulation *S. aureus* biofilm development. Chapter 3 describes the effect of Ca^{2+} on biofilm architecture in *bap*-positive, *bap*-negative, and *bap*-isogenic mutant of *S. aureus* bovine isolates. Biofilm assay showed that Ca^{2+} inhibited the biofilm growth of *bap*-positive V329 and *bap*-negative SA10 and in a dose-dependent manner whereas other *S. aureus* strains did not show any inhibition, however, biofilm inhibition in SA10 was found to be due to planktonic growth inhibition at ≥ 5 mM Ca^{2+}. Studies with increasing NaCl concentration and Mg^{2+} suggested that the interaction of Bap and Ca^{2+} is specific. CSLM studies at various concentration of Ca^{2+} on Bap positive *S. aureus* V329 showed a significant change (positively or negatively) in all biofilm parameters at ≥ 5 mM Ca^{2+}. The inhibition effect of Ca^{2+} on V329 and SA10 biofilms disappeared in the presence of chelating agent EDTA, which confirmed that biofilm inhibition was due to the presence of Ca^{2+}.

With increasing number of reports on protein-based staphylococcal biofilms, it is speculated that protease based dispersion method would be highly effective. In Chapter 4 studies showed that Proteinase K can emulate the naturally produced proteases and can be used to enhance the biofilm dispersal through cleavage of surface proteins i.e. Bap- dependent *S. aureus* biofilm. Results using Proteinase K to inhibit and to disperse preformed *S. aureus* biofilm indicated this approach would be useful in general and has wider applicability. However, it was also shown that Proteinase K did not affect the biofilm growth of *bap*-mutant M556 and SA392 strain. It was also shown that binding of Ca^{2+} to Bap did not render any protection against proteolytic activity of Proteinase K. Biofilm composition studies before and after Proteinase K treatment indicated that Bap might also be involved in eDNA retention in biofilm matrix that aid in biofilm stability. Generally, biofilm forms a physical barrier, limiting diffusion of antibiotics and thereby reducing its efficacy. A synergistic effect in antibiotic efficacy against *S. aureus* biofilm was observed when used with Proteinase K. CSLM studies showed surface to biovolume ratio and roughness coefficient significantly enhanced after proteinase K treatments which indicates that biofilm surface was more exposed for antibiotics action.

In Chapter 5 the observations on effect of c-di-GMP on *S. aureus* biofilm and cell morphology were described. The effect of c-di-GMP on Bap–mediated intercellular adhesion and biofilm formation in all available *S. aureus* strains was investigated. Results indicated that c-di-GMP apparently had no significant effect on *S. aureus* biofilm development as well as planktonic cells in all the strains tested. Interestingly, in this study, it was observed that c-di-GMP treated *S. aureus* cells were smaller in size as compared to their counterparts. Recent reports have

revealed that c-di-AMP plays important role in biofilm formation instead of c-di-GMP, hence no further experiments were performed in this line.

In Chapter 6, cloning work of *bap* in *E. coli*, its over-expression, and purification of His-tagged Bap protein was described**.** The two specific aims of this study were i) to clone full length bap long gene (~6.8 Kb) in *E. coli*; ii) the heterologous expression of the Bap protein to facilitate *in vitro* study of the Bap protein. The full *bap* gene (~6.8 kb) was amplified using long range PCR kit and cloned in expression vector pET21b(+) in *E. coli* stbl2 and in BL21 (pLysS) for its over-expression. DNA sequencing of the cloned gene confirms 100% identity with *bap* gene of *S. aureus* V329 *in-situ*. Successful expression of full length of Bap protein in *E. coli* BL21 (pLysS) was confirmed by the SDS-PAGE and Western blotting using Anti-His tag antibody. Sequence of expressing full Bap protein was confirmed using MALDI-TOF and found to be identical to Bap *in vivo*.

