Functional analysis of the accumulation associated protein (Aap) of *Staphylococcus epidermidis*

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Abstract

S. epidermidis is one of the primary opportunistic pathogens associated with indwelling medical devices such as intravenous catheters and artificial heart valves and joints. *S. epidermidis* is also a permanent commensal resident on human skin and mucus membranes providing a large potential reservoir for the contamination of medical implants. Persistent colonisation of implants occurs via biofilm formation and infected implants must usually be replaced. The surface-bound protein, accumulation associated protein (Aap), is one of the main biofilm promoting surface molecules on *S. epidermidis*. Aap is a LPXTG protein with a repetitive B-region, thought to promote biofilm formation as well as providing a stalk structure to project the A-domain away from the cell surface. Aap is expressed in lateral tufts of fibrils on the surface of a sub-population of strain NCTC 11047 and, here, similar sub-populations are shown to be present in other *S. epidermidis* strains. In order to determine the function of specific domains of Aap in adhesion and biofilm formation Aap constructs with and without the A-domain and with varying numbers of B-repeats were expressed on the surface of *Lactococcus lactis* MG1363 and *Staphylococcus aureus*. The expression of Aap with the A-domain on the surface of *L. lactis* increased corneocyte adhesion 20-fold compared to *L. lactis* carrying Aap without an A domain. Several *S. epidermidis* isolates also used the A-domain of Aap to adhere to corneocytes, emphasizing the role of Aap in skin adhesion. In addition, Aap promoted adhesion to polystyrene although only the A-domain additionally promoted adhesion to tissue culture treated polystyrene. Furthermore, biofilms were cultivated under flow conditions and analyzed by confocal microscopy. Aap, with the A-domain on the surface of both *L. lactis* and *S. aureus*, enhanced microcolony formation suggesting a potential role for the A-domain in the early stage of biofilm formation. The A-domain of Aap is therefore multifunctional because, in addition to mediating adhesion to corneocytes it can promote initial attachment to polystyrene and functions in the early accumulation stage of biofilm formation.

Declaration

With the exception of the results presented in figure 3.2 of chapter 3 of this thesis none of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Copyright statement

Thesis organisation and structure

The results presented in this thesis are written in the style of the international peer reviewed *Journal of Bacteriology*. Therefore, the results chapters in this thesis can be viewed as standalone works. However, the reference style, page numbers and figure numbers have been modified to conserve continuity between chapters. More detailed methodology is described in Chapter 2 and a single reference list has been compiled at the end of this thesis.

The results chapters have been accepted or will be submitted for publication in the *Journal of Bacteriology* as follows:

Chapter 3:
Robin L. Macintosh, Jane L. Brittan, Ritwika Bhattacharya, Howard F. Jenkinson, Jeremy Derrick, Mathew Upton, Pauline S. Handley. 2009. The terminal A-domain of the fibrillar accumulation associated protein (Aap) of Staphylococcus epidermidis mediates adhesion to human corneocytes. (Accepted)

Chapter 4:
Robin L. Macintosh, Jeremy Derrick, Pauline S. Handley, Mathew Upton. 2010. Heterologous expression of the accumulation associated protein (Aap) of *Staphylococcus epidermidis* predicts a role for the A-domain in adhesion and early biofilm formation. (To be submitted in September 2010)

Authors Jane Brittan and Howard Jenkinson were involved with the construction of protein expression plasmids as detailed in Chapter 2 (section 2.5.1) of this thesis. Ritwika Bhattacharya generated the results for figure 3.2 as part of a Master of Science degree under the supervision of Dr Pauline Handley. Jeremy Derrick, Mathew Upton and Pauline Handley were supervisors of this project.

Except where stated all practical studies and written text is the work of Robin L. Macintosh.

List of abbreviations

a.a	Amino acid
Aap	Accumulation Associated Protein
agr	Accessory gene regulator locus
AMP	Antimicrobial peptide
AtlE	The major autolysin of *S. epidermidis*
Bap	Biofilm associated protein (*S. aureus*)
Bhp	Bap homologous protein (*S. epidermidis*)
BHY	Brain heart infusion with 0.5 % yeast extract
ClfA	Clumping factor A (*S. aureus*)
b.p.	Base pair
HI	Heart infusion media
BSA	Bovine serum albumin
CoNS	Coagulase negative staphylococci
c.v.	Column Volumes
CV	Crystal violet
$_{dd}H_2O$	Double-distilled water
DNA	Deoxyribonucleic acid
Embp	Extracellular matrix binding protein
Fbe	Fibrinogen binding protein
FBS	Foetal bovine serum
Fg	Fibrinogen
Fib^+	Cell population with Aap fibrils
Fib^-	Cell population without Aap fibrils
FIVAR	Found in various architecture domain
Fn	Fibronectin
FnBPA & FnBPB	Fibronectin Binding Protein A & B (of *S. aureus*)
GM17	M17 media supplemented with 0.5 % glucose
His_6	6 x Histidine residues

LB	Luria Bertani broth
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LTA	Lipo-teichoic acid
NAG	N-acetyl glucosamine
NAM	N-acetyl muramic acid
MRSA	Methicillin resistant *S. aureus*
MSCRAMMs	Microbial surface components recognizing adhesive matrix molecules
MT	Methylamine tungstate
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBP	Penicillin-binding protein
PBS	Phosphate buffer saline
PBS-gel	PBS with 0.02 % gelatine
PBS-NGS	PBS-gel with 2 % normal goat serum
PCR	Polymerase chain reaction
PGA	Poly-γ-glutamic acid
PIA	Polysaccharide intercellular adhesin
Pls	Plasmin sensitive surface protein of *S. aureus*
Psl	Polysaccharide component of *P. aeruginosa* biofilm matrix
PMSF	Phenylmethylsulphonylfluoride
PS/A	Capsular polysaccharide adhesin
PSM	Phenol soluble modulin
rAap	Recombinant Aap
$rAap_{A\text{-}dom}$	Recombinant Aap A-region
$rAap_{A\text{-}rep}$	Recombinant Aap A-region short repeats
$rAap_{B\text{-}rep}$	Recombinant Aap B-repeat
$rAap_{6.5B\text{-}rep}$	Recombinant Aap B-region with 6.5 B-repeats
rpm	Revolutions per minute
rtPCR	Reverse transcription PCR

SasG	*Staphylococcus aureus* surface protein G
Sdr	Serine-aspartate dipeptide repeats
SDS	Sodium dodecyl sulphate
Ses	*Staphylococcus epidermidis* surface protein
SSP	Staphylococcal surface protein
TA	Teichoic acid
TEM	Transmission electron microscopy
TSB	Tryptone soya broth
WT	Wild type population
WTA	Cell wall teichoic acids

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Finally, I would not have had the oportunity to start, let alone complete, this PhD without the wisdom, love and support provided by my Mum and Dad and Casi.

Chapter 1:

General Introduction

1.1 *Staphylococcus epidermidis*

S. epidermidis is an increasingly prevalent cause of blood stream infections due to colonisation of indwelling medical devices (Rupp and Archer, 1994) but it is primarily a commensal resident on human skin. The bacterium is able to survive in two distinct niches: human skin and the surface of medical devices inside the host. The focus of this study was to determine the role that the cell surface accumulation associated protein (Aap), plays in adhesion of *S. epidermidis* in these two niches.

1.1.1 *S. epidermidis* and the skin microflora

S. epidermidis is the most frequently isolated skin bacterium and is found most commonly on the head and axillae and in the nares (Kloos and Musselwhite, 1975) although it is also a transient member of the oral microflora (Jackson *et al.*, 1999). Other staphylococci are frequently isolated from the human skin and coagulase-negative staphylococci (CoNS), specifically *S. epidermidis*, *S. haemolyticus* and *S. hominis*, are the most common. On a healthy adult there are between 10 and 10^5 colony forming units of CoNS per cm^2 of skin (Kloos, 1980) and approximately 40 species of CoNS share the skin environment with a plethora of other microorganisms (Roth and James, 1988). Besides staphylococci, commonly isolated and culturable bacteria include *Corynebacterium* spp. and *Propionibacterium* spp. Interestingly the latter is an obligate anaerobe or microaerophile and exists mainly in the hair follicles where local oxygen concentrations are thought to be very low due to oxygen consumption by the skin and facultative anaerobes . Most skin bacteria, however, cannot be cultured and a recent metagenomic study showed the true diversity of bacterial species present on the skin (Gao *et al.*, 2007). Skin samples from six healthy adults contained 91 different bacterial genera although 68 % of these genera were found on only one adult indicating the high level of interpersonal variation in skin microflora. The study also indicated the transient nature of most skin colonisers as only staphylococci, corynebacteria,

propionibacteria and streptococci were consistently found in multiple skin samples from the same individuals taken months apart.

The skin provides a harsh environment for bacteria; constantly changing temperature, humidity and salinity and exposure to detergents and host antimicrobial peptides (AMPs) present challenges for bacterial survival. Additionally the perpetual self renewal of the skin epithelium makes permanent bacterial attachment to the host problematic. The specific bacterial adaptations and mechanisms involved in skin colonisation are not fully understood but *S. epidermidis* possesses a wide variety of surface expressed molecules, some of which are likely to have important roles in survival and adhesion on the skin.

1.1.1.1 Structure of the skin epithelium

The human epithelia (reviewed by Candi *et al.*, 2005) comprises several layers of keratinocytes that progress from the basal layer of dividing cells through the spinous, granular and upper granular layers until they become terminally differentiated keratinocytes, corneocytes, in the outermost layer of the epithelium. This migration of keratinocytes towards the skin surface is accompanied by changes in the cells' surfaces. Whereas keratinocytes from the basal layer express proteins such as keratin intermediate filaments K5 and K14 which protrude through the plasma membrane, keratinocytes from the granular layer express mainly K1 filaments and no K5 or K14 filaments. In contrast, corneocytes are essentially dead cells with a very tough and ridged cell surface comprising a cross linked envelope of proteins and lipids and no plasma membrane. *S. epidermidis* requires the ability to attach to at least some of these keratinocyte surface molecules but none have been identified.

1.1.1.2 Bacterial adhesion to the skin

The bacterial cell surface molecules involved in adhesion to the skin have not been identified for *S. epidermidis* but a plethora of surface proteins are known to mediate adhesion to a variety of host matrix proteins. For example, the cell wall

associated proteins SdrG (Nilsson *et al.*, 1998) and EMPB (Williams *et al.*, 2002) promote adhesion to fibrinogen and fibronectin respectively (see section 1.5). No host matrix-binding proteins have been reported to mediate adhesion to skin but there are several *S. epidermidis* genes encoding surface proteins of unknown function (Bowden *et al.*, 2005) that may adhere to keratinocytes surface ligands. The close relative of *S. epidermidis*, *S. aureus*, is known to use at least five different cell surface proteins to attach to epithelial cells from the anterior nares (Corrigan *et al.*, 2009; Roche *et al.*, 2003b). This high degree of functional redundancy between surface proteins highlights the importance of adhesion to the life styles of staphylococci that colonise the epithelium.

1.1.1.3 S. epidermidis survival on the skin

The harsh environmental conditions experienced on the skin and the presence of AMPs are overcome by *S. epidermidis* via a number of known mechanisms and there may be more unknown genes associated with bacterial survival. For example, the bacterial surface polymer poly-γ-glutamic acid (PGA) (Kocianova *et al.*, 2005) along with the presence of eight sodium pumps allow growth in high salt concentrations (Gill *et al.*, 2005). PGA has a dual role in bacterial survival as it also provides protection from at least two antimicrobial peptides derived from human skin (Kocianova *et al.*, 2005). In addition a Gram positive antimicrobial peptide sensing system, *aps* (Li *et al.*, 2007b), that was discovered in *S. epidermidis* is responsible for up-regulating a variety of antimicrobial peptide resistance mechanisms. These mechanisms can involve covalent modifications of normally anionic bacterial surface molecules (Peschel *et al.*, 1999; Peschel *et al.*, 2001), in order to repel cationic antimicrobial peptides, or they may involve removal of AMPs from the bacterial cell membrane (Li *et al.*, 2007a).

The presence of *S. epidermidis* on the skin is not considered harmful and may even be beneficial in maintaining a healthy complement of skin microflora by outcompeting more harmful bacteria (Lina *et al.*, 2003). In support of this theory *S. epidermidis* produces small peptides, called phenol soluble modulins (PSMs),

which interact with human AMPs found on the skin to enhance killing of some other Gram positive bacteria (Cogen *et al.*, 2010a; Cogen *et al.*, 2010b).

1.1.2 *S. epidermidis* causes medical device related infection

For healthy individuals colonisation of the skin by *S. epidermidis* does not usually pose a threat. However, *S. epidermidis* is an opportunistic pathogen causing nosocomial infections associated with indwelling medical devices (Rupp and Archer, 1994) and the bacterium is widely regarded as one of the most common cause of nosocomial sepsis (Garza-Gonzalez *et al.*, 2009). Infections are caused by contamination of implanted medical devices and then persistent colonisation of the implanted device in spite of host immune defences and antibiotics. Although other CoNS are considered opportunistic pathogens, most notably *S. haemolyticus*, *S. saprophyticus* and *S. lugdunensus*, by far the most prevalent cause of disease is *S. epidermidis* (Rogers et al., 2009).

1.1.2.1 Mode of infection

The source of nosocomial *S. epidermidis* infection is likely to be the individuals' own microflora (Costa *et al.*, 2004; Haas *et al.*, 2005) which may adhere to an implant surface during implantation. Infection is associated with a wide range of medical devices such as prosthetic joints, intravenous catheters and artificial heart valves (Uckay *et al.*, 2009). However, very little is known about the bacterial surface molecules required for adherence to the surfaces of such implants. Once inside the host bacteria evade the immune system via the formation, on the surface of the implant, of a multilayered community of dividing bacteria embedded in a secreted polymeric matrix, a biofilm (Costerton *et al.*, 1999). The formation of a biofilm allows *S. epidermidis* to persist in the host despite production of antibodies to *S. epidermidis* proteins (Pourmand *et al.*, 2006) possibly in part due to the protective nature of molecules that comprise the biofilm matrix.

1.1.2.2 Consequences of infection

The type of infection resulting from contamination of medical devices differs depending on the type of implant. *S. epidermidis* colonisation of intravenous catheters, pacemakers or artificial heart valves usually leads to septicaemia or endocarditis (Arber *et al.*, 1994; Edmond *et al.*, 1999) whereas infection of cerebrospinal fluid shunts often leads to a variety of symptoms including fever, loss of consciousness and peritonitis (Wang *et al.*, 2004a). Infections of prosthetic joints usually give less severe symptoms such as loosening of the joint and inflammation (Zimmerli *et al.*, 2004). Antibiotic treatment of *S. epidermidis* device-related infections is often ineffective due to natural resistance and the ability of bacterial to persist in a biofilm which provides protection from some types of antibiotics (Rupp and Archer, 1994). Often the device must be removed in order to clear the infection.

1.1.3 Virulence factors of *S. epidermidis*

S. epidermidis, unlike the closely related *S. aureus*, does not rely on toxin production for virulence. Persistence in the host and immune evasion are the pathogenic strategies employed by *S. epidermidis*. Therefore, in addition to antibiotic resistance genes, molecules expressed on the cell surface that promote adhesion and biofilm formation are the key virulence determinants of *S. epidermidis*. Many of these surface molecules are also important for commensal colonisation of the skin and this has lead to *S. epidermidis* being termed the accidental pathogen (Otto, 2009).

1.1.3.1 Toxins

Although their role in infection is unclear almost all *S. epidermidis* strains are thought to produce PSMs which may be pro-inflammatory (Mehlin *et al.*, 1999; Vuong *et al.*, 2004a). One of these peptides, PSMγ (also known as δ-toxin) has cytolytic properties (Scheifele *et al.*, 1987) and several others share significant sequence homology to *S. aureus* PSMs that can lyse human neutrophils (Wang

et al., 2007). However, aggressive toxin production is not usually associated with *S. epidermidis* virulence and PSMs may have an unrelated role in competition with other microbes on the skin as they have been shown to enhance killing of *S. aureus* and Group A streptococci (Cogen *et al.*, 2010a; Cogen *et al.*, 2010b). The primary role of PSMs is unclear and it has been suggested that they may also play a role in the release of bacteria from *S. epidermidis* biofilms (Vadyvaloo and Otto, 2005).

1.1.3.2 Antibiotic resistance

The beta-lactam antibiotic methacillin is the first choice for treatment of staphylococcal infection and it acts by covalently binding to the cell wall synthesizing enzyme, (penicillin binding protein (PBP)), causing deformation of the cell wall and lysis. Staphylococcal resistance to methacillin is conferred by the *mecA* gene which encodes a PBP, PBP2a, with reduced affinity for methacillin (Hartman and Tomasz, 1984). The *mecA* gene is contained within a mobile genetic element termed SCC*mec* of which there are thought to be around 10 different types that differ slightly in their nucleotide sequence (Garza-Gonzalez *et al.*, 2009). The SCC*mec* type IV element of some methacillin resistant *S. aureus* (MRSA) strains including a community acquired MRSA was acquired via horizontal gene transfer from *S. epidermidis* (Hanssen and Ericson Sollid, 2006).

In cases of infection where methacillin is ineffective vancomycin is often used which inhibits cell wall synthesis by binding to peptidoglycan precursor molecules. However, intermediate levels of resistance have been reported (Schwalbe *et al.*, 1987) indicating the need for new classes of antibiotics to treat staphylococcal infection. The oxizalidinone class is now regarded as the last line of defence and the only member of this class currently in use has been used to successfully treat *S. epidermidis* infection (Gill *et al.*, 2002). However, the occurrence of linezolid resistance in *S. epidermidis* strains (Kelly *et al.*, 2008;

Potoski *et al.*, 2006) highlights the need for proper regulation of the use of such important drugs.

1.1.3.3 Adhesion and biofilm formation

Many *S. epidermidis* strains have the ability to adhere to abiotic surfaces and to host matrix proteins that rapidly coat implants after infection (Francois *et al.*, 1998) as well as the ability to form biofilms. These adhesive properties are the main virulence factors of *S. epidermidis* as they allow persistence in the host. *S. epidermidis* expresses a plethora of molecules that are, at some time, expressed on the cell surface including polysaccharides, proteins, teichoic acids and extracellular DNA (Otto, 2009). Many of these are known to be important in adhesion and biofilm formation, and thus, virulence. However, many molecules thought to be expressed on the bacterial surface are poorly characterized and some have no known function. An understanding of the function(s) of the surface molecules of *S. epidermidis* is essential to understand the mechanisms of adhesion and biofilm formation and to develop therapeutic approaches to clear *S. epidermidis* infections.

The following introductory sections will discuss in more detail the process of biofilm formation and the molecules involved in adhesion as this active field of research is the basis for the experimental work presented in this thesis.

1.2 Bacterial biofilms

Biofilms are of crucial importance to *S. epidermidis* infections as they provide protection from the host immune system and some antibiotics. However, this mode of growth is common throughout bacterial genera and is not always associated with disease. This section will define a general model for bacterial biofilm formation and examine specific characteristics of some important biofilms. *S. epidermidis* biofilms are discussed in section 1.6.

1.2.1 General model of biofilm formation and structure

Biofilms have been defined several times but perhaps most concisely as “a structural community of bacterial cells enclosed in a self produced polymeric matrix and adherent to an inert or living surface” (Costerton *et al.*, 1999). Each definition differs slightly with respect to the species of bacterium and the environment in which they exist but, in general, there are three essential components, a surface, bacteria and a polymeric matrix (Dunne, 2002). There are also differing models for formation depending on species and environment but they follow same basic stepwise progression: 1. primary attachment of cells to a surface, 2. accumulation of cells in multiple layers, 3. maturation, and 4. detachment and dispersal of planktonic cells (Fig. 1.1).

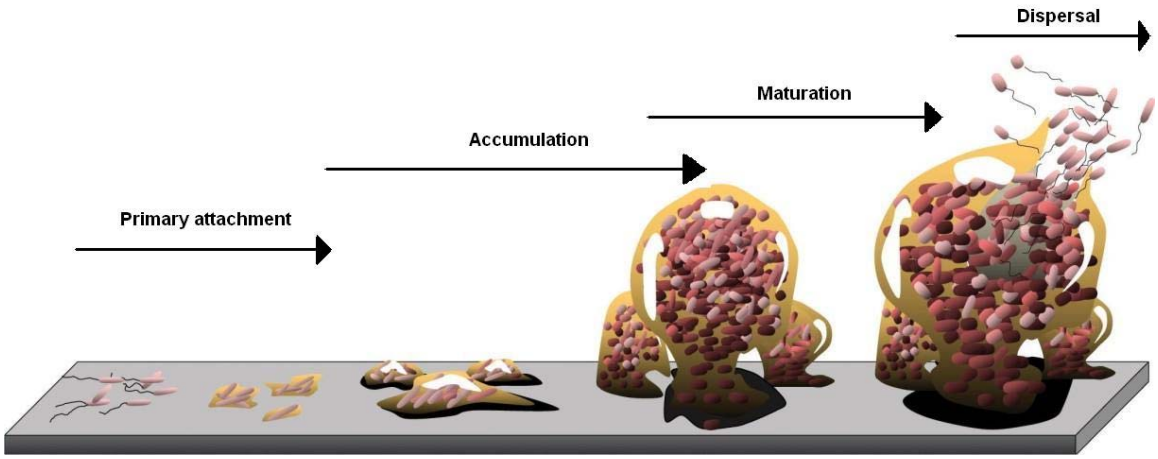

Figure 1.1: Model of biofilm progression showing distinct phases and microcolony structure. Adapted from Monroe *et al.*, (2007).