7.3 Scope for future work:

7.3.1 Testing anti-biofilm peptide

An amyloidogenic peptide, -STVTVTF- in C-repeat of the Bap from *S. epidermidis* was reported to be responsible for protein-protein interaction (Lembré et al. 2014). During bioinformatic analysis in Chapter 2, it was found to be conserved in all the biofilm-associated proteins studied so far. Therefore, it is hypothesized that a synthetic peptide having similar sequence -STVTVTF- may be tested. This can mask the amyloidogenic peptide motif in Bap proteins and interfere with its normal functioning. This hypothesis needs to be tested and validated.

7.3.2 Anti-Bap antibodies for various applications

During the bioinformatics studies as discussed in Chapter 2, an antigenic region, “DVTSDATQVTGQAEPNST”, was predicted in the primary sequence of tandem repeats which can be tested for developing a vaccine or antibody based detection kits for detection of Bap/Bap like-proteins harbouring *S. aureus* strains.

7.3.3 Biofilm inhibition in *S. aureus* strain SA10

In Chapter 3 while studying Ca^{2+} mediated biofilm inhibition in V329 it was understood that it is modulation of Bap functioning. Among the *bap* negative strains the significant variation in SA10 biofilm growth in the presence Ca^{2+} was observed in two different media. In TSB supplemented with glucose SA10 showed significant inhibition in the presence of Ca^{2+}, however, when similar experiment was conducted in the TRIS-G minimal medium it did not form a good biofilm as in TSB-G medium which is a rich medium. Moreover the effect of Ca^{2+} mediated biofilm inhibition was also not observed. This observation remain unexplained and needs further investigations to understand the basic mechanism behind such observation.

7.3.4 Application of Proteinase K based treatment regime

Recent studies as well as this thesis work (Chapter 4), on enzyme based *S. aureus* biofilm disruption has emerged as a promising strategy to combat biofilm-related persistent infections, since enzyme based antibiotic treatment enhances the antibiotic sensitivity of microbial biofilm (Nguyen and Burrows 2014). Among such enzymes DNase I, Dispersin B and Proteinase K are commercially produced. Dispersal mechanisms using such enzymes could be utilized in the prevention of biofilm formation which are particularly associated with

implanted medical devices (Svensäter et al. 1997). Several studies have found that pre-treatment of polymeric surfaces (Mah and O'Toole 2001) or local delivery of dispersal agents from the implanted device (Rasmussen 2000) should prevent biofilm development. While these approaches sound promising, there are several concerns that need to be thoroughly addressed before clinical trials. For example, if the antibiotic treatment along with the Proteinase K fails to fully eradicate the dispersed microbial cells, it might result in acute infections. Thus, more studies are to be performed to confirm dispersal mechanisms in relevant animal models, before treating *S. aureus* biofilm infections in clinical set ups. Apart from this, there is a need to study the effect of a mixture of degradative enzymes on various components of biofilm matrix.

7.3.5 Biophysical characterization of purified Bap proteins

As described in Chapter 6, the objective of the cloning and over expressing of Bap gene was to isolate and purify full length Bap protein in controlled manner under inducible promoter. Having optimized the condition for over-expression and purification of Bap protein from *E. coli*, further biophysical characterization, such as secondary structure analysis by circular dichroism and tertiary structure studies with X-ray crystallography or NMR (nuclear magnetic resonance) will be a valuable a contribution.

References

Lembré P, Vendrely C, Di Martino P. 2014. Identification of an amyloidogenic peptide from the Bap protein of *Staphylococcus epidermidis*. Protein Peptide lett. 21:75-79.

Mah T-FC, O'Toole GA. 2001. Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol. 9:34-39.

Nguyen UT, Burrows LL. 2014. DNase I and proteinase K impair *Listeria monocytogenes* biofilm formation and induce dispersal of pre-existing biofilms. Int J Food Microbiol. 187:26-32.

Rasmussen B. 2000. Filamentous microfossils in a 3,235-million-year-old volcanogenic massive sulphide deposit. Nature. 405:676-679.

Svensäter G, Larsson UB, Greif E, Cvitkovitch D, Hamilton I. 1997. Acid tolerance response and survival by oral bacteria. Oral Microbiol Immun. 12:266-273.