Primary attachment may be mediated by non-specific factors such as hydrophobic interactions as well as specific ligand binding receptors on the cell surface. The accumulation phase involves multiplication of bacteria and formation of structures called microcolonies. This requires inter-cellular adhesion and is accompanied by production of extracellular polymers (polysaccharides, proteins and DNA) that comprise the matrix of the biofilm. Maturation phase involves the increasing accumulation of bacteria as well as increasing structural

complexity and segregation of bacteria into physiologically heterogeneous layers (see section 1.2.2). Dispersal allows planktonic cells to be released into the environment where they may seed new biofilms elsewhere (Costerton *et al.*, 1999).

Exactly how biofilms provide a protective environment for bacteria in the host is unclear but components of the extracellular biofilm matrix may give some protection from phagocytosis and other elements of the immune system. The thickness of the biofilm may protect cells from reactive oxygen species or antibiotics as cells in the outer layers of the biofilms may adsorb or titrate such chemicals protecting the cells beneath. Also, slowly metabolising cells found in biofilms may be more resistant to classes of antibiotic that only affect actively dividing bacteria.

1.2.2 Biofilms of diverse bacterial genera

The exact composition, structure and human significance of biofilms are dependent on the constituent bacterial species and the environment where the biofilm forms. The following three examples of types of biofilms demonstrate the diversity and complexity of biofilms found in the environment.

1.2.2.1 Pseudomonas aeruginosa biofilms

The most thoroughly studied biofilms are those of *P. aeruginosa* and some of the most complex biofilm structures are demonstrated by this species. *P. aeruginosa* is an opportunistic pathogen and the primary cause of chronic infection and mortality in cystic fibrosis patients (Koch and Hoiby, 1993). The biofilms formed by this bacterium may contribute to the persistence of the organism in the cystic fibrosis lung.

At least *in vitro P. aeruginosa* biofilms comprise large mushroom like structures (Fig. 1.2) which are dependant on cell-cell communication as quorum sensing

mutants are unable to produce the same structures despite retaining the ability to form a thin biofilm (Davies *et al.*, 1998). When *P. aeruginosa* is grown under flow conditions in the presence of glucose the cells divide into motile and non-motile subpopulations (Klausen *et al.*, 2003a). The non-motile cells form small microcolonies (the mushroom stalk) initially and then motile cells migrate to the top of these microcolonies to form the mushroom cap. Interestingly, when grown in the presence of citrate no motile cells are produced and the biofilm does not develop mushroom-like structures (Klausen *et al.*, 2003b).

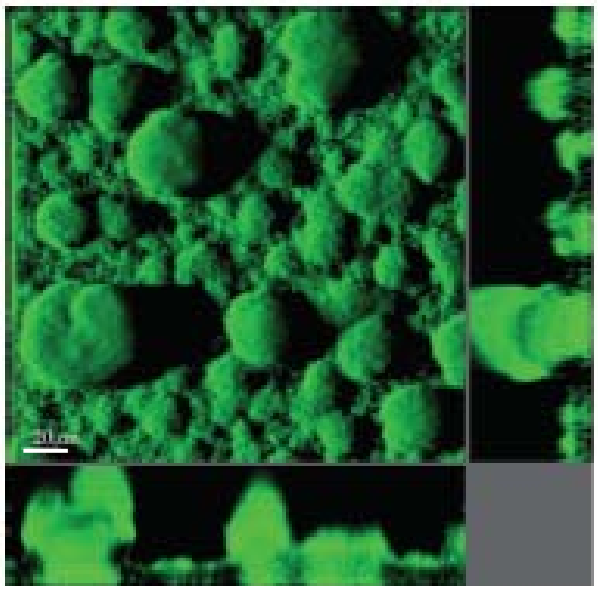

Figure 1.2: Biofilm of *P. aeruginosa* grown under flow conditions and visualized used green fluorescent protein and confocal microscopy. Image shows top down and side views of the biofilm including elaborate mushroom-like microcolonies. The scale bar is 20 μm. adapted from Bjarnsholt *et al.*, (2010).

The bacteria comprising the mushroom-like structures are not homogenous. Dead cells and extracellular DNA are found primarily in the stalk region of the mushroom-like structure, and this may contribute to the migration of motile cells, whereas live cells are found mainly in the outer layers of the top of the structure (Allesen-Holm *et al.*, 2006). In mature biofilms cells on the outer layers of the mushroom cap are embedded in a polysaccharide matrix (Psl) whereas the centre of the structure is free of Psl and contains motile cells (Ma *et al.*, 2009).

This stage of maturation is likely to immediately precede the dispersal stage where motile bacteria are thought to break free from the mushroom cap and leave a hollow cavity (Purevdorj-Gage *et al.*, 2005). In addition to the mechanisms and processes described here *P. aeruginosa* has several additional systems to sense environmental changes and adapt the biofilm structure and composition accordingly as reviewed recently (Harmsen *et al.*, 2010).

Chemical gradients exist within biofilms and in *P. aeruginosa* biofilms it has been shown that oxygen concentrations are dramatically reduced at the base of microcolonies compared to the outer layers (Xu *et al.*, 1998). The oxygen gradient can be sufficient to limit almost all protein synthesis to the outermost layers. The very slow metabolism of bacteria deep within the biofilm is likely to be responsible for resistance to some antibiotics that target dividing cells.

1.2.2.2 Interspecies biofilms

Biofilms often comprise several bacterial genera and there is evidence to suggests that biofilms on medical devices, containing *S. epidermidis*, could also contain other bacterial genera. Two PCR based studies have shown there to be a wide variety of bacterial genera present in biofilms on infected hip joints (Dempsey *et al.*, 2007; Riggio *et al.*, 2010). In both studies several hundred clones were identified from up to ten infected joints and *Lysobacter* spp comprised the majority of the clones. However, the proportion of the biofilm biomass represented by specific clones and the importance of these clones to the infection process is not known. *Lysobacter* spp. may have spread haematogenously from the mouth, where it may be a resident (Leung *et al.*, 2003), as has been suggested for *Propionibacterium acnes* (Tunney *et al.*, 1998). Very little is known about the interspecies aspect of biofilms on medical devices in contrast to the relatively well studied and complex biofilms of the human mouth.

Approximately 100 – 200 species of bacteria are found in a healthy human mouth (Paster *et al.*, 2006) and, due to the constant movement of saliva and swallowing, these bacteria grow in biofilms attached to tooth enamel and the soft tissue of the gums. Oral interspecies biofilms are more commonly referred to simply as dental plaque but they are in fact some of the most complicated microbial communities as discussed in two recent reviews (Jakubovics, 2010; Kolenbrander *et al.*, 2010). Primary colonizers such as members of the genera *Streptococcus* and *Actinomyces* create a distinct microenvironment allowing secondary colonizers to grow.

Dependency of some species of bacteria for others has been demonstrated in numerous studies, for example, *Streptococcus oralis* and *Actinomyces naeslundii* only grow in biofilms supplied with flowing saliva when co-culture and not on their own (Palmer *et al.*, 2001). Also, some oral pathogens such as *Porphyromonas gingivalis* are known to require co-culture in *in vitro* flow biofilms with relatively harmless bacteria (Periasamy and Kolenbrander, 2009). The reasons for inter-dependency in oral biofilms could be due to several factors. *Fusobacterium nucleatum* can co-aggregate with several other species and may provide a bacterial bridge to physically link different species of bacteria together allowing biofilm formation (Kolenbrander *et al.*, 2006). Requirement for a range of catabolic enzymes to generate nutrients from saliva may also be important as increased numbers of bacterial species caused an increase in total bacterial number in a chemostat due to a greater variety of catabolic enzymes (Bradshaw *et al.*, 1994). Additionally, *S. gordonii* DL1 is known to require *A. naeslundii* for sufficient arginine biosynthesis (Jakubovics *et al.*, 2008). Intriguingly, this may be due to signaling between the two species as the expression of 23 *S. gordonii* DL1 genes were affected by co-aggregation with *A. naeslundi* and this required physical contact between the two species. Of the 23 genes whose expression was altered, nine genes required for arginine metabolism were up-regulated and *S. gordonii* could only grow at low arginine concentrations when coaggregated with *A. naeslundi* (Jakubovics *et al.*, 2008). Furthermore, the interspecies

signaling molecule, AI-2 (the *luxS* quorum sensing signal molecule), is required for microcolony formation in a *S. gordonii*, *P. gingivalis* co-cultured biofilm (McNab *et al.*, 2003). Also, AI-2 can affect the formation of hyphae by *Candida albicans* (Bamford *et al.*, 2009) suggesting that signaling between bacteria and yeast may also occur in the interspecies biofilms of the oral environment.

Interspecies signaling has never been reported in medical device related biofilms involving *S. epidermidis*. However, the wide variety of bacterial genera recently reported in joint infections (Dempsey *et al.*, 2007; Riggio *et al.*, 2010) raises the possibility that such signaling may be important to biofilm development.

1.2.2.3 Biofilms in industry and the environment

Biofilms are also common in the environment outside human hosts and they help to allow bacteria to colonise a wide variety of niches. On particularly harsh environments such as the surface of exposed rocks, where levels of ultra violet light could be lethal to bacteria, a pigment is produced on the surface of the biofilm that absorbs ultra violet light and protects bacteria within (Gorbushina and Broughton, 2009). Biofilms are also present closer to home and can be found in abundance in kitchen sinks (Furuhata *et al.*, 2010) where the biofilm prevents bacteria from being washed down the drains. Biofilms can allow bacteria to exploit nutrient sources, for example, by forming on plant roots in the rhizosphere (Nazir *et al.*, 2010) or on the surface of rocks coated in algae (Gorbushina and Broughton, 2009).

The ability of bacteria to colonise environments through biofilm formation is not just a problem in infection of medical devices but also in several industrial processes. Biofilm formation on marine structures such as boats and oilrigs causes biofouling, the process of degradation of artificial structures caused or enhanced by microorganisms, is a serious concern to many industries (Dobretsov *et al.*, 2006). Biofilms formed by *Bacillus cereus* and *Serratia*

marcescens are also found in oil pipelines where they are thought to contribute to degradation of the pipe material (Rajasekar *et al.*, 2010). Some biotechnology industries, however, have capitalized on the biofilm mode as discussed in a recent review (Rosche *et al.*, 2009) and biofilms are already used in the treatment of waste water. The persistence and adherence of bacterial biofilms, the very characteristics that make them problematic for humans, are beneficial in processes that use bacteria as biological catalysts. In these processes biofilms protect bacteria from potential harmful reactants and their adherent nature is ideal for continuous processes involving the constant flow of reactants. It is clear that furthering our understanding of biofilm formation will help in the development of new biotechnologies as well as aiding in the fight against disease.

In comparison to many of the biofilms described in this section little is known about *S. epidermidis* biofilms. Their structure is not well characterized but they are not thought to produce elaborate mushroom-like microcolonies seen for *P. aeruginosa*. In addition, quorum sensing in *S. epidermidis* biofilms is not fully understood and the possible implications of interspecies interactions requires further study. However, as discussed in section 1.6, recent reports suggest *S. epidermidis* biofilms are likely to show similar levels of complexity to biofilms described here.

1.3 The surface of *S. epidermidis*

The key aim of this study is to determine and investigate the functions of the surface protein, Aap, in adhesion and biofilm formation and this requires knowledge of other cell surface molecules. The purpose of this section is to give an overview of the different types of molecules that comprise the cell surface of *S. epidermidis* (Fig. 1.3). Some of these molecules have specific adhesive or biofilm-related functions and these functions will be discussed in detail in the introductory sections on *S. epidermidis* adhesion and biofilm formation. The cell

surface as a whole will be described here in order to present the context in which specific adhesive molecules such as Aap function.

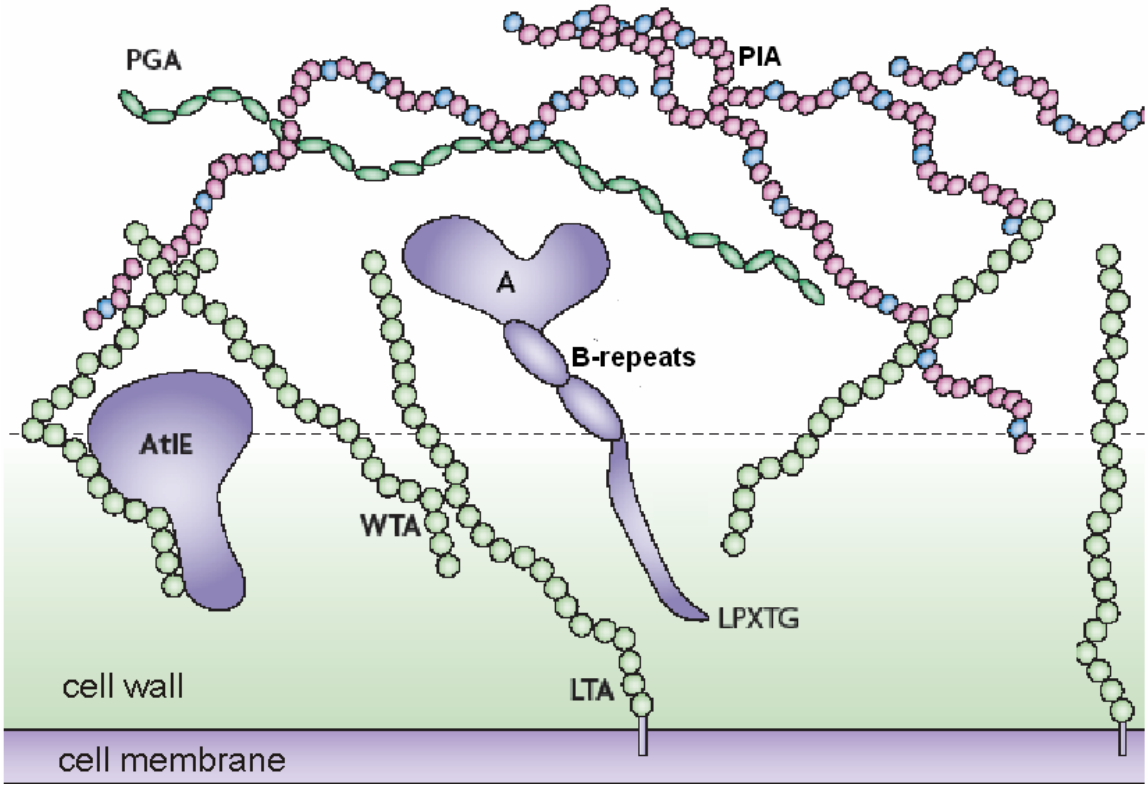

Figure 1.3: Schematic of a section of the *S. epidermidis* cell surface showing the different classes of surface molecules described in this section. Green colour denote negative charge and the blue circles denote positive charge. Shown are the cell membrane, cell wall peptidoglycan (from the membrane to the dashed line), wall and lipo-teichoic acids (WTA & LTA respectively), polysaccharide intercellular adhesin (PIA, acetylated and de-acetylated glucosamine residues are shown in pink and blue respectively), poly-γ-glutamic acid (PGA), the non-covalently bound cell wall protein AtlE (incorrectly shown associating with WTA), a covalently bound LPXTG protein with B-repeats and A-domain. Figure adapted from Otto *et al.*, (2009).

1.3.1 Peptidoglycan

Like all Gram positive bacteria *S. epidermidis* has a thick cell wall composed of long chains of alternating N-acetyl muramic acid (NAM) and N-acetyl glucosamine (NAG) linked by a β 1-4 glycosidic bond. These chains are cross linked with short peptide linkers to give the cell wall a rigid structure. The thick

cross-linked cell wall gives Gram positives more resistance to mechanical and osmotic stress compared to Gram negative bacteria. In the case of *S. epidermidis* this is likely to help facilitate survival on the skin.

Although the basic structure of peptidoglycan is conserved throughout bacteria the specific composition of peptide cross-links varies. *S. epidermidis* and *S. aureus* share the same peptide cross link. NAM subunits are covalently linked to a peptide of D-Ala-D-iGln-L-Lys-D-Ala-D-Ala (iGln is isoglutamine). A pentaglycine peptide then links the L-lysine of one NAM unit to the D-alanine at the fourth position of a different NAM unit with the resulting loss of the fifth D-alanine (Strominger and Ghuysen, 1967). The flexible nature of the pentaglycine peptide allows a high degree of cross-linking with approximately 90 % of NAMs cross-linked to neighboring NAG-NAM chains (Labischinski, 1992). This high degree of cross-linking gives staphylococci their resistance to lysis.

1.3.2 Teichoic acids

Teichoic acids (TAs) are polymers of either glycerolphosphate or ribitolphosphate and are present in Gram positive cell walls. *S. aureus* cells walls contain primarily poly-ribitolphosphate whereas poly-glycerolphosphate is found in *S. epidermidis* (Endl et al., 1983). In *S. epidermidis* the glycerol residues are sometimes modified with D-alanine, glucose, NAG or glucose which is its self modified with alanine (Sadovskaya *et al.*, 2004). TAs can be linked to the cell wall peptidoglycan (wall TAs) or to lipids within the cell membrane (lipo-TAs) and the catheter isolate *S. epidermidis* RP62A also produces extracellular TA (Tojo *et al.*, 1988).

The function of TAs is unclear but they give peptidoglycan an overall negative charge and modifications may be important in regulating several properties of the cell surface as reviewed by Neuhaus & Baddiley (2003) and Swoboda *et al.*, (2010). TAs bind divalent cations such as Ca^{2+} and Mg^{2+} and this interaction is

inhibited by D-alanyl modifications which reduce the overall negative charge of TA. The extent of D-alanyl modification is decreased when bacteria are grown in the presence of increased NaCl, temperature and pH resulting in increased divalent cation content in the cell wall. TA may also regulate the shape and rigidity of the cell wall in response to changing growth conditions. As D-alanylation of TAs reduces the negative charge of the cell surface the extent of this modification may influence adhesion of bacteria to different surfaces.

1.3.3 Polysaccharide

The only polysaccharide identified in *S. epidermidis* is a poly N-acetyl glucosamine termed polysaccharide intercellular adhesin (PIA) (Mack *et al.*, 1994) that is bound to the cell surface and forms part of the extracellular matrix. Around 20 % of the NAG residues in PIA are deacetylated giving the molecule an overall positive charge (Mack *et al.*, 1996) which is probably required for attaching the molecule to negatively charged components of the cell surface. The function of this molecule in *S. epidermidis* biofilm formation is discussed in detail in section 1.6.1. PIA may also have a role in immune evasion distinct from its biofilm role as *S. epidermidis* mutants not expressing PIA were more susceptible to killing by human AMPs and phagocytosis by macrophage (Vuong *et al.*, 2004c).

1.3.4 Pseudo-polypeptide

The surface expressed PGA which has a role in *S. epidermidis* survival on the skin (see section 1.1.1) is produced by the *cap* locus, first discovered in *Bacillus anthracis* (Little and Ivins, 1999). The polymer consists of glutamic acid residues linked via their α-amine and γ-carboxyl groups by an isopeptide bond and PGA is therefore anionic. In the only study of its kind PGA was expressed by all 74 *S. epidermidis* strains tested suggesting the polymer may be ubiquitously expressed (Kocianova *et al.*, 2005). Also, 11 of 22 strains representing 16 coagulase-negative species expressed PGA but *S. aureus* does not contain the *cap* locus. In addition to functions associated with skin survival PGA also

protects *S. epidermidis* from neutrophil phagocytosis (Kocianova *et al.*, 2005) and is up-regulated in *S. epidermidis* biofilms (Yao *et al.*, 2005a).

1.3.5 Wall anchored LPXTG proteins (including accumulation associated protein, Aap)

Many Gram positive cocci express a class of cell wall associated proteins that are covalently linked to peptidoglycan via a C-terminal LPXTG motif (Schneewind *et al.*, 1995). LPXTG proteins share a common modular domain architecture (Navarre and Schneewind, 1999) with a N-terminal signal sequence directing the protein for export via the Sec pathway usually followed by a non-repetitive A-domain which often contains a ligand binding site. Following this is usually a repetitive region, the B-repeat region, which may form a stalk-like structure in some LPXTG proteins. In the case of Aap the number of B-repeats varies between strains and *aap* genes with 3 - 17 B-repeats have been reported (Rohde *et al.*, 2007). Nearer the C-terminus is the LPXTG motif adjacent to which is a hydrophobic membrane spanning region and a short region of positively charged amino acids. After export through the Sec apparatus the positively charged region remains in the cell (Fig. 1.4) and the LPXTG protein is transiently anchored in the membrane before being released by a sortase enzyme which cleaves the protein between the T and G residues (Mazmanian *et al.*, 2001). The same enzyme then catalyses the formation of a peptide bond between the carboxyl group of the T and the free amino group of a G from the pentaglycine crosslink in the cell wall peptidoglycan. A proline rich sequence is often located immediately adjacent to the LPXTG motif the purpose of which may be to feed the protein through the peptidoglycan so it is exposed on the cell surface.