LIST OF PUBLICATIONS, CONFERENCES AND WORKSHOPS

JOURNAL PUBLICATIONS

Accepted

[1]. **Sudhir K. Shukla,** T. Subba Rao. Effect of Calcium on *Staphylococcus aureus*biofilm architecture: A Confocal Laser Scanning Microscopic Study. Colloid Surf B: Biointerfaces 2013; 103:448-454.

[2]. **Sudhir K. Shukla,** T. Subba Rao. Dispersal of Bap-mediated *Staphylococcus aureus* biofilm by proteinase K. The Journal of Antibiotics 02/2013; 66(2):55-60.

[3]. **Sudhir K. Shukla,** T. Subba Rao. Calcium-Mediated Modulation of Staphylococcal Bacterial Biofilms. Indian Journal of Geo-Marine Sciences 11/2014; 43(11):2107-2116.

[4]. **Sudhir K. Shukla,** T. Subba Rao. Biofilm removal by targeting biofilm-associated extracellular proteins in bovine mastitis *Staphylococcus aureus* isolates. Indian Journal of Medical Research (accepted).

Communicated

[1]. **Sudhir K. Shukla,** T. Subba Rao*. In silico* Molecular Characterization of Biofilm-Associated-Proteins by Homology Modelling to Identify Antigenic Regions of Bap. (Under Review PLoS One).

[2]. **Sudhir K. Shukla,** T. Subba Rao. Heterologous Expression and purification of a 238 kDa large Biofilm Associated Surface Protein (Bap) in *Escherichia coli*. Protein Expression and Purification. (Communicated).

[3]. **Sudhir K. Shukla**, T. Subba Rao. An Improved Crystal Violet Assay for Biofilm Quantification in 96-Well Microtitre Plate. Journal of Microbiological Methods. (Communicated).

Oral Presentations:

[1]. **Sudhir K. Shukla,** T. Subba Rao. *Calcium-Mediated Modulation of Staphylococcal Bacterial Biofilms*. International Conference on Advanced Technologies for Management of Ballast Water and Biofouling (MABB-2014), Chennai, India, 4-7 March 2014.

Posters:

[1]. **Sudhir K. Shukla,** T. Subba Rao. Designing of an anti-biofilm peptide against Bap protein expressing *Staphylococcus aureus* biofilms. International Conference on Medicinal Chemistry, MEDCHEM, 10-11, Sept 2015.

[2]. **Sudhir K. Shukla,** T. Subba Rao. *Effect of Proteinase K on Staphylococcus aureus Biofilms*. International Conference on Advanced Technologies for Management of Ballast Water and Biofouling (MABB-2014), Chennai, India, 4-7 March 2014.

[3]. **Sudhir K. Shukla,** T. Subba Rao. *Antimicrobial activity of N-acetyl cysteine against biofilm forming bacteria*. International Conference on "Microbial World: Recent Innovations and Future Trends", 22-25, November 2012.

WORK SHOP ATTENDED

Hands – on workshop on Biophysical and Biotechnological Research Techniques for Scientist and Scholars. AU-KBC Research Centre, Life Sciences Division, Anna University, Chennai, India, 25-27 July 2011.

APPENDICES

8.1 Reagents

0.8% Agarose

Agarose	0.8 g
Distilled water	100 ml

(For 1% add 1 g of agarose in 100 mL)

10% Ammonium persulfate

Ammonium persulfate	0.1 g
Distilled water	1.0 ml

(Store at -20 ºC about a week)

5X Bradford's reagent

Coomassie Brilliant blue G250	50 mg
95% ethanol	25 ml
85% Phosphoric acid	50 ml
Distilled water	25 ml

(For assays dilute to 1X and use)

0.2% Crystal violet

Crystal violet	0.2 g
Distilled water	100 ml

70% Ethanol

Absolute ethanol	70 ml
Distilled water	30 ml

(Similarly for 90 and 96% ethanol use 90 mL and 96 mL of absolute ethanol respectively and make up the volume to 100 mL)