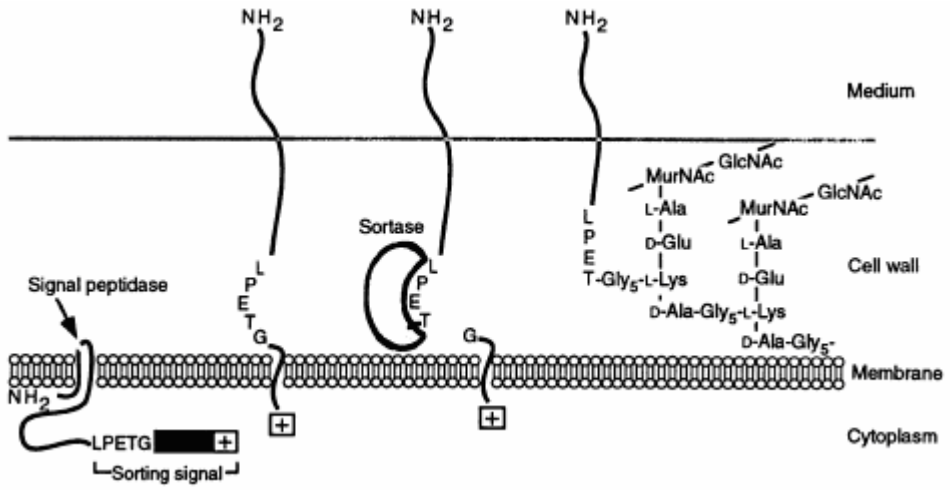

Figure 1.4: The export and anchoring of an LPXTG protein to cell wall peptidoglycan by a sortase enzyme. Figure taken from Schneewind *et al.*, (1995).

The *S. epidermidis* RP62A genome codes for 11 putative LPXTG proteins (Fig. 1.5) (Bowden *et al.*, 2005). The function of five of these has been at least partially characterized and they promote adhesion to a specific host matrix protein or biofilm formation. Two LPXTG proteins (SdrF and G) have previously been described as part of the Sdr sub-class of LPXTG proteins present in *S. epidermidis* and *S. aureus* (McCrea *et al.*, 2000) characterised by the presence of a serine aspartate repeat region near the C-terminus. Screening of several *S. aureus* genomes has identified 21 LPXTG proteins (Roche *et al.*, 2003a) including the archetypal LPXTG protein, Protein A.

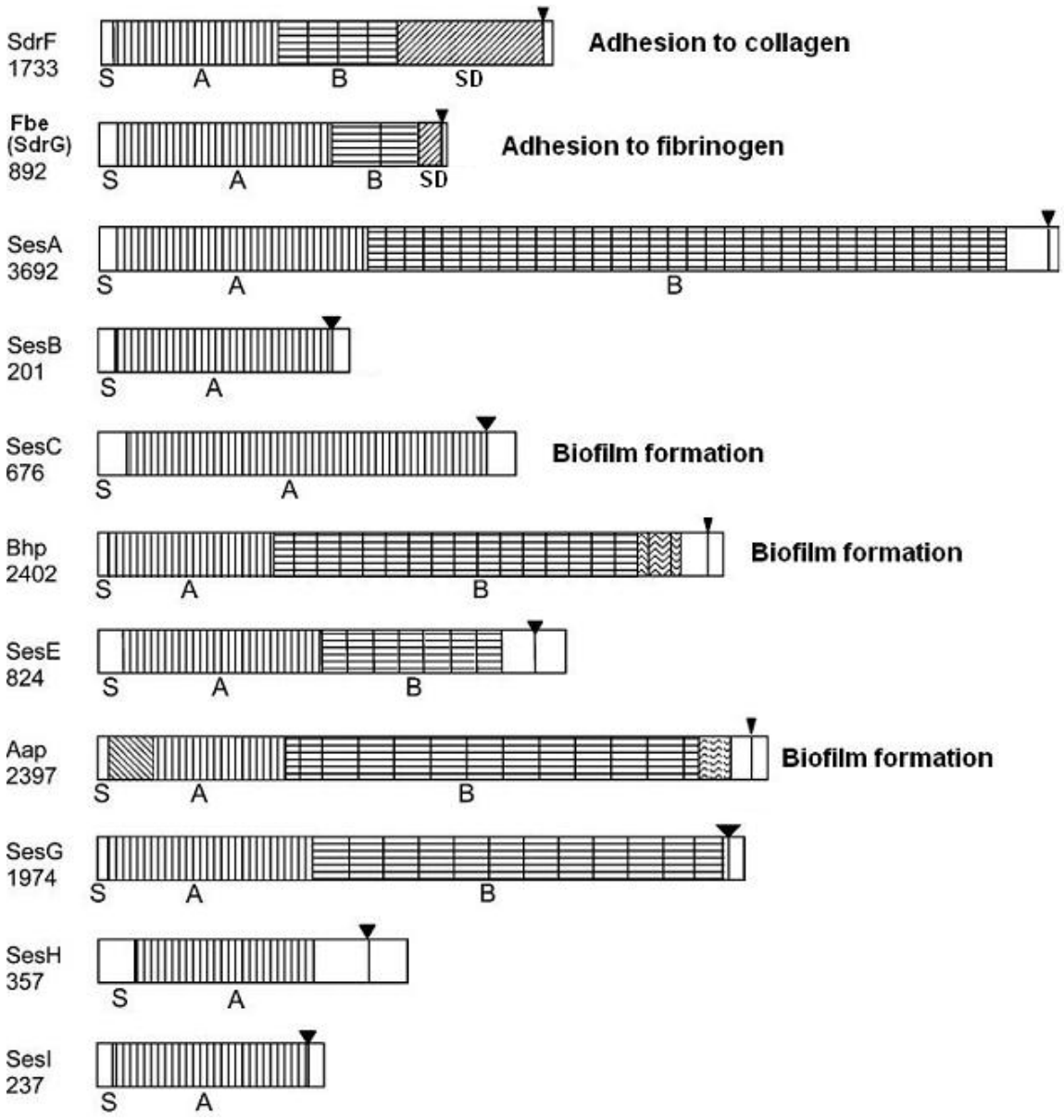

Figure 1.5: The LPXTG proteins of *S. epidermidis* RP62A. The size (in amino acids), domain organisation and function (where known) are shown. S, Sec signal sequence; A, A-domain; B, B-repeat region; SD, serine aspartate repeat region. Triangle above each schematic shows the LPXTG motif, vertical lines show the A-domain, horizontal lines show the B-repeat region, diagonal lines denote other short repeat regions and wavy lines denote short imperfect repeats. Figure adapted from Bowden *et al.*, (2005).

Not all LPXTG genes are present in all *S. epidermidis* strains (Bowden *et al.*, 2005). Numerous attempts have been made to identify LPXTG genes that are indicative of either commensal or invasive strains but results from different studies often contradict, for example the *aap* gene was found in only 27 % of disease isolates in one study (Bowden *et al.*, 2005) and 100 % in another (Rohde *et al.*, 2007). Regulation of individual LPXTG genes is poorly understood but they are broadly thought to be under the control of the quorum sensing locus, *agr* (see section 1.5.6). However, different LPXTG proteins were found to be up or down- regulated respectively during exponential and stationary phases of growth (Bowden *et al.*, 2005) suggesting individual genes may be affected differently by the *agr* locus.

1.3.6 Non-LPXTG cell wall proteins

Cell wall associated proteins such as the autolysin, AtlE (Heilmann *et al.*, 1997), and the large extracellular matrix binding protein, EMBP (Williams *et al.*, 2002), also have roles in adhesion and biofilm formation. However, these proteins do not contain LPXTG motifs and it is not clear how they remain attached to the cell wall. Previous suggestions that AtlE was anchored to the cell wall via TAs appear to have been unfounded as a recent study (Schlag *et al.*, 2010) showed that wall-TAs inhibit the binding of the *S. aureus* autolysin, AtlE, to the cell surface. Wall-TAs were found to be present predominantly in old peptidoglycan away from the septum where new peptidoglycan is produced and it was proposed that this distribution of wall-TAs is responsible for the localization of Atl close to the septum. AtlE was found to adhere to isolated peptidoglycan but the mechanism of attachment remains unclear. EMBP contains FIVAR (found in various architecture) domains which are found in two cell wall associated proteins from *S. aureus* and *Bacillus sp* GL1 and may adhere to peptidoglycan but this has not been proven (Christner *et al.*, 2010). A second autolysin, Aae (Heilmann *et al.*, 2003), is known to bind non-covalently to peptidoglycan via its

LysM domain which is responsible for non-covalent cell wall attachment of other proteins involved in cell wall metabolism (Scott and Barnett, 2006).

1.3.7 Microbial surface components recognising adhesive matrix molecules (MSCRAMMs)

The acronym MSCRAMMs refers to any bacterial surface molecule that promotes adhesion to a component of the human extracellular matrix. Such a classification is useful for *S. epidermidis* surface molecules as host matrix proteins rapidly coat medical devices after implant. MSCRAMMs are therefore vital for *S. epidermidis* infections. The importance of MSCRAMMs was highlighted in a study (Guo *et al.*, 2007) showing the LPXTG protein, SdrG, which adheres to fibrinogen (Nilsson *et al.*, 1998), was required for increased virulence in a rat model of central venous catheter infection.

1.3.8 Fibrillar tufts containing Aap

S. epidermidis strains NCTC 11047 and RP62A are known to express dense tufts of fibrils (Fig. 1.6) that are approximately 120 nm in length (Banner *et al.*, 2007). The tufts are visible by negative staining in the TEM on a proportion of cells and they labeled densely with antiserum raised to the LPXTG protein Aap indicating Aap is a major constituent of the tufts (Banner, 2007). The function of the tufts on NCTC 11047 is unclear as other molecules may be present in the tufts but they may contribute to cell surface hydrophobicity and may have a role in adhesion (see section 1.4.4).

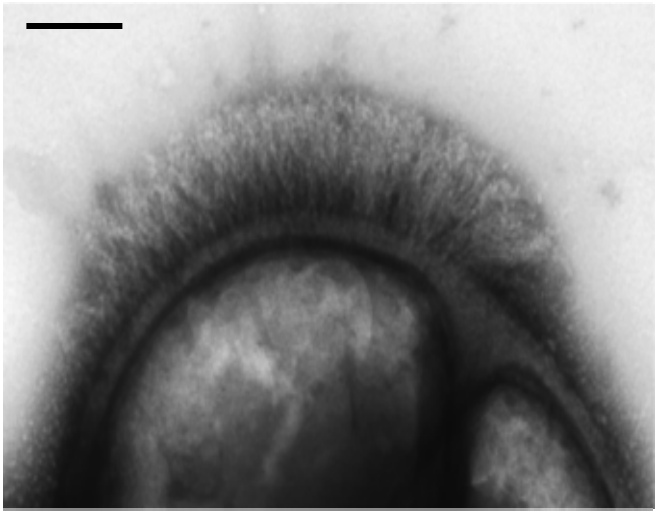

Figure 1.6: Transmission electron micrograph showing a tuft of densely packed fibrils, to one side of the septum, containing Aap on the surface of *S. epidermidis* NCTC11047. The cells were negatively stained with methylamine tungstate before imaging in the transmission electron microscope (TEM). The scale bar is 100nm. Adapted from Banner *et al.*, (2007).

Fibrillar tufts are common on oral streptococci and have been reported on *Streptococcus oralis* (Jameson et al., 1995), *Streptococcus sanguis* (Handley et al., 1985) and *Streptococcus cristatus* (Handley et al., 2005). The tufts vary in length and width but in most cases the tuft phenotype correlates with greater cell surface hydrophobicity. *S. cristatus* adhere to other oral bacteria via their tufts (Handley *et al.*, 2005) suggesting tufts of other streptococci may also have adhesive roles although this has not been proven.

The tuft phenotype suggests that there must be a mechanism for targeting proteins to certain regions of the cell wall. A membrane-associate protein complex found in *Streptococcus pyogenes* and termed the ExPortal is responsible for targeting the HtrA cell wall-anchored protein to a single region on the cell wall (Rosch and Caparon, 2005). The ExPortal comprises a highly localised concentration of the Sec translocons and anionic phospholipids (Rosch *et al.*, 2007) and is thought to recognise a specific N-terminal sequence of specific proteins that are targeted for export. No similar structures have been reported in staphylococci.

In summary, the surface of *S. epidermidis* (Fig. 1.3) comprises a complex mix of polymers with very different structural compositions and functions. The vast majority of these surface molecules are known to, or are predicted to, contribute to the adhesive abilities of *S. epidermidis* strains or the ability of strains to form biofilms. However, many surface molecules have no known function and more are only partially characterized. It is essential to understand the functions of surface proteins and molecules such as Aap in detail in order to develop therapeutic strategies to treat or prevent *S. epidermidis* infection.

1.4 Adhesion of *S. epidermidis* to abiotic surfaces

Adhesion to abiotic surfaces is likely to be primarily dependant on the physiochemical properties of the bacterial cell surface and the abiotic surface itself (Rijnaarts *et al.*, 1995; Vacheethasanee *et al.*, 1998). The overall net charge or hydrophobicity of the abiotic and bacterial surface can result in either attractive or repulsive forces. However, repulsive forces may be overcome by van der Waals interactions between bacterial surface molecules and the abiotic surface resulting in bacterial attachment.

The net negative charge of the cell surface is largely due to the TA content of the cell wall and the extent of modification with D-alanine (Gross *et al.*, 2001) which adds positive charge to TAs. D-alanylation of TAs increases adhesion of *S. aureus* to glass and polystyrene. Hydrophobicity of the cell surface is thought to increase with increased expression of generic cell wall proteins and increased cell surface hydrophobicity correlates with increased adhesion of *S. epidermidis* to polystyrene (Vacheethasanee *et al.*, 1998). Besides the general effect of generic surface proteins on the hydrophobicity of the cell surface a few specific *S. epidermidis* surface molecules have been identified that can significantly increase adhesion to abiotic surfaces.

1.4.1 Polysaccharide mediator of adhesion

The *S. epidermidis* surface polysaccharide PIA is now widely regarded as identical to the previously discovered capsular polysaccharide adhesin (PS/A) (Mack *et al.*, 2006; Tojo *et al.*, 1988). PIA of *S. epidermidis* is highly adherent to silastic (silicone-elastomer) catheter material. In two of three adherent strains tested, purified PIA and an antibody raised against it were able to block bacterial adherence to the catheter material (Tojo *et al.*, 1988). PIA negative transposon mutants were deficient in primary adherence to silastic catheter material (Muller *et al.*, 1993). These findings suggest that, in at least some strains of *S. epidermidis*, PIA mediates initial adherence to silastic catheters. However, PIA does not appear to promote adherence to all polymer types as there was no difference between adhesion of a PS/A negative mutant and the PIA positive parent strain to polyethylene (Higashi *et al.*, 1998).

1.4.2 *Staphylococcus* surface protein (Ssp)

Two surface epitopes on *S. epidermidis* strain 354 were discovered that corresponded to two bands on SDS PAGE (Timmerman *et al.*, 1991), later termed *Staphylococcus* Surface Proteins 1 and 2 (Ssp1 and Ssp2) (Veenstra *et al.*, 1996). These bands appeared to be derived from the same protein as tryptic digest experiment suggested that Ssp2 (250 kDa) was a break-down product of Ssp1 (280 kDa) (Veenstra *et al.*, 1996). The Ssp proteins were found to promote adhesion to polystyrene and this may have been due primarily to Ssp1 rather than Ssp2 (Timmerman *et al.*, 1991; Veenstra *et al.*, 1996). These results suggest that Ssp is a surface protein capable of mediating attachment to polystyrene. Immunogold labelled electron micrographs were interpreted as showing the protein was part of a fimbria like structure extending further than 1 µm from the cell surface (Timmerman *et al.*, 1991). It should however be noted that these micrographs were of a poor quality and do not show conclusively that Ssp is present in large fimbria-like structures. Unfortunately no further functional

studies have been performed on this potentially important surface protein and no corresponding gene was identified.

1.4.3 Bifunctional autolysin involved in adhesion

The *S. epidermidis* cell wall degrading enzyme, AtlE (Heilmann *et al.*, 1997), and the *S. aureus* homologue, AtlA (Oshida *et al.*, 1995) are known to be proteolytically processed via an unknown mechanism to give approximately five SDS PAGE bands. The 52 kDa and 60 kDa bands contain glucosaminidase and amidase activity respectively and the 60 kDa band also promotes adhesion to polystyrene. Exactly how AtlE promotes adhesion to polystyrene is unclear but a recent study has suggested that it may allow release of genomic DNA which can become attached to the cell surface and promote adhesion of bacteria (Qin *et al.*, 2007).

1.4.4 Evidence for involvement of Aap in tufts

S. epidermidis NCTC 11047 comprises two subpopulations, one of which expresses dense fibrillar tufts containing Aap whereas the other subpopulation does not express tufts or Aap (Banner *et al.*, 2007). The wild-type (WT) strain was reported to contain approximately 25 % tufted and 75 % non-tufted cells as counted in the TEM with negative staining. However, tufts can only be detected on bacterium lying in certain orientations with the tuft projecting out to the side of the cell. If the tufts project directly up or down they cannot be detected by negative staining in the TEM and this method gives an underestimate of the number of tufted cells. Additional work, presented in a University of Manchester PhD thesis (Banner, 2007), using immunogold labelling of Aap in the TEM showed tufts were present on 75 % of cells. In this present thesis I have used flow cytometry to give the most accurate count of cells expressing Aap. However, flow cytometry gives no indication of the presence or absence of tufts and only detects surface expression of Aap.

Repeated subculture and enrichment for either hydrophobic or hydrophilic cells using a hydrocarbon affinity assay resulted in two stable populations of cells that comprised either 100 % tufted cells, the Fib^+ population, or no tufted cells, the Fib^- population (Banner, 2007; Banner *et al.*, 2007). Reverse transcription PCR (rtPCR) experiments on both populations showed Aap expression to be regulated at the post transcriptional level as the Fib^- population contained Aap mRNA. The Fib^+ population of cells was more hydrophobic and more adhesive to polystyrene than the Fib^- population, suggesting Aap may promote adhesion although it is not clear whether other molecules are present on Fib^+ but not Fib^- cells (Banner *et al.*, 2007). The fibronectin-binding protein, EMBP (see section 1.5.4), is likely to be present on the Fib^+ but not the Fib^- cells as Fib^+ cells adhered in greater number to the fibronectin-coated wells of a microtiterplate (Banner, 2007). Work performed by myself but not presented in this thesis showed Aap was not responsible for the adhesion of Fib^+ cell to fibronectin. Fibronectin adhesion assays with *Lactococcus lactis* that expressed heterologous Aap (strains and Aap constructs are detailed in chapters 3 & 4) showed that Aap is not a fibronectin adhesin (data not shown). It was one of the aims of this study to determine whether or not Aap could mediate adhesion to polystyrene and to further investigate the expression of Aap on subpopulations of other *S. epidermidis* strains.

1.5 Adhesion of *S. epidermidis* to host proteins and cells

Adhesion to host proteins is likely to be important for pathogenesis of *S. epidermidis* as host matrix proteins coat implanted medical devices (Francois *et al.*, 1998) which *S. epidermidis* colonises. Host protein interactions are mediated by specific bacterial surface proteins and this section will examine the literature relating to these adhesins. In addition, evidence linking Aap to host cell adhesion will be discussed.

1.5.1 Fibrinogen (Fg) binding by *S.epidermidis*

Fg is a component of the extra-cellular matrix and has a well established role as part of the immune system. It is a 340 kDa glycopeptide hexamer comprising two sets of α, β and γ chains. As part of haemostasis, thrombin cleaves the α and β chains to release A and B fibrinopeptides which act as a signal to immune cells attracting them to the area. Fg also rapidly coats the surfaces of indwelling medical devices and Pei and Flock (2001a) found Fg to be the only matrix component present on haemodialysis tubing from all patients tested.

The Fg-binding protein of *S. epidermidis*, Fbe (119 kDa), was identified using phage display (Nilsson *et al.*, 1998) and the *fbe* gene was present in 40 out of 43 clinical isolates of *S. epidermidis* suggesting an important role for this protein in pathogenesis. Indeed, antibodies raised to Fbe blocked *S. epidermidis* adhesion to Fg-coated catheters (Pei and Flock, 2001b). Sequence comparisons showed that Fbe had similar overall organisation to the *S. aureus* Fg-binding MSCRAMM clumping factor A (ClfA) with some regions showing high degrees of similarity (Nilsson *et al.*, 1998). The greatest degree of similarity between ClfA and Fbe was found to be in the N-terminal A-region, responsible for Fg-binding in both proteins (McDevitt *et al.*, 1995; Nilsson *et al.*, 1998). However, *S. epidermidis* does not aggregate in the presence of Fg as *S. aureus* does (McDevitt *et al.*, 1995) and Fbe binds to the β-chain of Fg (Davis *et al.*, 2001) as opposed to the γ-chain that ClfA binds.

1.5.1.1 The "dock, lock and latch" mechanism for staphylococcal MSCRAMMs

A high resolution crystal structure of an N-terminal fragment of Fbe in complex with a Fg peptide analogue has been published (Ponnuraj *et al.*, 2003). The authors of this study proposed a binding mechanism, termed the "dock, lock and latch" mechanism, and give some evidence to suggest that the mechanism could be common to a number of Gram positive MSCRAMMs.

The A-domain of Fbe was rapidly proteolytically degraded by a contaminating protease at the N-terminus to yield a resistant fragment. The proteolytically degraded N-terminus was termed N1 (not shown in Fig. 1.7) and the resistant fragment was found to be composed of two IgG like folds termed N2 and N3 (Fig. 1.7).

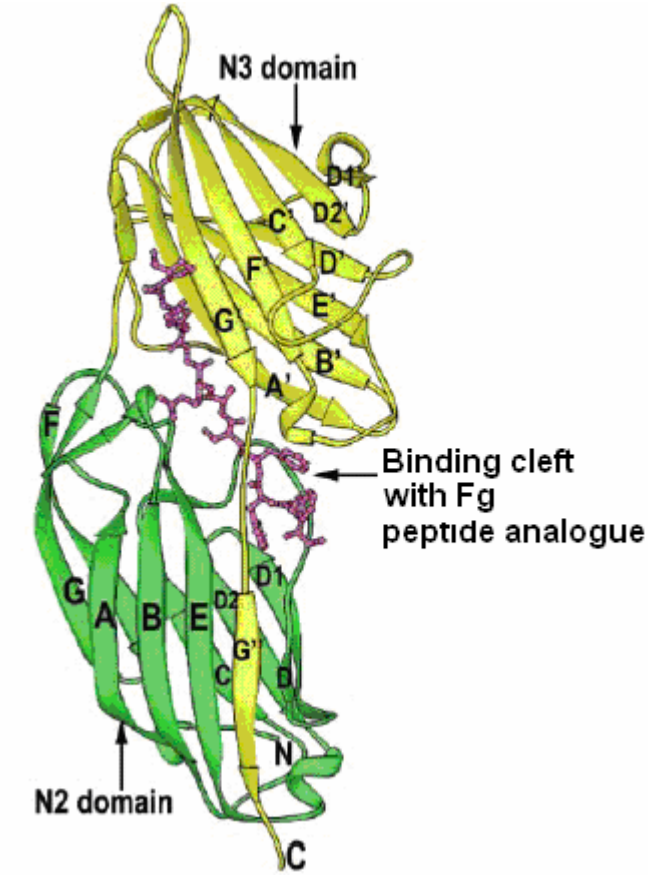

Figure 1.7: Crystal structure of a recombinant fragment of Fbe in the "latched" position with bound Fg peptide. The N-terminal N2 domain is green, the N3 domain is yellow and the Fg peptide is purple. Adapted from Ponnuraj *et al.*, (2003)

The authors proposed that the ligand firstly binds in the cleft between N2 and N3 (the "dock") bringing about a conformational change in the C-terminus. The C-terminal moves to cover the binding cleft and makes a number of backbone hydrogen bonds with the peptide ligand (the "lock"). Finally the rest of the C-terminal strand (labeled G' in Figure 1.7) interacts with the N2 region and complements a β-strand stabilizing the overall structure (the "latch"). The

authors propose that this “dock, lock and latch” model may be a common mechanism amongst Gram positive cell-wall-anchored adhesins. The theory is based on the finding that a short motif, present at the back of the binding cleft (TYTFTDYVD), and another present on the C-terminal “latch” (comprising alternating small and large amino acids) are present in many Gram positive adhesins. This theory is yet to be substantiated by other structural studies into Gram positive MSCRAMMs.

Fbe is the most studied *S. epidermidis* surface adhesin and the only one for which the binding mechanism is known. It is hoped that such detailed knowledge of the way in which adhesins functions will directly aid drug discovery processes such as rational drug design and lead to new and effective treatments for implant device related infections.

1.5.2 Vitronectin binding by *S. epidermidis*

Vitronectin is a 75 kDa glycoprotein with important roles in haemostasis and cell motility. It may also play a part in *S. epidermidis* infections by coating implanted medical devices. AtlE promotes adhesion to vitronectin (Li *et al.*, 2001) and polystyrene (Heilmann *et al.*, 1997) suggesting AtlE could play an important role in pathogenesis. Indeed AtlE was shown to be important in a rat central venous catheter infection model (Rupp *et al.*, 2001). More recently another bifunctional autolysin, Aae, was discovered (Heilmann *et al.*, 2003) that bound to vitronectin as well as fibronectin (Fn) and Fg.

1.5.3 Collagen binding by *S. epidermidis*

Collagen makes up a large part of the extra cellular matrix and, when exposed to plasma, can activate a variety of host immune cells. *S. aureus* expresses a collagen binding protein called Cna (Patti *et al.*, 1992) which is important for virulence as demonstrated in an animal model of septic arthritis (Patti *et al.*, 1994b).

GehD of *S. epidermidis* was originally identified as a lipase (Longshaw *et al.*, 2000). However, Bowden *et al.,* (Bowden *et al.*, 2002) showed the secreted protein to be bi-functional by demonstrating it binds to collagen I, II and IV and providing evidence that it may mediate adherence of *S. epidermidis* to immobilised collagen. GehD is not an LPXTG protein and it is not clear how the protein adheres to the bacteria surface. There is a degree of functional redundancy among *S. epidermidis* adhesins and the LPXTG protein SdrF also mediated adhesion to collagen (Arrecubieta *et al.*, 2007). Unusually for LPXTG proteins the collagen binding region on SdrF is in the stalk-like B-repeat region and not in the terminal A-domain. SdrF is likely to play an important part in device related infections as antibodies raised to the B-region of SdrF significantly reduced infection in a mouse model of device related infection.

It is possible that further collagen adhesins have yet to be discovered on the surface of *S. epidermidis*. In both the GehD (Longshaw *et al.*, 2000) and SdrF (Arrecubieta *et al.*, 2007) studies adhesion of *S. epidermidis* to collagen coated surfaces was not completely abolished by treatment of cells with GehD or SdrF antibodies and the strain used in the GehD study did not express the SdrF protein. The possibility of further functional redundancy among cell surface adhesins highlights the need for further functional studies into *S. epidermidis* surface proteins.

1.5.4 Fibronectin binding by *S. epidermidis*

Fn is a large homodimeric glycoprotein with the two protomers joined by disulphide bonds at the C-terminus. Each protomer comprises a number of distinct domains connected to each other by flexible linker regions. These domains are in turn made up of several repeating units the most common being the type III Fn repeat which binds integrins. Many types of bacteria including *S. aureus* exploit this modular composition to invade host cells by binding to one

type of module while the host integrin binds to another (Schwarz-Linek *et al.*, 2006). The *S. aureus* Fn binding proteins (FnBPA and FnBPB) are necessary for opsonization (Dziewanowska *et al.*, 1999) and Fn was found to act as a molecular bridge linking the bacteria with the host cell. Although *S. epidermidis* is not able to invade host cells under physiological conditions it has been shown to bind immobilised Fn (Delmi *et al.*, 1994; Herrmann *et al.*, 1988) and this is likely to be important for the colonization of medical implants.

To date one *S. epidermidis* surface adhesin for Fn, EMBP, has been reported (Williams *et al.*, 2002) in a phage display experiment. A recombinant protein including the binding domain of EMBP also bound plasminogen and heparin but not fibrinogen, collagen, laminin or vitronectin. EMBP contains FIVAR domains that adhere to type III fibronectin repeats (Christner *et al.*, 2010). Unlike the Fn binding proteins of *S. aureus* which use Fn as a molecular bridge to invade human cells, EMBP could not fulfill this function as the type III Fn repeats must be free to adhere to integrins on human cells.

1.5.5 Aap and adhesion to host cells

An interesting theme arising from studies into *S. epidermidis* adhesins is multifunctionality, where one molecule can perform two distinct functions, and Aap may exemplify this theme. Aap was first identified due to its role in the accumulation stage of biofilm formation (Schumacher-Perdreau *et al.*, 1994) which will be discussed in section 1.6.2. However, evidence for a second role for Aap was provided more recently via a study into *S. aureus* adhesion to nasal epithelial cells (Roche *et al.*, 2003b). The structural organisation of Aap is very similar to that of two *S. aureus* surface adhesins, SasG and Pls and the A-domains of these LPXTG proteins contain a region of high sequence similarity (59 % identical amino acid sequence). SasG mediates adhesion to nasal epithelial cells as the protein conferred the ability to bind to nasal epithelial cells when heterologously expressed on *L. lactis* (Roche *et al.*, 2003b). Recombinant

A-region proteins of SasG, Pls and Aap were added exogenously to the binding assay and were found to inhibit binding implying that Aap may also bind to nasal epithelial cells via the same unknown host receptor. This suggests a possible adhesive role for Aap in nasal colonisation. *S. aureus* primarily colonises the nares whereas *S. epidermidis* is primarily found on the skin and it was one of the primary aims of this study to determine whether Aap also mediates adhesion to skin derived corneocytes.

1.5.6 Regulation of adhesins

S. epidermidis infections involve adhesion to a variety of substrates (skin, medical devices, host proteins and neighbouring bacteria in a biofilm) at different time points as the infection progresses. The regulation of surface proteins and adhesins involved in distinct stages of this process must, therefore, be under tight control. However, regulation of specific *S. epidermidis* adhesins is unclear and much of the current understanding comes from studies involving *S. aureus*. In *S. epidermidis* and *S. aureus* there are several partially characterised global regulator mechanisms including two quorum sensing systems, *agr* and *luxS*, an alternative sigma factor, σ^B, a family of global transcription factors, the SarA family, and several two-component systems responsible for sensing the external environment as discussed in a review by Novick (Novick, 2003). These regulatory mechanisms involve considerable cross talk making specific regulatory networks for individual genes difficult to unravel. The majority of adhesive cell surface proteins in both *S. epidermidis* and *S. aureus* are thought to be under the control of the main quorum sensing locus *agr* (accessory gene regulator).

1.5.6.1 The agr *locus*

The agr locus was first described in *S. aureus* (Novick *et al.*, 1995) and encodes two transcripts, RNA II and RNA III, under the control of promoters P2 ad P3 respectively (Morfeldt *et al.*, 1995). RNAII contains four genes encoding an auto-

inducing peptide, AgrD, which is processed and exported by AgrB and then detected at the cell surface by the two component system AgrA and AgrC (Fig. 1.8.). AgrA, when activated, enhances transcription of both RNAII and RNAIII by binding the P2 and P3 promoter region thus completing the auto-induction feed-back loop. RNAIII is unusual in that it is a regulatory RNA that modulates transcription of a wide variety of virulence genes and also encodes the delta-toxin. The 514 bp RNAIII molecule has a complex structure that is conserved throughout staphylococci (Benito *et al.*, 2000). RNAIII is thought to act as an antisense RNA to several mRNA transcripts from specific virulence genes and also to mRNA of several transcription factors whose targets include virulence factors. RNAIII, for example, is thought to bind to the translation initiation regions of mRNA encoding the *S. aureus* LPXTG protein, Protein-A (Novick *et al.*, 1993), and the transcription factor, Rot (Geisinger *et al.*, 2006), preventing translation and targeting the mRNA for degradation. The overall effect of an increase in transcription of RNAIII is an up-regulation of exotoxins and a down-regulation of surface proteins (Novick, 2003).

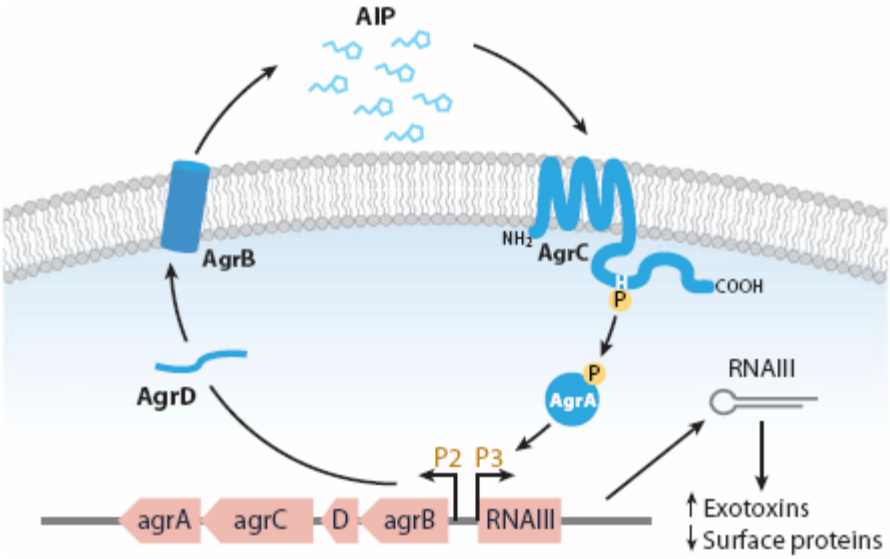

Figure 1.8: Diagram demonstrating the *agr* system. The *agr* operon is shown in pink arrows, the Agr proteins are shown in blue and the phosphate that forms part of the AgrC, AgrA two-component system is shown as a P in a yellow circle. P2 and P3 are the two promoters driving transcription of RNA II and RNA III respectively. AIP is the auto-inducing peptide. Figure taken from Novick & Geisinger (2008).

Agr dependant up-regulation of adhesive surface proteins at low cell density and down regulation at high cell density appears to fit the model for *S. epidermidis* infection. At the start of an infection when cell density is low surface proteins are expressed at a high level to allow attachment to devices and host matrix proteins. Once a biofilm becomes established and cell density increases, adhesive surface proteins are down-regulated as they are no longer required and may provide targets for the host immune system. However, the reality may not be this straightforward as some *S. epidermidis* LPXTG proteins are more prevalent in the cell wall at late exponential phase compared to early exponential phase (Bowden *et al.*, 2005).

A family of transcription factors, the SarA family, are global transcriptional regulators present in both *S. aureus* and *S. epidermidis* (Cheung *et al.*, 2008). In *S. aureus* SarA levels peak during late exponential growth and fall throughout stationary phase. SarA is thought to up-regulate the *S. aureus* Fn-binding LPXTG proteins, FnbA and FnbB, in an agr independent way (Cheung *et al.*, 1999) giving expression of FnbA and FnbB at higher cell densities than proteins regulated by agr. The *S. epidermidis* SarA homologues may be responsible for regulation of some surface proteins in this species.

Difficulties in determining regulatory mechanisms for LPXTG proteins are partly due to the cross talk between different global regulatory networks but further research into the pattern of expression of surface proteins during infection will give insight into their function in the disease process.

1.6 Biofilms of *S. epidermidis*

Biofilms of *S. epidermidis* are key to this bacterium's ability to cause serious nosocomial infection yet surprisingly little is known about the process of biofilm formation in this species and the surface molecules and mechanisms involved. The exopolysaccharide PIA has long been the primary molecule of interest as it

is known to comprise a major part of the extracellular matrix. In some strains where the *ica* operon is disrupted by insertion of a transposon biofilm formation is abolished (Mack *et al.*, 1994; Ziebuhr *et al.*, 1999). This may have lead to a simplistic view of *S. epidermidis* biofilm formation whereby expression of PIA was considered necessary and sufficient for biofilm development. More recent studies have identified proteins whose absence or inhibition resulted in a decrease in biofilm formation in some strains (see following sections). However, these studies involved little or no work into the specific stages of biofilm progression and maturation and this may have contributed to a simplistic view of *S. epidermidis* biofilms. However, there is no reason to assume *S. epidermidis* biofilms are any less complex than those of oral bacteria and *P. aeruginosa* (see section 1.2.2). This was highlighted by transcriptomics experiments performed on *S. epidermidis* biofilms at different stages in development which demonstrated genome-wide changes in gene expression at several different stages of biofilm progression suggesting different surface molecules are involved at different stages (Yao *et al.*, 2005a). This section will discuss the current knowledge of surface molecules involved in biofilm formation and the regulatory mechanisms involved.

1.6.1 Role of polysaccharide intercellular adhesin (PIA) in biofilm formation

PIA was originally discovered as a hexosamine antigen (Mack *et al.*, 1992) involved in accumulation phase of biofilm formation (Mack *et al.*, 1994) and was later found to consist primarily of a β-1,6-linked N-acetylglucosamine (Mack *et al.*, 1996) (Fig. 1.9a). Transposon mutants lacking PIA were deficient in biofilm formation but not in primary attachment to polystyrene implicating PIA in the accumulation stage (Mack *et al.*, 1994). PIA production in strains isolated from infection is common (Rohde *et al.*, 2007) and to my knowledge all PIA producing *S. epidermidis* strains in the literature can produce biofilms suggesting it has a central role in biofilm formation and virulence in most strains. Homologues of

this molecule have more recently been found to promote biofilm formation in other bacteria (Darby *et al.*, 2002; Wang *et al.*, 2004b) supporting the idea that this molecule is an important part of the biofilm matrix.

The biosynthetic operon responsible for PIA synthesis contains 4 synthetic genes *icaA, icaB, icaC* (Heilmann *et al.*, 1996b) and *icaD* (Gerke *et al.*, 1998) and an upstream negative regulator, *icaR*, that represses transcription by binding to the promoter of *icaA* (Conlon *et al.*, 2002) (Fig. 1.9b). The mechanism by which PIA promotes intercellular adhesion is unknown. It has been suggested that simple ionic interactions between positive and negative charges within the polysaccharide could play a role but an unidentified specific surface receptor protein for PIA has not been ruled out (Mack *et al.*, 2006).

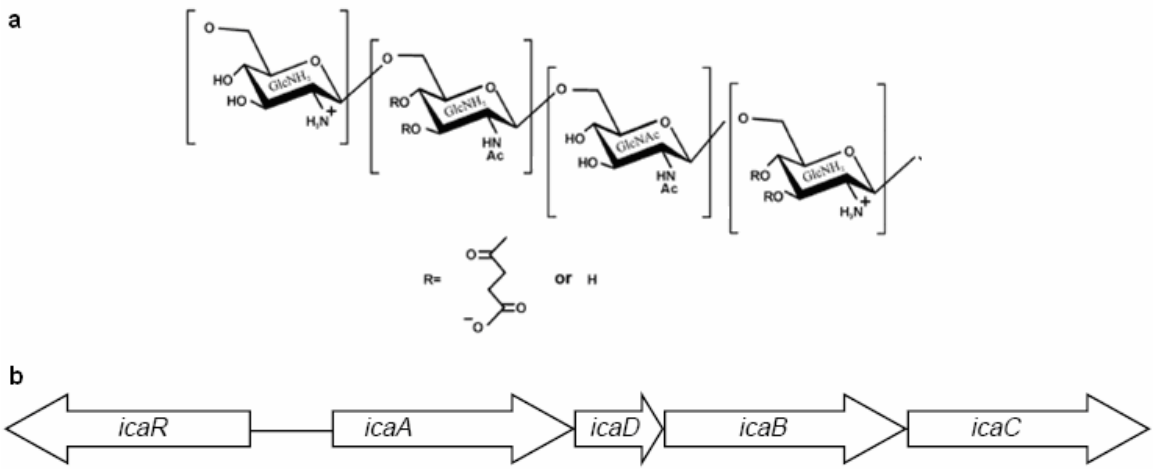

Figure 1.9. PIA structure and the *ica* operon. (a) Schematic representation showing the four different repeating units of modified N-acetyl glucosamine that make up the PIA polymer. Taken from Mack *et al.*, (2006). (b) Schematic representation of the *ica* operon. Taken from O'Gara & Humphreys (2001).

Regulation of the *ica* operon is independent of the major quorum sensing system, *agr* (Vuong *et al.*, 2003). However, the *luxS* quorum sensing system is known to repress *ica* transcription and decrease biofilm formation (Xu *et al.*, 2006). The global regulatory proteins SarA (Tormo *et al.*, 2005b) and SarZ (Wang *et al.*, 2008) both up-regulate *ica* transcription independently of

icaR and probably through binding to the *icaA* promoter directly. Additionally the alternative sigma factor, σ^B, up-regulates transcription of *ica* via repression of *icaR* (Knobloch *et al.*, 2004). A further possible mechanism of regulation involves an insertion sequence, IS256, which can cause phenotypic variation by inserting into the *ica* operon abolishing production of PIA (Ziebuhr *et al.*, 1999). Whether or not this can be regarded as a true regulatory mechanism is the subject of some debate as the excision of IS256 occurs far less frequently than insertion (10^{-8} excisions per cell generation compared to 10^{-6} insertions). However, excision is precise and does results in the restoration of PIA production and, interestingly, occurs independently of transposase activity (Hennig and Ziebuhr, 2008).