2X Gel loading dye

0.05% Bromophenol blue	0.25 ml (from 2% stock)
0.05% Xylene cyanol	0.25 ml (from 2% stock)

70% Glycerol	7 ml (from 100% stock)
Distilled water	2.5 ml

20% Glucose

Glucose	20 g
Distilled water	100 ml

50% Glycerol

Glycerol	50 ml
Distilled water	50 ml

0.88 % NaCl

Sodium chloride	0.88 g
Distilled water	100 ml

1X PBS

Potassium dihydrogen orthophosphate	0.2 g
Potassium chloride	0.2 g
Disodium hydrogen orthophosphate	1.15 g
Sodium chloride	8.0 g
Distilled water	1000 ml

5% Phenol

Phenol	5 g
Distilled water	100 ml

0.85% saline

Sodium chloride	0.85 g
Distilled Water	100 ml

50X TAE

Tris base	242 g
Acetic acid, glacial	57.1 ml
0.5 M EDTA, pH 8.0	100 ml
Distilled water	to 1000 ml

(To prepare 1X TAE, dilute 1 ml of 50X TAE to 50 ml with distilled water)

Acrylamide / Bisacrylamide (37.5 : 1)

Acrylamide	38.93 g
Bisacrylamide	1.07 g
Distilled water	to 100 ml

(Filter through a 0.45 µm filter membrane and store at 4 ºC)

BSA (2 mg/ml)

Bovine Serum Albumin	2 mg
Distilled water	1 ml

Cell lysis solution (per ml)

20 mM Tris-Cl	20 µl (from 1M stock)
2 mM EDTA	4 µl (from 0.5 M stock)
1.2% Triton-X 100	12 µl
Lysozyme	20 mg
Distilled water	964 µl

Ethidium bromide

Ethidium bromide	10 mg
Distilled water	10 ml

Hexamine/hydrochloric acid solution

Hexamine	30 g
50% Hydrochloric acid	1000 ml

SDS detergent solution

SDS	6 g
Distilled water	1000 ml

8.3 Culture Media

Triptic Soy Broth medium

Tryptic Soy Broth powder	30.0 g/L
Distilled water	1000 ml
Agar-Agar	15 g/L

Final pH (at 25 ºC) 7.3 ± 0.2

Boil to dissolve the medium completely and sterilize by autoclaving at 15 lbs pressure (121 ºC) for 20 minutes. For plates add 15 g/L agar and sterilize.

Tris minimal medium

A) Tris 17

Tris base, 0.5 M	60.57 g/L
Sodium chloride, 0.8 M	46.75 g/L
Ammonium chloride, 0.2 M	10.69 g/L
Sodium sulphate, 0.03 M	4.26 g/L
Potassium chloride, 0.2 M	14.91 g/L
Calcium chloride, 0.002 M	0.29 g/L
Magnesium chloride, 0.01 M	2.03 g/L

B) Tris 8

Sodium dihydrogen phosphate, 1%	1g/100 ml

C) Trace elements

Zinc sulphate hexahydrate, 5 mM	143.77 mg/L
Manganese chloride tetrahydrate, 5 mM	98.96 mg/L
Cobalt chloride tetrahydrate, 8 mM	190.34 mg/L
Copper chloride dihydrate, 1 mM	17.05 mg/L
Nickel chloride hexahydrate, 1 mM	23.77 mg/L
Sodium moybdate dihydrate, 1.5 mM	26.29 mg/L
Boric acid, 10 mM	61.83 mg/L
Ferrous sulphate	in trace

Mix 100 ml of Tris 17 solution, 4 ml of Tris 8 solution and 800 ml of distilled water. Adjust to a final pH of 7.2 ± 0.2 (at 25 ºC). Make up the volume to 1000 ml with distilled water, sterilize by autoclaving at 15 lbs pressure (121 ºC) for 20 minutes. At the time of use add 100 µl/L of filter sterilized trace elements and the required amount of glucose from a 20% stock solution.