1.6.2 Role of Aap in biofilm formation

PIA was long thought to be essential for biofilm formation but Aap is now known to promote biofilm independently of PIA and it is common in clinical isolates lacking the *ica* operon (Rohde *et al.*, 2007). Aap is thought to be one of the main surface proteins responsible for biofilm formation, and therefore virulence, in *ica*$^-$ strains.

In *S. epidermidis* RP62A (*ica*$^+$) it was suggested that a ~140 kDa (size predicted from SDS PAGE) version of Aap (Hussain *et al.*, 1997), but not a ~200 kDa version of Aap expressed by a transposon mutant of the same strain (Sun *et al.*, 2005), promoted biofilm formation. It was also suggested that Aap might act as a receptor for PIA to promote intercellular adhesion. However, Aap was later found to promote biofilm formation in a PIA independent manner (Rohde *et al.*, 2005). An *ica*$^-$ catheter isolate, strain 5179, expressed Aap as two high molecular weight SDS PAGE bands (~200 and 260 kDa) and did not form a biofilm. Repeated enrichment for biofilm producing cells resulted in a biofilm producing revertant strain termed 5179-R1 which expressed a third Aap band at ~140 kDa. N-terminal sequencing was performed on this ~140 kDa band and it

was found to be a proteolytically truncated version of Aap lacking the A-domain and comprising only the B-region (Fig. 1.10). The authors did not present the N-terminal sequence for the other two Aap bands and they report that previous attempts to do so failed due to N-terminal blockade. However, a more recent study (Suh *et al.*, 2010) gave an alternative explanation. A sophisticated mass spectrometry approach showed that a single SDS PAGE band of the Aap homologue, SasG, contained multiple N-termini that differed by as much as 81 amino acids. This may also be true for Aap and could explain the failure of N-terminal sequencing of two Aap bands as multiple N-termini would make N-terminal sequencing impossible.

Based on the N-terminal sequence of the ~140 kDa Aap band a proteolytic cleavage model was proposed whereby the A-domain of Aap has to be removed in order for the B-repeats to promote biofilm formation. In support of this model, expression of recombinant full-length Aap on the surface of strain 1585 (*ica*$^-$, *aap*$^-$), did not result in biofilm formation whereas expression of the B-region only produced a biofilm (Rohde *et al.*, 2005).

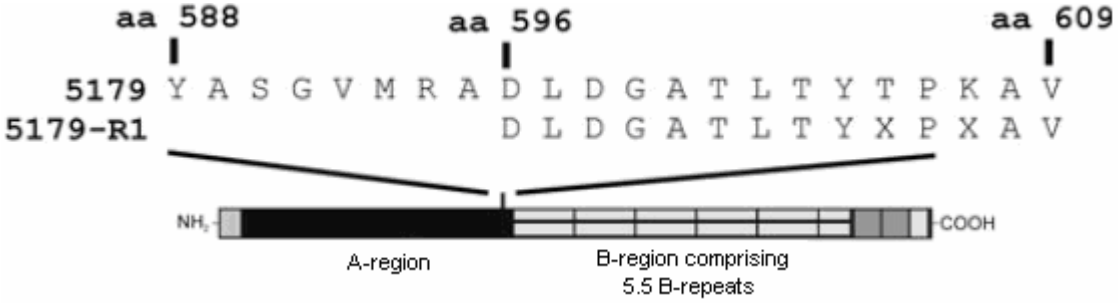

Figure 1.10: Domain organisation of Aap showing the proteolytic cleavage site in *S. epidermidis* 5179-R1. The N-terminus of the 140 kDa protein of *S. epidermidis* 5179-R1 is aligned to the predicted amino acid sequence of Aap from *S. epidermidis* 5179. The V at amino acid position 609 is the first amino acid of the B-region. Abbreviations: aa, amino acid. Figure taken from Rohde *et al.*, (2005).

The proteolytic cleavage model appeared to fit with results from previous Aap studies involving strain RP62A (Hussain *et al.*, 1997; Sun *et al.*, 2005) where

~140 kDa Aap (possibly comprising only the B-region) but not a ~200 kDa version (possibly full-length Aap) could promote biofilm formation but there are some discrepancies. Strain RP62A contains *aap* with 12.5 B-repeats (Bowden *et al.*, 2005) whereas 5179 contains only 5.5 (Rohde *et al.*, 2005) and their molecular weights, predicted from the DNA sequences, are 256 kDa and 160 kDa respectively. Therefore, the B-regions from the two strains would not both be expected to migrate to 140 kDa as proposed by the proteolytic cleavage model. In addition, more recent analysis of Aap from strain RP62A shows that it expresses Aap as two high molecular weight bands at ~230 and ~280 kDa both of which contain at least part of the A-domain (Bowden *et al.*, 2005). This has been confirmed in our laboratory where the same strain was reported to form a thick biofilm suggesting that Aap complete with the A-domain is functional in this strain (Banner *et al.*, 2007). Furthermore, the Aap homologue from *S. aureus*, SasG, promoted biofilm formation when expressed with the A-domain (Corrigan *et al.*, 2007). In this study SasG with 5, 6 and 8 B-repeats promoted biofilm formation but 4, 2 and 1 repeats were insufficient suggesting a minimum length of 5 B-repeats for functional SasG.

A more recent mechanistic study into the function of Aap found that B-regions could dimerize in 1 mM $ZnCl_2$ (Conrady *et al.*, 2008). However, the high concentrations of Zn^{2+} used in the study and the subsequent discovery that the cell wall protein AtlE (also involved in biofilm formation, see section 1.6.5) contains a Zn^{2+} ion in it's active site (Zoll *et al.*, 2010) show the need for further testing of this hypothesis. Results presented in chapter 4 of this thesis address this controversial model.

The regulatory mechanisms that control transcription of *aap* are unknown but the protein appears to be more abundant in the cell wall of late exponential phase bacteria compared to early exponential phase (Bowden *et al.*, 2005) suggesting the gene may not follow the normal pattern of down-regulation by *agr* (see section 1.5.6). Possibly contradictory to this were the results of a

transcriptomics study of *S. epidermidis* biofilms at different developmental stages (Yao *et al.*, 2005a). This study found *aap* to be down-regulated at the transcriptional level in late stage biofilms suggesting its role may be confined to early stages of biofilm development and may not be involved in the maintenance of *S. epidermidis* biofilms.

1.6.3 Extracellular matrix binding protein (EMBP) promotes biofilm formation

EMBP is a surface protein that mediates adhesion to fibronectin (Williams *et al.*, 2002) and therefore already has a role in the initial adhesion stage of biofilm formation on indwelling devices. The protein is not expressed by most strains under normal culture conditions but when calf serum is added to the medium EMBP is expressed as a ~1 MDa protein (Christner *et al.*, 2010). EMBP promoted biofilm formation in a *ica*$^{-}$, *aap*$^{-}$ negative strain showing that it functions independently of PIA and Aap. Alternating G-related albumin binding (GA) and FIVAR domains present in the C-terminal half of the molecule are thought to be required for intercellular adhesion. Given that this gene is present in a high proportion of clinical isolates EMBP may be an important virulence factor.

1.6.4 Bap homology protein (Bhp)

Biofilm associated protein (Bap) is a *S. aureus* surface protein identified in a transposon mutant deficient in biofilm formation and adhesion to polystyrene (Cucarella *et al.*, 2001). Bap was found to reduce initial adherence to host matrix proteins by interfering with MSCRAMMs (Cucarella *et al.*, 2002) but it has also been found to promote biofilm formation and long term persistence in animal models (Cucarella *et al.*, 2001). This suggests that although Bap hinders the initial stage of biofilm formation on immobilised host matrix proteins at can promote the accumulation stage. More recent work has focused on the

regulation of Bap and the gene's transcription was found to be phase-variable (Tormo *et al.*, 2007).

S. epidermidis contains a homologue of Bap termed the Bap homologous protein (Bhp). One study has implicated a role for Bhp in biofilm formation (Tormo *et al.*, 2005a) by disrupting the Bhp gene by transposon mutagenesis and showing that *S. aureus* Bap could compliment this mutation.

1.6.5 Autolysin E (AtlE) and extracellular DNA

The bifunctional autolysin AtlE which is responsible for maintenance of the cell wall and adhesion to vitronectin (see sections 1.3.6 and 1.5.2 respectively) is also thought to have a role in accumulation. Mature biofilms of several *S. epidermidis* were found to contain high concentrations of extracellular DNA in the biofilm matrix and attached to the surface of cells and biofilms could be disrupted with DNase I (Qin *et al.*, 2007). An isogenic mutant of AtlE was unable to produce extracellular DNA implicating this protein in secretion of a component of the biofilm matrix. DNA is also thought to comprise a large part of the biofilm matrix in other bacteria including *S. aureus* (Rice *et al.*, 2007) and *P. aeruginosa* (Whitchurch *et al.*, 2002).

1.6.6 *S. epidermidis* surface protein C (SesC) and biofilm formation

The LPXTG protein SesC is the surface protein most recently associated with biofilm formation (Shahrooei *et al.*, 2009). Polyclonal antibodies raised to SesC were able to prevent biofilm formation *in vitro* and were also an effective treatment in a rat model of catheter infection. The antibodies also reduced adhesion of bacteria to fibrinogen. It is difficult to determine the role of SesC specifically from this study as antibodies binding to SesC on the cell surface may also have sterically hindered other surface molecules in close proximity. Further studies involving expression of cloned SesC are required.

1.6.7 Quorum sensing, phenol soluble modulins (PSMs) and biofilm dispersal

Very little is known about biofilm dispersal in *S. epidermidis* in contrast to *P. aeruginosa* biofilms which release motile cells from mushroom-like structures (see section 1.2.2). It is not known whether detachment of *S. epidermidis* cells from biofilms is important for device related infections but studies into possible dispersal mechanisms *in vitro* suggest dispersal may occur *in vivo*. Dispersal is believed to require a reduction in adhesive factors and the production of molecules that can disrupt cell-cell interaction (Otto, 2009). PSMs are amphipathic molecules and therefore act as detergents to disrupt electrostatic and hydrophobic interactions. They are also up-regulated by the *agr* system (Vuong *et al.*, 2004a) which is known to be active in the upper layers of *S. epidermidis* biofilms (Vuong *et al.*, 2004b) where the density of metabolically active cells is greatest. Agr-dependent PSM production could disrupt cell-cell contacts in this region of the biofilm and promote cell detachment whereas *agr* signaling would be expected to down-regulate many adhesive cell surface proteins. In addition to the *agr* quorum sensing system the *luxS* system also affects *S. epidermidis* biofilms and is predicted to be active in the upper layers similar to *agr* (Xu *et al.*, 2006). The l*uxS* system which down-regulates PIA production could contribute to the reduction in adhesive forces in the upper layer of biofilms and thus promote detachment.

The discovery of quorum sensing in specific regions of *S. epidermidis* biofilms and possible mechanisms for cellular detachment serve to highlight the complexity of biofilms formed by this bacterium. The complexity is not adequately represented by the standard biofilm assay where cells are incubated in 96-well plates for 24 hours after which the wells are washed and stained. The intensity of subsequent staining gives a measure of the thickness of the biofilms. This method ignores the multiple stages involved in biofilm formation, does not take into account flow conditions and results in rapid oxygen depletion in the medium. The use of this over simplistic assay to analyse a very complex

process limits the interpretation than can come from such experiments. It is only possible to identify genes or molecules that are involved in a stage of the biofilm forming or disruption process. Studies involving more sophisticated models such as the flow-cell which allows bacteria to grow under flow conditions and allows imaging of biofilm structure by confocal microscopy or even animal models are required to further our understanding of biofilm formation and virulence in *S. epidermidis*.

1.7 Aims and objectives

This introduction has shown that *S. epidermidis* is an important nosocomial pathogen and the incidence of infections caused by this organism are increasing. It has also focused on the key virulence factors which are the large number of surface molecules that contribute to adhesion and biofilm formation. The last decade has seen a dramatic increase in the number of studies linking surface molecules with aspects of the disease process but many surface molecules remain uncharacterised or their functions are incompletely understood. This thesis will concentrate on the surface protein Aap. This fibrillar protein is known to be expressed in tufts on a subpopulation of bacteria in some strains, it may have a role in adhesion to epithelial cells and it is known to promote biofilm formation although its precise role in this process is not fully understood. The main aim of this study is to determine the functions of Aap in adhesion and to further characterize its role in biofilm formation. The main objectives are to:

1. Examine *S. epidermidis* strains for the presence of subpopulations of cells expressing Aap
    a. Use flow cytometry to accurately determine the proportion of cells in strain NCTC 11047 that express Aap
    b. Screen other wild-type strains for subpopulations and determine the ratio of Aap positive to Aap negative cells.

2. Generate a heterologous expression model for functional studies of Aap
    a. Clone several *aap* constructs with varying numbers of B-repeats and a truncated A-domain using the pUB1000 surface expression vector
    b. Express *aap* constructs in the surrogate host *L. lactis* to give relatively high and low levels of Aap surface expression.
    c. Prove Aap is expressed as fibrils on the surface of *L. lactis*

3. Use the *L. lactis* heterologous expression model to characterize the role of Aap in adhesion to corneocytes from human skin.
    a. Test the ability of the *L. lactis* surrogate host to adhere to corneocytes when expressing Aap with or without the terminal A-domain
    b. Use recombinant Aap domains to test the role of specific Aap domains in adhesion to corneocytes.
    c. Assess the importance of Aap fibril length on adhesion to corneocytes.
    d. Determine whether or not wild-type *S. epidermidis* strains rely on Aap for adhesion to corneocytes.

4. Determine the function of Aap in adhesion to polystyrene.
    a. Test the ability of the *L. lactis* surrogate host to adhere to polystyrene when expressing Aap.
    b. Express Aap on the surface of a *S. aureus* surrogate host to determine the effect of Aap expression on polystyrene adhesion in a staphylococcal background.
    c. Determine the effect of limited proteolysis on cell surface Aap and the effect that this has on polystyrene adhesion.

5. Test the ability of different domains of Aap to promote the accumulation stage of biofilm formation independently, on *L. lactis*, and in the presence of other staphylococcal surface molecules, on *S. aureus*.
    a. Test the theory that Aap B-regions dimerise to promote the accumulation stage of biofilm formation by culturing the *L. lactis* surrogate host in a biofilm assay under flow conditions.
    b. Determine the ability of full length Aap to promote accumulation when expressed on the *L. lactis* surrogate host in the absence of other Staphylococcal biofilm promoting molecules.

c. Determine the ability of the Aap B-region and full length Aap to promote biofilm formation when expressed on the *S. aureus* surrogate host to determine the contribution of the Staphylococcal cell surface to Aap function.
d. Test for *S. aureus* surface ligands that may bind Aap by attempting to block Aap-dependant biofilm formation with recombinant Aap domains.

Chapter 2:
Materials and methods

2 Materials and Methods

The following sections give detailed descriptions of the materials and methods used in results chapters 3 and 4 and this has lead to some unavoidable repetition of the methods sections within the results chapters. This chapter should be referred to when more detailed information is required. A comprehensive list of bacterial strains and plasmids is presented in Table 2.1 and a list of primers can be found in Table 2.2.

Table. 2.1. List of bacterial strains and plasmids used in this study. Aap$^+$ and Aap$^-$ are used to denote strains that express or do not express Aap respectively.

Strains	Description	Reference
S. epidermidis		
NCTC 11047 WT	nasal isolate Aap$^+$	
NCTC 11047 Fib+	sub population Aap$^+$	(Banner *et al.*, 2007)
NCTC 11047 Fib-	sub population Aap$^-$	(Banner *et al.*, 2007)
RP62A	intravenouse catheter isolate Aap$^+$	(Christensen *et al.*, 1985)
JBN 3, 8, 9 & 10	nasal isolates Aap$^+$	This study
JBJ 1, 4 & 5	joint infection isolates Aap$^+$	This study
JBC 7, 9 & 13	catheter infection isolates Aap$^+$	This study
JBS 4, 5, 14	skin isolates Aap$^+$	This study
L. lactis MG1363	surrogate host for Aap expression	(Gasson, 1983a)
S. gordonii DL1	intermediate cloning host	
S. aureus RN4220	Surrogate host for Aap expression, Restriction deficient mutant of strain 8325-4	
E. coli M15 pREP4 pQE30Xa	cloning and expression of rAap constructs	Qiagen
Plasmids		
pUB1000	*L. lactis* cell-wall-expression vector carrying erythromycin resistance	(Heddle *et al.*, 2003)
pUB100*aap6high*	pUB1000 containing *aap* gene with 6 B-repeats giving high level of expression	This study
pUB1000*aap6highT*	pUB1000 containing truncated version of *aap6* with no A-domain giving high level of expression	This study
pUB1000*aap2*, *4*, *5*, *6* & *7*	pUB1000 containing *aap* genes with 2, 4, 5, 6 or 7 B-repeats giving lower level of expression	This study
pQE30Xa rAapA-dom, sh-A-reps, B-rep & 6.5B-rep	expression of rAap proteins	This study

Table 2.2. List of primers used in this study. Underlined sequences show the restriction enzyme cleavage sites.

Primers	Description	Reference
	16S DNA sequencing of *S. epidermidis* isolates	
16SR	CCGTCAATTCGTTTCAGTTT	(Lane, 1991)
	cloning region of short repeats within A-domain	(Roche *et al.*,
raap157-857F	CCGGGATCCGCAGAAGAAAAACAAGTTGATC	2003b)
	Cloning region of short repeats within A-domain	
raap157-857R	CGGAAGCTTGATAGTTGGAACATTCGGTGCTTC	This study
	cloning aap into pUB1000	
aapFSalI	TACGCTGTCGACCCAATTACACAAGCTAATCAAAATGATAG	This study
	cloning aap into pUB1000	
aapRBamHI	TGTCGGATCCAAATTATTTTTCATTACCTTTTTTACGACG	This study
	sequencing of aap inserts	
pUB1000F	CCGTTGTCAGGTGTTTACGCT	This study
	sequencing of aap inserts	
pUB1000R	CTTTTGGTGTCTCAGGTTTGT	This study
	cloning truncated aap into pUB1000	
aapTFSalI	TACGCTGTCGACAGAGCTGATTTAGATGGTGC	This study
	cloning truncated aap into pUB1000	
aapTRSalI	TACGCTGTCGACAGCGTAAACACCTG	This study
	checking size of B-region	(Rohde *et al.*,
Aap53-608 r.c.	CATTGACATACACTCCTAAGC	2007)
	checking size of B-region	
aapR	CCAAATATGAACAATGATCCG	This study
	cloning rAap B-region	
F-BregBamHI	CCGGGATCCACATTGACATACACTCCTAAAGCA	This study
	cloning rAap B-region	
R-BregHindIII	CGGAAGCTTTGTTGGACCATACTCAACAATTTCG	This study

2.1 Bacterial strains and culture conditions.

2.1.1 *S. epidermidis* strains

S. epidermidis JB strains were isolated previously in our laboratory from the anterior nares (JBN strains) or from the skin of the forehead (JBS strains) of healthy volunteers or were donated by the University of Manchester Medical Microbiology Culture Collection, having been isolated from intravenous catheters (JBC strains) or cases of hip-joint infection (JBJ strains). Isolates were confirmed previously in our laboratory as *S. epidermidis* by both API 20 staph tests (Biomerieux Industry) and 16S rDNA sequence determination using the 16SR primer shown in table 2.2 (Lane, 1991).

S. epidermidis NCTC 11047 (ATCC 14990) is a nasal isolate that was previously found to comprise 2 sub-populations based on the presence or absence on the cell surface of lateral tufts of fibrils comprising Aap (Banner *et al.*, 2007). Previous work in our laboratory enriched for cells stably expressing the tufts (Fib^+) and cells lacking the tufts (Fib^-). The *aap* gene from NCTC 11047 was sequenced previously in our laboratory and the sequence was submitted to the GenBank database (MH 587132). Genomic DNA was extracted from *S. epidermidis* RP62A and was used as a PCR template for cloning a recombinant protein comprising 6.5 B-repeats of Aap ($rAap_{6.5B\text{-}rep}$). The genome of this strain has been sequenced (accession number NC 002976). All *S. epidermidis* strains were cultured statically in tryptic soya broth (TSB, Oxoid) at 37 °C.

2.1.2 Surrogate host strains

Streptococcus gordonii DL1 was used as an intermediate host for the cloning of pUB1000*aap* constructs and was cultured statically at 37°C in brain heart infusion broth (Oxoid) supplemented with 0.5 % yeast extract (BHY). *Lactococcus lactis* MG1363 was used as a surrogate host for Aap expression (Gasson, 1983a). The genome of this strain has been sequenced (accession

number NC 009004). This strain was cultured statically at 30 °C in M17 broth (Oxoid) supplemented with 0.5 % (w/v) glucose (GM17). *S. aureus* RN4220 (Kreiswirth *et al.*, 1983) was also used as a surrogate host for expression of Aap from pUB1000aap constructs. It is a restriction deficient mutant of strain 8325-4 (Novick, 1967) and was cultured statically at 37 °C in either TSB or heart infusion broth (HI, Oxoid) supplemented with glucose (0.5 % w/v). Routine stationary phase cultures of all strains were incubated for 18 h. Erythromycin (Sigma) (5 µg/ml) was added to media when culturing strains containing pUB1000.

2.1.3 Recombinant protein expression strain

Expression of $rAap_{A\text{-}dom}$, $rAap_{sh\text{-}A\text{-}reps}$ and $rAap_{B\text{-}rep}$ and cloning and expression of $rAap_{6.5B\text{-}rep}$ proteins were performed in *E. coli* M15 pREP4 pQE30Xa (Qiagen) cultured in Luria-Bertani broth 37 °C with aeration (200 r.p.m). Kanamycin (25 µg/ml) and ampicilin (100 µg/ml) were added to media when culturing *E. coli* containing pREP4 and pQE30Xa respectively.

2.2 Flow cytometry.

Fluorescent labelling of bacteria and flow cytometry was performed using a method based on that of Humphries *et al.* (Humphries *et al.*, 2003). Bacteria from 18 h stationary phase cultures were diluted with fresh media to an OD_{600} of 0.3 and cells from 10 ml of this suspension (~$5x10^8$ cells) were harvested by centrifugation (3,000 x g, 12 min). Cells were washed three times in phosphate buffered saline (PBS; Sigma) containing 0.02 % gelatine (PBS-gel) to remove media components and any caspsual that may have been present on some strains. Gelatine was added as a blocking agent to reduce nonspecific labelling by antibodies. After the final wash cells were resuspended in 0.5 ml of a blocking solution comprising PBS-gel containing 0.2 % normal goat serum

(Sigma, PBS-gel-NGS) and incubated for 30 min at room temperature with gentle rotation to allow goat antibodies to block epitopes on the cell surface that may have reacted with some antibodies. Rabbit anti-Aap A-domain antiserum (Banner *et al.*, 2007) was then added to the suspension at a dilution of 1:250 and incubated for a further hour to allow specific antibodies to bind to epitopes on Aap A-domains. Cells were washed in PBS-gel three times to remove unbound antibodies and resuspended in PBS-gel-NGS containing a 1:250 dilution of R-phycoerythrin conjugated donkey anti-rabbit IgG antibody (Abcam). This was incubated for 1 h to allow fluorescent antibodies to bind to the anti-Aap antibodies already bound to Aap molecules. Cells were washed to remove unbound fluorescent antibodies and resuspended in 1 ml of PBS. This was then diluted 1:100 in PBS to give ~$5x10^6$ cells ml^{-1}. This cell solution was then loaded into a Beckman Coulter Cyan ADP flow cytometer. Forward and side scatter were used to detect intact bacteria and the fluorescence (excitation at 488 nm and absorption at 530 nm) of 30,000 events (bacteria) was measured. Summit V4.3 software (Dako, Denmark) was used to analyse the data and calculate the proportion of bacteria expressing Aap on the cell surface (fluorescent bacteria). For statistical analysis, flow cytometry was repeated with samples from three independent experiments. Results were also confirmed using antiserum specific to the B-region of Aap at the same dilution as for antiserum specific to the A-domain.

To show that antiserum did not bind non-specifically to Fib^+ cells *S. epidermidis* strain NCTC 11047 (Table 2.1) was incubated with pre-immune serum instead of anti-Aap antiserum (data not shown). Using the pre-immune serum no fluorescent labelling was detected above the background level seen for bacteria that were not incubated with any fluorescent antibody. *S. epidermidis* strain (JBJ8), that does not contain the *aap* gene, did not show fluorescent labelling when cells were incubated with anti-Aap antiserum. This showed that the anti-Aap antiserum did not cross react with other surface molecules (data not shown). In addition, Western Blot of cell wall proteins from the clinical isolate

strains (Table 2.1 & section 3.4.1) was performed using anti-Aap antiserum (data not shown). Only two high molecular weight bands (both Aap) were detected on each strain indicating that antiserum did not cross react with any other surface proteins present on these strains.

2.3 Quantification of bacterial adhesion to corneocytes.

2.3.1 Preparation of cells and adhesion

Corneocytes were harvested from both hands of up to four healthy volunteers by gentle agitation of the hand inside a laboratory glove (nitrile powder-free exam glove, Kimberly Clark, USA) containing 20 ml PBS for 2min. The collection of corneocytes had full ethical approval from the University of Manchester ethics board. The corneocytes were harvested by centrifugation (3,000 x g, 10 min) and washed three times and resuspended to an OD_{440} of 0.35 (~7.0 $x10^4$ corneocytes ml^{-1}) in PBS. Stationary phase bacterial cultures were harvested by centrifugation (3,000 x g, 12 min) and washed 3 times and resuspended to an OD_{490} of 0.08 (7.0$x10^6$ cfu ml^{-1}) for *S. epidermidis* strains and 0.6 (4.2$x10^7$ cfu ml^{-1}) for *L. lactis* strains. Equal volumes (2.5 ml) of bacteria and corneocytes were then mixed in a Falcon tube (50 ml, Corning) and rotated (200rpm, to avoid cells settling) at 37 °C for 2 h for adhesion to occur.

2.3.2 Quantification of adhesion

The suspension of bacteria and corneocytes carefully applied to the top of 5 ml of a solution of 6 % (w/v) dextran (~100,000 Da, Sigma) and 0.9 % NaCl in a 15 ml falcon tube (Corning). The tube was centrifuged (1,200 *x g*, 5 min) to pellet the corneocytes and leave the unbound bacteria in a band higher up the dextran solution. The top 5 ml was discarded from the tube by pipetting and the remaining solution was centrifuged at (3,300 x g, 5 min). The resulting pellet containing corneocytes, bound bacteria and some remaining unbound or weakly

bound bacteria was resuspended in 1 ml PBS and applied to the top of another dextran solution (5 ml). This process was performed three times in order to remove all unbound bacteria. Finally, the pellet containing corneocytes and bound bacteria was resuspended in double distilled water ($_{dd}H_2O$, 100 µl) and samples (50 µl) applied to microscope slides. The slides were left to dry at room temperature over night (~ 18 h). The slides were Gram stained in order to stain the bound bacteria dark purple and the corneocytes pale pink. The number of bacteria per corneocyte were counted for 30 corneocytes using a light microscope with a 100 x objective lens. To test reproducibility, three batches of cells were tested in the assay and two slides were counted (2 x 30 corneocytes) for each batch.

2.3.3 Inhibition of adhesion using recombinant proteins

For blocking experiments the corneocytes were preincubated with 40 µl recombinant Aap (rAap) domain proteins (see below) in PBS before addition of bacteria. Final concentrations of 0.05, 0.2 or 1.0 µM were used for each protein and 40 µl of PBS was used for a negative control. Corneocytes and rAap were preincubated for 20 min at 37 °C at 200 rpm before 2.5 ml of bacterial suspension was added and the assay was performed as described above.

2.4 Construction of recombinant His_6-tagged fusion proteins.

2.4.1 rAap constructs and cloning strategy

rAap proteins corresponding to a single B-repeat ($rAap_{B\text{-}rep}$) and the region of short repeats within the A-domain ($rAap_{sh\text{-}A\text{-}reps}$) were constructed previously in our laboratory using genomic DNA from *S. epidermidis* NCTC 11047 (accession number HM 587132) and the expression vector pQE30Xa (Table 2.1). The construct $pQE30Xa_{A\text{-}dom}$ (Table 2.1), used for expression of a rAap protein corresponding to the full A-domain ($rAap_{A\text{-}dom}$), was a kind gift from Prof. Tim

Foster, Trinity College, Dublin to our laboratory and was generated using genomic DNA from *S. epidermidis* RP62A.

In addition to the above a rAap protein corresponding to 6.5 B-repeats was constructed. The *aap* gene of *S. epidermidis* RP62A was used as template DNA and the gene fragment was cloned and expressed using the pQE30Xa expression vector and *E.coli* M15 pREP4 (Qiagen). This expression vector allows DNA to be inserted down stream of a *lacZ* promoter which is repressed by the *lac* repressor, *lacI*, contained in the pREP4 DNA sequence. Addition of IPTG to M15 pREP4 pQE30 cultures inhibits the LacI repressor protein allowing transcription from the *lacZ* promoter. The pQE30Xa sequence immediately upstream of the DNA insert site and downstream of the ATG start codon encodes six histidine residues (His_6) meaning that the inserted DNA is fused at the N-terminus to a His_6 tag allowing purification of the recombinant proteins with immobilised Ni^{2+} ions.

2.4.2 Construction of pQE30Xa$_{6.5B\text{-}rep}$

Genomic DNA was extracted from RP62A using a Qiamp DNA min kit (Qiagen) with some modifications. Bacteria from 3 ml of a stationary phase culture were harvested by centrifugation in a table-top microcentrifuge (13,000 r.p.m., 30 s). The cell pellet was resuspended in 144 µl of lysis buffer (20 mM Tris HCl pH8.0, 2 mM EDTA, 1.2 % Triton) and 36 µl of lysostaphin (1 mg/ml in $_{dd}H_2O$) was added. The suspension was incubated at 37 °C for ~ 20 min until the solution became clear. Then 20 µl proteinase K (Qiagen) was added and the manufacturers protocol was followed. Primers F-BregBanHI and R-BregHindIII (Table 2.2) that contained HindIII and BamHI recognition sites were used to PCR amplify a 5 kb region including the full B-region of *aap* (12.5 B-repeats) and the product size was confirmed by gel electrophoresis (0.8 % agarose gel). B-region DNA from a 50 µl PCR was purified using a PCR purification kit (Qiagen) according to the manufacturers instructions. DNA was treated with HindIII and

BamHI restriction enzymes (Roche, 10 U each, 1 h, 37 °C) and purified to remove small DNA fragments and enzymes using a PCR purification kit (Qiagen). Vector DNA (pQE30Xa) was cleaved with the same restriction enzymes and the linearised plasmid was purified with a PCR purification kit (Qiagen). DNA concentrations were determined by measuring the A_{260} using a nano-drop (PEQLAB) and the insert and vector DNA were mixed at a ratio of 1:1 and ligated using T4 DNA ligase (Roche, 18 h, 4 °C) according to manufacturers instructions.

2.4.3 Preparation of competent *E. coli* M15 pREP4

The method used to prepare competent cells was based on that of Mandel and Higa (Mandel and Higa, 1970). A stationary phase culture of *E. coli* M15 pREP4 (1 ml aliquot) was inoculated into 100 ml fresh pre-warmed LB broth and incubated with shaking (37 ^{o}C, 200 r.p.m.) until the OD_{600} reached 0.5. The culture was cooled on ice for 5 min, and transferred to a sterile, round-bottom centrifuge tube. The cells were then collected by centrifugation (3,000 x *g*, 10 min, 4 °C) and were re-suspended gently in ice cold 100 ml of sterile transformation buffer 1 (TFB1; 100 mM RbCl, 50 mM $MnCl_2$, 30 mM potassium acetate, 10 mM $CaCl_2$, 15 % glycerol, pH 5.8). The cell suspension was then incubated on ice for 30 min. Cells were collected by centrifugation (3,000 x *g*, 10 min, 4 °C) and re-suspended in 4 ml of ice cold sterile transformation buffer 2 (TFB2; 10 mM 3-(N-morpholino)propanesulfonic acid (MOPS), 10 mM RbCl, 75 mM $CaCl_2$, 15 % glycerol, adjusted to pH 6.8 with KOH). Cells were incubated on ice for 2 h. Competent cells were then aliquoted into chilled 1.5 ml microfuge tubes in 100 µl volumes and were snap-frozen in liquid nitrogen and stored at -80 ºC. Before transformation cells were allowed to thaw for at least 15 min on ice.

2.4.4 Transformation of *E. coli* M15 pREP4 with pEQ30Xa$_{6.5B\text{-}rep}$

An aliquot of ligation mix (2 µl) was added to an aliquot (50 µl) of thawed competent *E. coli* M15 pREP4 and incubated on ice for 15 min. The cells were then heat shocked (42 °C, 90 s) and 0.5 ml ice cold LB medium was added immediately afterwards. Cells were incubated on ice for 5 min and were then incubated at 37 °C for a further 1 h. Cells were then spread on LB agar plates and incubated at 37 °C. Hundreds of transformant colonies were clearly visible after 18 h. Colonies were screened for the correct insert size by colony PCR using QE2F and QE2R primers (Table 2.2). All of the colonies tested (80) contained an insert that was smaller than the expected 5 kb (the full *aap* B-region). The transformant with the largest insert size (2.8 kb) was selected and the plasmid purified using a plasmid mini-prep kit (Qiagen). This was then partially sequenced using QE2F and QE2R primers that annealed to the vector DNA at either side of the insert site and were directed across the insert. The inserted sequence was found to be that of the *aap* B-region except the 5th repeat was fused to the 11th repeat at approximately the midpoint of each repeat. Repeats 6-10 were lost apparently due to homologous recombination. This appeared to have occurred for almost every transformant tested and 6 full and one partial repeat was the maximum number of repeats that could be obtained. This construct was termed pQE30X*rAap*$_{6.5Brep}$ (Table 2.1).

2.4.5 Small scale expression of rAap$_{6.5B\text{-}rep}$

To ensure that *E. coli* M15 pREP4 pQE30Xa$_{6.5B\text{-}rep}$ expressed rAap$_{6.5B\text{-}rep}$ in sufficient quantity and that the protein was not degraded a 10 ml culture was induced to express rAap and the cell lysate was analysed by SDS PAGE. A stationary phase culture of *E. coli* M15 pREP4 pQE30Xa$_{6.5B\text{-}rep}$ was inoculated into 10 ml of fresh LB medium and incubated at 37 °C with shaking (200 r.p.m.) until the OD_{600} reached ~ 0.5. Expression of rAap was then induced by addition of IPTG (final concentration 0.5 mM) and the culture was incubated for a further 4 h. The bacteria were then harvested by centrifugation (3,000 x g, 10 min) and

lysed by resuspending the pellet in lysis buffer (50 mM Tris-HCl pH 8.0, 2 % SDS, 5 mM EDTA) and heating at 100 °C for 2 min. After centrifugation in a table-top microcentrifuge (13,000 r.p.m., 5 min) the supernatant of the induced and also an uninduced culture were analysed by SDS PAGE (4 – 20 % precast gradient gel, NuSep). The induced culture gave one intense band at 120 kDa that was not present in the uninduced culture. This band was confirmed as the B-region of Aap by Western Blotting (see below) using antiserum specific to a single B-repeat of Aap which was generated previously for our lab (Banner *et al.*, 2007).

2.4.6 Large scale expression of rAap proteins

All the rAap proteins ($rAap_{A\text{-}dom}$, $rAap_{sh\text{-}Areps}$, $rAap_{B\text{-}rep}$ and $rAap_{6.5B\text{-}rep}$) were expressed in 2 L cultures of M15 pREP4 containing the appropriate $pQE30Xa_{rAap}$ construct (Table 2.1). When the culture reached an OD_{600} of 0.5 IPTG was added (0.5 mM) and the culture incubated for a further 4 h. After incubation cells were harvested by centrifugation (3,000 x g, 12 min, 4 °C) and the cell pellet frozen at ¯20 °C for storage.

2.4.7 Purification of rAap proteins

The cell pellet was then thawed and resuspended in 30 ml of ice cold lysis buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 1 mM phenylmethylsulphonyl fluoride [PMSF, Sigma]). The cell suspension was then loaded into a large French Press cell (Thermo) which had been cooled to 4 °C. Bacteria were lysed by 2 rounds of French Pressing and the sample was kept on ice as much as possible. The lysate was then centrifuged (15,000 x g, 20 min, 4 °C) to remove cell debris and the supernatant containing soluble proteins was kept.

Ni^{2+} ion chromatography was used to purify the rAap proteins. A HisTrap column (5 ml column volume [cv], Amersham biosciences) was connected to a peristaltic pump and equilibrated with 5 cv equilibration buffer (50 mM Tris-HCl

pH 8.0, 300 mM NaCl) at a flow rate of 5 ml/min. The soluble fraction of the cell lysate was then applied to the column followed by 5 further cv of equilibration buffer. In order to remove any *E. coli* proteins from the column that may have contained several His residues a wash step was performed with 5 cv of wash buffer (equilibration buffer containing 10 mM imidazol for $rAap_{A\text{-}dom}$, $rAap_{sh\text{-}Areps}$ and $rAap_{B\text{-}rep}$ and 40 mM imidazol for $rAap_{6.5B\text{-}rep}$). Elution of rAap was performed with 10 ml of elution buffer (equilibration buffer containing 250 mM imidazol). All column fractions were collected and samples were analysed by SDS PAGE. The $rAap_{6.5B\text{-}rep}$ was present at low levels in the 40 mM imidazol wash fraction along with larger amounts of other unidentified *E. coli* proteins. In the elution fraction, however, $rAap_{6.5B\text{-}rep}$ was present at a high concentration and was estimated (by SDS PAGE with coomassie staining) to be ~ 80 % pure. The elution fractions of $rAap_{A\text{-}dom}$, $rAap_{sh\text{-}Areps}$ and $rAap_{B\text{-}rep}$ contained respective rAap proteins at greater than 90 % purity (estimated by SDS PAGE). The elution fractions were dialysed into PBS overnight at 4 °C. The solutions were then concentrated using a Vivaspin concentration column (Sartorius) with a 10 kDa molecular weight cut off.

The $rAap_{A\text{-}dom}$ and $rAap_{sh\text{-}A\text{-}reps}$ proteins were analysed by SDS PAGE and they migrated more slowly than was expected based on the gene sequences (data not shown). The $rAap_{A\text{-}dom}$ and $rAap_{sh\text{-}A\text{-}reps}$ proteins were predicted to be 61 kDa and 27 kDa respectively whereas they migrated to ~ 15 kDa and ~ 50 kDa respectively. This was not due to post translational modification of the proteins as their masses (accurately determined by electrospray mass spectrometry) were consistent with the gene sequences (data not shown). The slow migration is unlikely to be due to oligomer formation or due to the proteins maintaining secondary structure on the SDS PAGE gel because running the gel in the presence of 8 M urea did not alter the migration of rAap. The slow migration is probably due to the unusually high proportion of acidic amino acids in the A-repeats as slow migration was previously reported for proteins containing high proportions of such amino acids (Kingsley *et al.*, 2004).

2.4.8 Determination of protein concentration

Protein concentration was determined by the Bradford assay using a Protein Assay Kit I (Biorad) and standard curve using BSA according to the manufacturers instructions. This was confirmed by measuring the absorption of rAap solutions at 280 nm (A_{280}) and converting this to protein concentration by the method of Gill and Von Hippel (Gill and von Hippel, 1989). This method relies on the fact that only tryptophan, tyrosine and cysteine amino acids absorb light at a wavelength of 280 nm. The molar extinction co-efficient (E, $M^{-1}cm^{-1}$) of the protein is therefore equal to the sum of the number of each amino acid multiplied by the respective molar extinction co-efficient of that amino acid as follows:

$$\mathbf{E = [n(Tyr) \times E(Tyr)] + [n(Trp) \times E(Trp)] + [n(Cys) \times E(Cys)]}$$

where n(amino acid) is equal to the number of that amino acid in the protein sequence and E(amino acid) is the molar extinction co-efficient of that amino acid. The concentration was calculated from the A_{280} of each protein using the Beer-Lambert Law:

$$\mathbf{Concentration = A / Eb}$$

where A is equal to the A_{280} of each protein, E is the molar extinction co-efficient and b is the path length. Aliquots of rAap were snap frozen in liquid nitrogen in the presence of 10 % glycerol and stored at ˉ80 °C.

2.5 Expressing recombinant Aap in L. lactis MG1363.

2.5.1 Cloning strategy and construction of pUB1000*aap6high*

Aap of *S. epidermdis* NCTC 11047 was expressed on the surface of *L. lactis* MG1363 using the expression vector pUB1000 (Heddle *et al.*, 2003). The pUB1000 vector contains a cell-wall-protein expression cassette comprising a constitutive lactococcal promoter fused to the N-terminal signal sequence of the cell-wall-associated protein *sspA* from *S. gordonii*. Cloning *aap* into the BamHI/SalI cloning site of pUB1000 generated a fusion between the signal sequence of *sspA* and the *aap* gene such that the fusion protein was correctly directed for export through the Sec pathway in *L. lactis* MG1363. The readily transformable *S. gordonii* DL1 was used as an intermediate cloning host to generate the pUB1000*aap* constructs (Table 2.1) before electroporating them into *L. lactis* MG136.

The pUB1000*aap6high* construct (Table 2.1) was created in *S. gordonii* DL1 by Jane Brittan of Howard Jenkinson's laboratory at Bristol University. Confirmation of the correct *aap* insert in the pUB1000*aap6high* construct and confirmation of protein expression was performed by myself. The other pUB1000 constructs (Table 2.1) were generated by myself and electroporated into *L. lactis* MG1363 after a visit to Howard Jenkinson's laboratory with supervision from Jane Brittan.

2.5.2 Construction of pUB1000*aap* constructs

Genomic DNA from *S. epidermidis* NCTC 11047 was extracted as described above (section 2.4.2) and the *aap* gene (accession number HM587132) was PCR amplified using expand high fidelity long template DNA polymerase (Roche) according to manufacturers instruction. Primers aapFSalI and aapRBamHI (Table 2.2) were used to amplify a 7195 bp fragment of the *aap* gene from immediately downstream of the N-terminal signal sequence to the stop codon. The ~ 7195 bp PCR product was separated by agarose gel

electrophoresis (0.8 % agarose) and was purified from the agarose gel using a Gel Extraction Kit (Qiagen). The *aap* insert DNA and pUB1000 vector were then digested with SalI and BamHI (Roche) according to manufacturers instructions. Ligation was performed using a 1:1 ratio of insert and vector DNA and T4 ligase (Roche) according to the manufacturer's instructions.

2.5.3 Transformation of *S. gordonii* DL1

S. gordonii DL1 was used as an intermediate cloning host to generate pUB1000*aap* constructs that could then be electroporated into competent *L. lactis* MG1363. Competent *S. gordonii* DL1 bacteria were prepared according to a published method (Haisman and Jenkinson, 1991). A stationary phase *S. gordonii* DL1 culture was used to inoculate a fresh pre-warmed aliquot (5 ml) of BHY medium containing 1 % fetal bovine serum (Sigma) and 0.1 % glucose (BHY/FBS/Gluc). This was incubated for 90 min at 37 °C until the OD_{600} reached 0.3 and an aliquot (50 µl) was added to fresh pre-warmed BHY/FBS/Gluc. This was incubated for a further 60 min and then aliquots (800 µl) were placed in 1.5 ml tubes (Eppendorf). Either plasmid DNA or ligation mix was then added (~ 3 µl containing ~ 1 µg of DNA) and the tubes were incubated for 4 h at 37 °C. As a negative control 3 µl of sterile $_{dd}H_2O$ was added. Cell suspensions were then spread on BHY agar containing 5 µg/ml erythromycin and incubated for at least 24 h to allow growth of transformants.

2.5.4 Screening of *S. gordonii* DL1 transformants for expression of different sizes of Aap on the cell wall

In order to determine whether or not Aap was correctly targeted and anchored to the cell wall transformants were screened for Aap expression by SDS PAGE of cell wall proteins and Western Blot with antiserum specific to the A-domain of Aap (see below). The majority of the 20 transformants tested expressed Aap on the cell wall as two high molecular weight bands by SDS PAGE (Aap is

expressed as 2 high molecular weight bands in WT *S. epidermidis* strains). Five *S. gordonii* DL1 transformants were indentified that each expressed a different size of Aap.

2.5.5 Preparation of plasmids from *S. gordonii* DL1 (also used for *L. lactis* MG1363)

Plasmids were purified from clones expressing five different sizes of Aap using a miniprep kit (Qiagen) with some modifications. Stationary phase cultures of the transformants (7 ml) were harvested by centrifugation (3,000 x g, 10 min) and resuspended in 100 µl of a Tris-glucose solution (50 mM Tris-HCl pH 8.0, 25 % glucose [w/v]). To this suspension was added 100 µl of TES (50 mM Tris-HCl pH 8.0, 5 mM EDTA, 150 mM NaCl), 10 µl of RNase solution (10 mg/ml, Sigma), 10 µl of mutanolysin solution (10,000 U/ml, Sigma) which cleaves the β1-4 linkage of cell wall peptidoglycan, and 20 µl of Lysozyme solution (10 mg/ml, Sigma). This was incubated at 37 °C for 1.5 h to allow enzymatic digestion of the cell wall. Following this a miniprep kit (Qiagen) was used from stage 2 (addition of 250 µl buffer P2) of the manufacturer's instructions. Plasmid DNA was finally eluted in $_{dd}H_2O$ and stored at ¯20 °C. At this stage pUB1000 constructs containing *aap* were digested with BamHI and SalI to confirm the insert sizes. Further confirmation of the *aap* insert sequence was performed after electroporation of pUB1000*aap* constructs into *L. lactis* MG1363 (see below).

2.5.6 Preparation of competent *L. lactis* MG1363

Electrocompetent *L. lactis* MG1363 were prepared as described previously (Hartford *et al.*, 2001a; Wells *et al.*, 1993). Fresh pre-warmed GM17 medium containing 2.5 % glycine (100 ml, GM17G) was inoculated with a 2 ml aliquot of stationary phase MG1363 and incubated for ~ 6 h at 30 °C until the OD_{600} reached 0.5. The suspension was chilled on ice for 10 min and the cells were harvested by centrifugation (3,000 x g, 10 min, 4 °C). The cell pellet was washed twice in 20 ml of wash buffer (0.5 M sucrose, 10 % glycerol v/v, 4 °C). The pellet

was finally resuspended in 1 ml wash buffer and aliquots (40 µl) were snap frozen in liquid nitrogen and stored at ⁻80 °C.

2.5.7 Electroporation of *L. lactis* MG1363

Plasmids from *S. gordonii* DL1 pUB1000aap strains were electroporated into *L. lactis* MG1363 by a published method (Hartford *et al.*, 2001b; Wells *et al.*, 1993). Aliquots of electrocompetent *L. lactis* MG1363 were allowed to thaw on ice for 15 min before plasmid DNA from *S. gordonii* was added (~ 3 µl containing ~ 1 µg DNA). Cells and DNA were then transferred to a chilled electroporation cuvette (0.1 cm cuvette, BioRad) and electroporated (100 Ω, 50 µf, 1 kV). Immediately following electroporation recovery medium was added to the cuvette (950 µl, GM17G containing 0.5 M sucrose, 20 mM $MgCl_2$, 2 mM $CaCl_2$) and the cuvette was incubated on ice for 10 min and incubated for a further 2 h at 30 °C. Cells were then spread on GM17 agar containing 5 µg/ml erythromycin and incubated for at least 24 h at 30 °C to allow transformants to grow.

2.5.8 Determination of the number of B-repeats in each pUB1000*aap* construct and partial sequencing

Plasmids were extracted from MG1363 transformants by the same method used for *S. gordonii* DL1. The number of B-repeats in each *aap* gene were calculated from the lengths of the B-repeat regions determined by PCR of the insert regions using primers Aap53-608 r.c and aapR (Table 2.2), which anneal on either side of the B-repeat region. Primers pUB1000F/R (Table 2.2), which anneal either side of the BamHI/SalI cloning site, were used to confirm the sequence of the 5' and 3' ends of the *aap* gene. Plasmids were isolated that contained 2, 4, 5, 6 and 7 B-repeats and these were termed pUB1000*aap2*, *4* ,*5* ,*6* & *7* respectively. One construct was found to express Aap with 6 B-repeats at a higher cell surface density than the others (see below, 2.8) and this was termed pUB1000*aap6high*.

2.5.9 Generation of truncated pUB1000*aap* constructs lacking the A-domain

pUB1000*aap6highT*, a truncated derivative of pUB1000*aap6high* with the entire A-domain removed was generated using primers aapTFSalI and aapTRSalI (Table 2.2) and pUB1000*aap6high* as template DNA with expand high fidelity long template DNA polymerase (Roche). The forward primer annealed immediately upstream of the B-repeat region and the reverse primer annealed immediately upstream of the A-domain. The PCR product, comprising the full pUB1000 backbone and the B-region of *aap* with a SalI cut site at either end (8.8 kb), was digested with SalI (Roche) and self-ligated with T4 ligase (Roche) according to manufacturer's instructions. It was then electroporated directly into *L. lactis* MG1363 as described above. Primers pUB1000F and pUB1000R (Table 2.2) were used to confirm the correct insert sequence.

2.6 Western Blot of cell wall proteins

2.6.1 Extraction of cell wall proteins from *L. lactis* MG1363 and *S. gordonii* DL1

Cells wall proteins were extracted from stationary phase cultures of *S. gordonii* DL1 and *L. lactis* MG1363 (20 ml, OD_{600} ~ 2.5) using mutanolysin (Sigma) as described previously (Demuth *et al.*, 1996). Bacteria from stationary phase cultures (20 ml) were harvested by centrifugation (3,000 x g, 12 min) and washed once in PBS. Cells were resuspended in spheroplasting buffer (50 µl; 20 mM Tris-HCl pH 6.8, 10 mM $MgCl_2$, 26 % (w/v) raffinose.$5H_2O$) and the cell wall degrading enzyme mutanolysin (final concentration of 500 Uml^{-1}; Sigma) was added along with 1 mM PMSF. This suspension was incubated for 15 min at 37 °C to digest the cell wall and release cell wall bound proteins without lysing the cells. This suspension was then centrifuged in a table-top microcentrifuge (13,000 x g, 5 min) to remove the spheroplasts from solution. The supernatant, containing cell wall proteins was analysed by SDS PAGE.

2.6.2 Extraction of cell wall proteins from *S. aureus* RN4220

Cell-wall proteins were extracted from stationary phase cultures of *S. aureus* RN4220 (30 ml) by a similar method. Cells were washed once in PBS then resuspended in 50 µl spheroplasting buffer (20 mM Tris-HCl [pH 6.8], 10 mM $MgCl_2$, 26% [wt/vol] raffinose · $5H_2O$) containing lysostaphin (0.2 mg/ml) which cleaves the penta-glycine peptide cross-links in staphylococcal cell walls, lysozyme (0.4 mg/ml) and PMSF (1 mM) and incubated at 37 °C for 25 min to release cell-wall proteins without lysing the cells. Cell were then centrifuged (13,000 rpm for 5 min) and the supernatant containing soluble cell-wall proteins was analysed by SDS PAGE (8% acrylamide gel).

2.6.3 SDS PAGE and Western Blot

Cell wall proteins were separated by SDS PAGE (8 % gel) and blotted onto a PVDF membrane. Brief staining of the PVDF blot after blotting with Ponceau S (Sigma) was performed to ensure equal transfer of proteins from the different lanes of the SDS PAGE gel. The blot was destained in $_{dd}H_2O$ for 15 min. The membrane was then incubated with blocking buffer (6 % [w/v] skimmed milk [Oxoid] in PBS containing 0.05 % Tween 20 [Sigma]) overnight at 4 °C. Rabbit anti-Aap A-domain antiserum at a dilution of 1:5000 or anti-Aap B-repeat antiserum at a dilution of 1:1000 (Banner *et al.*, 2007) in blocking buffer was incubated with the blot at room temperature for 1 h. The blot was then washed 3 times (5 min for each wash) in PBS-Tween and a horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:35,000, Sigma) was then incubated with the blot. The blot was washed a further 4 times. Proteins were visualised using Western Lightning chemiluminescence reagent (PerkinElmer, UK) and photo graphic film.

2.6.4 SDS PAGE and Western Blot of trypsin treated cells

For analysis of cell wall proteins from trypsin treated *L. lactis* MG1363 pUB1000*aap6*, trypsin treatment was carried out as described previously (Veenstra *et al.*, 1996). A single stationary phase culture (20 ml) was split in to two 10 ml aliquots. To one aliquot 100 µl trypsin solution (10 µg/ml in 100 mM HCl, TPKC treated to remove chymotrypsin, Sigma) was added and, as a negative control, 100 µl HCl (100 mM) was added to the other aliquot. Both 10 ml aliquots were then were incubated at 37 °C for 10 min. Cells were then washed three times in PBS at 4 °C to remove trypsin and resuspended in spheroplasting buffer. The procedure described above was then followed (2.6.1).

2.7 Transmission electron microscopy.

2.7.1 Negative staining of bacteria and imaging in the TEM

Cell surface structures were analysed by negative staining with 2 % methylamine tungstate (MT, Agar Scientific) at pH 6.5 in the TEM as described previously (Banner *et al.*, 2007; Handley *et al.*, 1985). Copper grids (400 mesh, Agar Scientific) were coated with 0.2 % (w/v) formvar in chloroform and carbon-coated (Bio-Rad E6200 Carbon Coater). Prior to use grids were plasma-glowed (Plasma Barrel Etcher PT7150, Fisons, UK) at 20 mV for 15 s to render the grid surface hydrophilic. Stationary phase bacteria (5 ml) were harvested by centrifugation (3,000 x g, 10 min) and washed twice in $_{dd}H_2O$ and resuspended in 100 µl $_{dd}H_2O$. An aliquots of the suspension (10 µl) was applied to a grid and 10 µl MT (2 % [w/v], pH 6.5) was then applied also. Excess fluid was carefully drawn off the grid using blotting paper. The grid was inserted into a FEI Tecnai 12 electron microscope (FEI Company, Eindhoven, The Netherlands) and viewed at 100 kV at magnifications up to x 80,000 to visualise individual Aap fibrils.

2.7.2 Immunogold labelling of the bacterial cell surface

Immunogold negative staining was performed with anti-Aap A-domain antiserum and a secondary anti-rabbit IgG conjugated to 10 nm gold particles (Agar Scientific, UK) (Banner *et al.*, 2007; McNab *et al.*, 1999). Cells were harvested, washed twice and resuspended in $_{dd}H_2O$ as above (2.7.1). A grid was treated as above and inverted onto a drop (20 µl) of cell suspension and incubated at room temperature for 5 min to allow cells to attach. The grid was then inverted onto a drop of Anti-Aap A-domain antiserum (diluted 1:10) in immunogold buffer (0.05 M Tris-HCl pH 8.0 containing 1 % w/v ovalbumin, 0.1 % [w/v] gelatin and 0.05 % [v/v] Tween 20) and incubated at room temperature for 30 min. The grid was then washed 5 times by inverting the grid on 5 individual drops of immunogold buffer (5 min each drop). The grid was then incubated with secondary immunogold-conjugated antiserum for 30 min and washed 5 times with $_{dd}H_2O$. The grid was then negatively stained by adding MT and viewed in the TEM as above (2.7.1).

2.8 Whole-cell immunoblotting.

The method of Corrigan *et al.* (Corrigan *et al.*, 2007) was used to determine the relative surface density of Aap on *L. lactis* MG1363 pUB1000*aap* strains (Table. 2.1). Stationary phase cells were washed and resuspended to an OD_{600} of 2 in PBS. Doubling dilutions of bacterial suspensions (10 µl) were then spotted on to a PVDF membrane (Bio-rad). The membrane was blocked, probed with anti-Aap A-domain antiserum and developed as for Western blots (see above).

2.9 Electroporation of pUB1000*aap6high* and *aap6highT* into *S. aureus* RN4220

Aap was expressed on the cell surface of a second surrogate host, *S. aureus* RN4220. The pUB1000*aap6high* and *aap6highT* constructs from *L. lactis* MG1363 (see section 2.5.5) were electroporated into RN4220 and Aap was

expressed on the cell wall. Electrocompetent RN4220 cells were prepared and electroporated according to a previously published method (Kraemer and Iandolo, 1990).

2.9.1 Preparation of electrocompetent *S. aureus* RN4220

Fresh pre-warmed TSB medium (100 ml) was inoculated with a 2 ml aliquot of stationary phase RN4220 and incubated for ~ 3 h at 37 °C with shaking (200 r.p.m) until the OD_{600} reached 0.5. Cells were harvested by centrifugation (3,000 x g, 10 min) and resuspended in 100 ml of sucrose solution (0.5 M) before being harvested again. Cells were then resuspended in 50 ml sucrose solution and left on ice for 20 min to deplete the intracellular ion pool as much as possible. Cells were then harvested and resuspended in 10 ml sucrose solution and aliquots (200 µl) were snap frozen in liquid nitrogen for storage at $^{-}$80 °C.

2.9.2 Electroporation of *S. aureus* RN4220

Electrocompetent RN4220 cells were thawed at room temperature and ~ 2.5 µg (~ 5 µl) of plasmid DNA was added to 80 µl bacteria. This suspension was incubated at room temperature for 25 min in an electroporation cuvette (0.2 cm cuvette, BioRad). Cells were then electroporated (100 Ω, 25 µf, 2 kV) and 800 µl SMMP50 electroporation recovery medium (5.5 parts SMM buffer [1 M sucrose, 0.04 M maleic acid, 0.04 M $MgCl_2$ pH 6.5], 4 parts 7 % Antibiotic Medium 3 [Difco] and 0.5 parts 10 % BSA) was added immediately afterwards. Cells were then incubated for 3.5 h at 37 °C before cells were spread on TSB agar plates containing 5 µg/ml erythromycin. Hundreds of colonies appeared after 24 h incubation at 37 °C.

2.9.3 Confirmation of *aap* insert in pUB1000*aap6high* & *aap6highT*

Plasmid DNA was extracted from 3ml cultures of RN4220 using a miniprep kit (Qiagen) according to the manufacturer's instructions with the following

modification. In order to digest the cell wall cells were resuspended in 250 µl buffer P1 (Qiagen) containing 5 µl lysostaphin solution (5 mg/ml) and incubated for 30 min. The manufacturer's instructions were followed from this point and plasmid DNA was eluted in $_{dd}H_2O$. The plasmids were digested with BamHI and SalI (Roche) according to the manufacturer's instructions and the products were analysed by agarose gel electrophoresis to check the correct size of *aap* insert DNA. Primers pUB1000F and pUB1000R (Table 2.2), which anneal either side of the BamHI/SalI cloning site, were used to confirm the sequence of the 5' and 3' ends of the *aap* gene.

2.10 Quantification of adhesion to tissue culture treated 96-well plates

A previously published method (Banner *et al.*, 2007) was used to quantify adhesion to the model abiotic surface, tissue culture treated polystyrene, with some modifications. Stationary phase bacterial cultures were washed 3 times in PBS and resuspended to an OD_{490} of 1.0. Aliquots of cell suspensions (200 µl) were then added to flat bottomed tissue culture treated 96-well plates (Corning) and incubated at 37 °C for 2 h. For each experiment 8 wells were used. Wells were then washed 3 times with PBS (200 µl), dried at 42 °C for at least 3 h and stained with 4 % (v/v) crystal violet (CV) for 10 min. Excess CV was washed out of the wells with distilled water and ethanol (200 µl) was used to solubilise the remaining stain. OD_{530} of the well was then measured to quantify adhesion. Unmodified polystyrene surfaces are hydrophobic but tissue culture treatment involves the addition of polar chemical groups to render the surface hydrophilic.

2.10.1 Quantification of adhesion to unmodified polystyrene

Adhesion to unmodified polystyrene was quantified by a method described previously (Heilmann *et al.*, 1996a). Stationary phase bacterial cultures were washed 3 times in PBS and resuspended to an OD_{490} of 0.2. Aliquots (10ml)

were then added to unmodified polystyrene petri dishes (Sterilin Ltd, UK) and incubated at 37 °C for 2 h taking care not to disturb the dishes during this time. Dishes were then washed 3 times in PBS (10 ml) and dried for at least 3 h. Dishes were then stained with CV for 10 min and rinsed with distilled water to remove excess stain. Photographs of the bacteria were then taken at random positions on the dish (3 photographs for each dish) using a light microscope with x100 objective lens and digital camera. To calculate the number of bacteria per microscope view a grid was placed over the photographs and the number of bacteria in a random sample of ten squares were counted to calculate the number of bacteria per microscope view.

2.10.2 Polystyrene adhesion of typsin treated cells

For assays involving trypsin treated *L. lactis* MG1363 pUB1000aap6, trypsin treatment was carried out as described previously (Veenstra *et al.*, 1996). A single stationary phase culture (20 ml) was split in to two 10 ml aliquots. To one aliquot 100 µl trypsin solution (10 µg/ml in 100 mM HCl, TPKC treated to remove chymotrypsin, Sigma) was added and 100 µl HCl (100 mM) was added to the other aliquot. Both 10 ml aliquots were then incubated at 37 °C for 10 min. Cells were then washed three times in PBS at 4 °C to remove trypsin and were resuspended to an OD_{490} of 1.0. The adhesion procedure described above was then followed.

2.10.3 Inhibition of adhesion with recombinant proteins

For inhibition of adhesion, $rAap_{A\text{-}dom}$, $rAap_{sh\text{-}A\text{-}reps}$ and BSA at different concentrations in PBS were added to wells and incubated for 2 h at 37 °C. Wells were then washed three times in PBS to remove unbound rAap or BSA and bacteria were added as described above.

2.11 Mass spectrometry of SDS PAGE bands

The two Aap SDS PAGE bands of *L. lactis* MG1363 pUB1000*aap6high* were excised and passed on to a core facility with in the Faculty of Life Sciences (University of Manchester) for analysis of tryptic peptides in order to determine the size of the Aap molecule comprising each band. A previously described method was used (Rosenfeld *et al.*, 1992) with some modifications. SDS PAGE bands were treated with 55 mM iodoacetamide to alkylate Cystine residues and permanently prevent any di-sulphide bond formation and then 10 mM dithiothreitol was added to reduce any unreacted iodacetamide. Bands were then digested for 18 h with trypsin to completely digest the proteins within the bands. Digested samples were analysed by LC-MS/MS using a NanoAcquity LC (Waters, Manchester, UK) coupled to a LTQ Velos (Thermo Fisher Scientific, Waltham, MA). Peptides were separated on the liquid chromatograph column using an increasing concentration gradient of acetonitrile. Peptides were selected for fragmentation automatically in order to unambiguously identify the individual peptides from the masses of their respective B and Y ions produced during fragmentation. Peptide data were analysed using Scaffold 2 software (Proteome software Inc, USA) and aligned to the sequence of *aap* from *S. epidermidis* NCTC 11047 to determine the section of Aap that was present in the top SDS PAGE Aap band but not in the bottom band.

2.12 Biofilms cultured under flow conditions

Biofilms were cultivated in a flow cell system in order to more accurately recreate physiological conditions. I attended an 8 day course in Denmark (funded by a BBSRC Travel Award and the SGM) organized by DTU university who were one of the main developers of the system in order to learn the technique.

2.12.1 Set-up of flow cell apparatus

A flow chamber (DTU, Lyngby, Copenhangen, Denmark) with channel dimensions of 1 x 4 x 40 mm was used (Fig. 2.1).

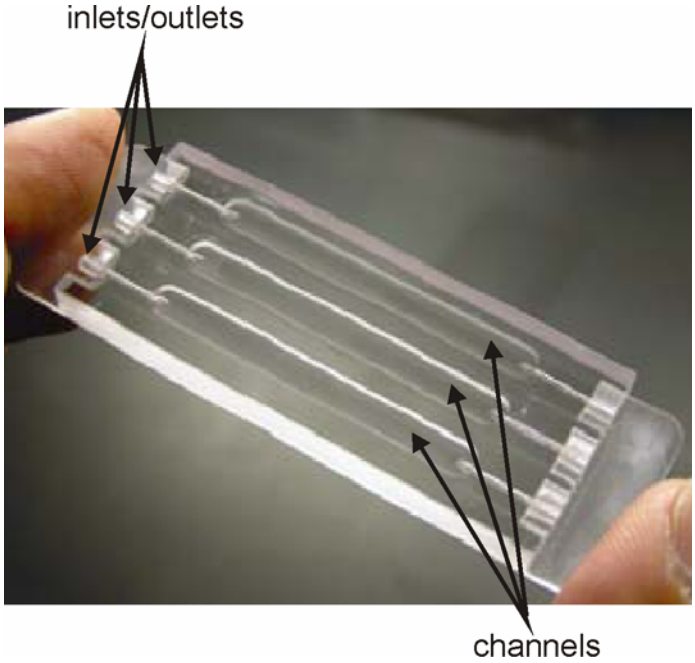

Figure 2.1. Diagram of a biofilm flow cell. Silicone tubing was attached to the inlets and outlets at either end. A microscope coverslip was glued to the top to enclose the channels.

The flow cell system was assembled as described previously (Christensen *et al.*, 1999). A 24 x 50 mm glass coverslip was used as substratum for biofilm growth and was glued (Super Silicone Sealant, 3M) to the flow chamber to enclose the three channels (Fig. 2.1. arrows) and was allowed to dry overnight. The flow-chamber outlets were then connected to a waste flask with silicone tubing (Altec Tubing). The inlets were connected to a Watson-Marlow 205S peristaltic pump via a bubble trap (DTU, Denmark). The purpose of the bubble trap was to prevent air bubbles entering the flow chamber. The system was sterilised by pumping hypochlorite (0.5 %, ~ 0.5 ml/min) through the system for at least 4 h as reported previously (Lappann *et al.*, 2006). The system was then rinsed with sterile H_2O four times, emptying the system between each rinse. The system was then filled with medium (GM17 for *L. lactis* MG1363 and 5 % TSB in AB

medium [15 mM $(NH_4)_2SO_4$, 34 mM $Na_2HPO_4 \cdot H_2O$, 22 mM KH_2PO_4, 51 mM NaCl, 1mM $MgCl_2$, 0.1 mM $CaCl_2$, 3 mM $FeCl_2$] (Clark and Maaløe, 1967) for *S. aureus* RN4220) and care was taken to remove all air bubbles that could disrupt the biofilm structure.

2.12.2 Inoculation of flow cells and culture conditions

Inocula were prepared as follows: *L. lactis* MG1363 stationary phase cultures were diluted in PBS to an OD_{490} of 1.0 whereas *S. aureus* RN4220 stationary phase cultures were diluted 1:100 into 5 % TSB diluted in AB medium in accordance with a previously published method for culturing staphylococcal biofilms under flow conditions (Qin *et al.*, 2007). Aliquots (200 µl) were then injected through the silicone tubing into the inlets of the flow-chamber using a hypodermic needle and syringe after which silicone sealant was used to seal up the tube. After inoculation flow chambers were incubated statically upside down (cover slip down) for 1 h to allow bacteria to attach to the cover-slip. Flow chambers were then inverted (cover slip up) and medium (GM17 for *L. lactis* MG1363 and 5 % TSB in AB medium for *S. aureus* RN4220) was pumped through the channels (0.2 mm/s) using a Watson-Marlow 205S peristaltic pump. All biofilms were cultured at 37 °C.

2.12.3 Biofilm Image acquisition and analysis

All images were taken using a Leica SP5 upright CLSM (Leica) using a x63/1.4 oil emersion objective lens. Bacteria were stained by injecting the green fluorescent nucleic acid stain SYTO 9 (8.7 µM in PBS, Invitrogen) into the channels slowly using a hypodermic needle and syringe. The tubing was clamped either side of the flow cell so it could be disconnected from the system. CLSM images were acquired at random positions roughly half way down the centre of the flow-chamber channels (9 images per channel). Imaris (Bitplane) was used to create top down and sagital views. The freely available COMSTAT

computer program (Heydorn *et al.*, 2000) was used to calculate the average microcolony volume.

2.13 Biofilms cultured in 96-well plates

Biofilms were cultured and quantified in 96-well plates by a previously published method (Christensen *et al.*, 1985). Stationary phase *S. aureus* RN4220 cells were diluted 1:100 in fresh HI media supplemented with glucose (0.5 % w/v) and aliquots (200 µl) were added to wells and incubated for 24 h at 37 °C. The wells were then washed with 200 µl PBS three times and stained with crystal violet as described above (2.10). The OD_{535} of the wells was measured to quantify the biofilms. For inhibition of biofilm formation rAap and BSA solutions in PBS were added to the wells containing bacteria 2 h after the start of incubation to ensure primary adhesion to the wells was not affected.

2.14 Statistical analysis.

Statistical analysis was performed using SPSS software (version 11.5). One-way ANOVA with Tukey post hoc test or a Student's T-test was used to determine statistical differences at the 0.05 level where relevant.

Chapter 3:

The terminal A-domain of the fibrillar accumulation associated protein (Aap) of *Staphylococcus epidermidis* mediates adhesion to human corneocytes.

3.1 Abstract

The opportunistic pathogen *Staphylococcus epidermidis* colonizes indwelling medical devices by biofilm formation, but is primarily a skin resident. In many *S. epidermidis* strains biofilm formation is mediated by a cell-wall-anchored protein, the accumulation associated protein (Aap). Here, we investigate the role of Aap in skin adhesion. Aap is a LPXTG protein with a domain architecture including a terminal A-domain and B-repeat region. *S. epidermidis* NCTC 11047 expresses Aap as localized, lateral tufts of fibrils on one sub-population of cells (Fib^+) whereas a second subpopulation does not express these fibrils of Aap (Fib^-). Flow cytometry showed that 72 % of NCTC 11047 cells expressed Aap and 28 % of cells did not. Aap is involved in adhesion of Fib^+ cells to squamous epithelial cells from the hand (corneocytes) as recombinant A-domain protein partially blocked binding to corneocytes. To confirm the role of the Aap A-domain in corneocyte attachment Aap was expressed on the surface of *Lactococcus lactis* MG1363 as sparsely distributed, peritrichous, fibrils. Expression of Aap increased corneocyte adhesion 20-fold compared to *L. lactis* carrying Aap without an A-domain. *S. epidermidis* isolates from catheters, artificial joints, skin and the nose also used the A-domain of Aap to adhere to corneocytes, emphasizing the role of Aap in skin adhesion. In addition, *L. lactis* expressing Aap with different numbers of B-repeats revealed a positive correlation between the number of B-repeats and adhesion to corneocytes, suggesting an additional function for the B-region in enhancing A-domain dependent attachment to skin. Therefore in addition to its established role in biofilm formation Aap can also promote adhesion to corneocytes, and is likely to be an important adhesin in *S. epidermidis* skin colonization.

3.2 Introduction

S. epidermidis is the leading cause of nosocomial infections associated with indwelling medical devices including intravascular catheters, cardiac pacemakers and artificial joints (Finch *et al.*, 1995; Rupp and Archer, 1994). The main virulence mechanism is biofilm formation which promotes persistence in the host, leading to infections such as bacteremia or endocarditis (Arber *et al.*, 1994). *S. epidermidis* is also a common commensal resident on the skin all over the human body and may be a transient member of the oral microflora (Jackson *et al.*, 1999; Murdoch *et al.*, 2004), Clinical evidence shows that commensal strains from the skin and mucous membranes can translocate to cause bacteraemia (Costa *et al.*, 2004). In addition, there have been recent reports of linezolid resistance in skin commensal strains of *S. epidermidis* (Kelly *et al.*, 2008; Potoski *et al.*, 2006). It is therefore important to study the bacterial factors involved in *S.epidermidis* colonisation of the skin as this is likely to provide a reservoir for contaminating medical devices.

Very little is known about how *S. epidermidis* colonises the skin, although many cell-wall-associated adhesins have been identified that are involved with adhesion, mainly to host matrix proteins. The *S. epidermidis* RP62A genome contains 11 putative LPXTG cell-wall-anchored proteins (Bowden *et al.*, 2005), a class of proteins common on Gram-positive cocci that often mediate adhesion to host proteins (Schneewind *et al.*, 1995). So far, only three of these have prescribed functions: the Bap-homology protein (Bhp) and the accumulation associated protein (Aap) are involved in biofilm formation (Cucarella *et al.*, 2001; Hussain *et al.*, 1997; Rohde *et al.*, 2005), and SdrG mediates adhesion to fibrinogen. In addition, *S. epidermidis* is known to express a variety of other non-LPXTG proteins such as the autolysins Aae, which promotes adhesion to vitronectin and the β-chain of fibrinogen (Heilmann *et al.*, 2003; Rupp *et al.*, 2001) and AtlE, which promotes adhesion to vitronectin (Heilmann *et al.*, 1997). The elastin binding protein (Ebp) (Park *et al.*, 1996; Zhang *et al.*, 2003),

extracellular lipase (GehD) (Bowden *et al.*, 2002), extracellular matrix binding protein (Embp) (Williams *et al.*, 2002) and staphylococcal surface proteins-1 and 2 (Ssp-1 and 2) (Veenstra *et al.*, 1996) mediate adhesion to elastin, collagen, fibronectin and polystyrene, respectively. Furthermore, teichoic acids have been shown to promote adhesion to fibronectin (Hussain *et al.*, 2001) and a polysaccharide termed PS/A or PIA (Mack *et al.*, 2006) promotes adhesion to a plastic used to make catheters (Tojo *et al.*, 1988). To date no work has been published linking any of these adhesins to colonisation of the skin.

We recently showed that one of the LPXTG cell-wall-anchored proteins, Aap on *S. epidermidis* NCTC 11047, is a thin, fibrillar protein that projects 120nm away from the cell wall in localised tufts (Banner *et al.*, 2007); this study investigated the possible role of Aap in mediating adhesion to human skin cells. Aap is an archetypal LPXTG protein with a Sec-dependent signal sequence and a 556 amino acid (aa) N-terminal A-domain, which comprises 10 imperfect repeats of 16 aa and a non-repetitive region. Proximal to the A-domain are several 128 aa B-repeats, the number of which varies between strains. For example *S. epidermidis* strains RP62A and NCTC 11047 have 12 full and one partial B repeat (Banner *et al.*, 2007; Gill *et al.*, 2005) compared to 5.5 B repeats in strain 5179 (Rohde *et al.*, 2005). Aap is important in biofilm formation but the A-domain must be cleaved for the B-repeats to promote intercellular adhesion in the accumulation phase of biofilm formation (Rohde *et al.*, 2005). Cell to cell adhesion is thought to rely on the Zn^{2+}-dependent dimerisation of B-repeat regions (Conrady *et al.*, 2008). In addition, Aap has been indirectly implicated in adhesion to nasal epithelial cells (NECs) (Roche *et al.*, 2003b) as the Aap homologue, SasG from *S. aureus*, was found to mediate adhesion to NECs and a recombinant protein derived from the A-domain of Aap ($rAap_{A\text{-}Dom}$) was able to block the adhesion of a surrogate host expressing SasG to NEC. It was therefore suggested that both SasG and Aap share a receptor on the host cell surface of NEC (37).

Not all cells in a wild type population of *S. epidermidis* NCTC 11047 express fibrillar tufts of Aap, as stationary phase cells contain a sub-population of cells with Aap fibrils (Fib^+ cells) , and a second sub-population of cells that have no tufts of fibrils and no Aap on the cell surfaces (Fib^- cells) (Banner *et al.*, 2007). The sub-populations were separated by 36 cycles of hexadecane enrichment to yield two stable populations (Fib^+ and Fib^-). The Fib^- subpopulation expressed only Aap mRNA but no Aap protein and it was proposed that fibril expression is regulated at the posttranscriptional level by an unknown mechanism (Banner *et al.*, 2007). The Fib^+ cells, expressing Aap, are also more hydrophobic and have greater affinity for polystyrene compared to Fib^- cells (Banner *et al.*, 2007). Therefore strain NCTC 11047 produces some cells which have the potential to form biofilms due to the presence of Aap and some which may lack the ability. Any adhesive functions mediated by Aap on the Fib^+ subpopulation would be absent in the Fib^- subpopulation.

Here, we present data which shows that the A domain of Aap on *S. epidermidis* NCTC 11047 mediates adhesion to corneocytes from the uppermost layer of the skin epidermis. The results suggest that Aap could play an important role in the colonization of human skin by *S. epidermidis.*

3.3 Materials and methods

3.3.1 Bacterial strains and culture conditions

Bacterial strains and plasmids are listed in Table 3.1. *S. epidermidis* JB strains were isolated from the anterior nares (JBN strains) or from the skin of the forehead (JBS strains) of healthy volunteers or were donated by the University of Manchester Medical Microbiology Culture Collection, having been isolated from intravenous catheters (JBC strains) or cases of hip-joint infection (JBJ strains). Isolates were confirmed as *S. epidermidis* by both API 20 staph tests (Biomerieux Industry) and 16S rDNA sequence determination using the primer shown in table 3.1 (Lane, 1991). *S. epidermidis* strains were cultured statically in tryptic soya broth (TSB; Oxoid) at 37 °C. *L. lactis* MG1363 (Gasson, 1983a) was

cultured statically at 30 °C in M17 broth (Oxoid) supplemented with 0.5 % (w/v) glucose (GM17). *S. gordonii* DL1 was cultured statically at 37°C in brain heart infusion broth (Oxoid) supplemented with 0.5 % yeast extract (BHY). Erythromycin (Sigma) (5 µg/ml) was added to media when culturing strains containing pUB1000.

Table. 3.1. Bacterial strains, plasmids and primers used in this study.

Strains	Description[a]	Reference
S. epidermidis		
NCTC 11047 WT	nasal isolate Aap$^+$	
NCTC 11047 Fib$^+$	sub-population Aap$^+$	(Banner *et al.*, 2007)
NCTC 11047 Fib$^-$	sub-population Aap$^-$	(Banner *et al.*, 2007)
RP62A	intravenous catheter isolate Aap$^+$	(Christensen *et al.*, 1985)
JBN 3, 8, 9 & 10	nasal isolates Aap$^+$	This study
JBJ 1, 4 & 5	joint infection isolates Aap$^+$	This study
JBC 7, 9 & 13	catheter infection isolates Aap$^+$	This study
JBS 4, 5, 14	skin isolates Aap$^+$	This study
L. lactis MG1363	surrogate host for Aap expression	(Gasson, 1983a)
S. gordonii DL1 (NCTC 7868)	intermediate cloning host	
Plasmids		
pUB1000	*L. lactis* cell-wall-expression vector carrying erythromycin resistance	(Heddle *et al.*, 2003)
pUB100*aap6high*	pUB1000 containing *aap* gene with 6 B-repeats giving high level of expression	This study
pUB1000*aap6highT*	pUB1000 containing truncated version of *aap6* with no A-domain giving high level of expression	This study
pUB1000*aap2*, *4*, *5*, *6* & *7*	pUB1000 containing *aap* genes with 2, 4, 5, 6 or 7 B-repeats giving lower level of expression	This study
Primers		
16SR	16S DNA sequencing of *S. epidermidis* isolates CCGTCAATTCGTTTCAGTTT	(Lane, 1991)
raap157-857F	cloning region of short repeats within A-domain CCGGGATCCGCAGAAGAAAAACAAGTTGATC	(Roche *et al.*, 2003b)
raap157-857R	cloning region of short repeats within A-domain CGGAAGCTTGATAGTTGGAACATTCGGTGCTTC	This study
aapFSalI	cloning *aap* into pUB1000 TACGCTGTCGACCCAATTACACAAGCTAATCAAAATGATAG	This study
aapRBamHI	cloning *aap* into pUB1000 TGTCGGATCCAAATTATTTTTCATTACCTTTTTTACGACG	This study
pUB1000F	sequencing of *aap* inserts CCGTTGTCAGGTGTTTACGCT	This study
pUB1000R	sequencing of *aap* inserts CTTTTGGTGTCTCAGGTTTGT	This study
aapTFSalI	cloning truncated *aap* into pUB1000 TACGCTGTCGACAGAGCTGATTTAGATGGTGC	This study
aapTRSalI	cloning truncated *aap* into pUB1000 TACGCTGTCGACAGCGTAAACACCTG	This study
Aap53-608 r.c.	checking size of B-region CATTGACATACACTCCTAAGC	(Rohde *et al.*, 2007)
aapR	checking size of B-region CCAAATATGAACAATGATCCG	This study

[a] Aap$^+$ and Aap$^-$ indicate strains that express or do not express the Aap protein respectively.

3.3.2 Flow cytometry

Fluorescent labelling of bacteria and flow cytometry was performed based on the method of Humphries *et al.* (Humphries *et al.*, 2003). Bacteria (~5x10^8 cells) from 18 h stationary phase cultures were washed three times in phosphate buffered saline (PBS; Sigma) containing 0.02 % gelatine (PBS-gel) and then incubated in PBS-gel containing 0.2 % normal goat serum (Sigma, PBS-gel-NGS) for 30 min. Rabbit anti-Aap A-domain antiserum (Banner *et al.*, 2007) was then added to the suspension at a dilution of 1:250 and incubated for a further hour. Cells were washed in PBS-gel three times and incubated in PBS-gel-NGS containing a 1:250 dilution of R-phycoerythrin conjugated donkey anti-rabbit IgG antibody (Abcam) for 1 h. Cells were washed and resuspended to ~5x10^6 cells ml^{-1} in PBS. A Beckman Coulter Cyan ADP flow cytometer and Summit V4.3 software (Dako, Denmark) was used to analyse 30,000 events (bacteria). For statistical analysis, flow cytometry was repeated with samples from three independent experiments.

3.3.3 Quantification of bacterial adhesion to corneocytes

Corneocytes were harvested from both hands of up to four healthy volunteers by gentle agitation of the hand inside a laboratory glove (nitrile powder-free exam glove, Kimberly Clark, USA) containing 20 ml PBS for 2min. The collection of corneocytes had full ethical approval from the University of Manchester ethics board. The corneocytes were washed three times and resuspended to an OD_{440} of 0.35 (~7.0 x10^4 corneocytes ml^{-1}). Stationary phase bacterial cultures (18h) were washed 3 times and resuspended to an OD_{490} of 0.08 (7.0x10^6 cfu ml^{-1}) for *S. epidermidis* strains and 0.6 (4.2x10^7 cfu ml^{-1}) for *L. lactis* strains. Equal volumes (2.5 ml) of bacteria and corneocytes were then mixed in a Falcon tube (50 ml, Corning) and rotated (200rpm, to avoid cells settling) at 37 °C for 2 h for adhesion to occur.

The suspension of bacteria and corneocytes was immediately applied to the top of 5 ml of a solution of 6 % (w/v) dextran (~100,000 Da, Sigma) and 0.9 % NaCl

in a 15 ml falcon tube (Corning). The tube was centrifuged at 1200 x *g* for 5 min to pellet the corneocytes and leave the unbound bacteria in a band higher up the dextran solution. The top 5 ml was discarded from the tube and the remaining solution was centrifuged at 3300 x *g* for 5 min. The resulting pellet was resuspended in 1 ml PBS and applied to the top of another dextran solution. This process was performed three times in order to remove all unbound bacteria. Finally, the pellet containing corneocytes and bound bacteria was resuspended in water (100 µl) and samples (50 µl) applied to microscope slides for counting the attached bacteria. After Gram staining, the number of bacteria per corneocyte was counted for 30 corneocytes. To test reproducibility, three batches of cells were tested in the assay and two slides were counted (2 x 30 corneocytes) for each batch.

For blocking experiments the corneocytes were preincubated with 40 µl of each recombinant Aap domain protein (see below) to give final concentrations of 0.05, 0.2 or 1.0 µM for each protein. Corneocytes were preincubated for 20 min at 37 °C at 200 rpm before 2.5 ml of bacterial suspension was added and the assay was performed as described above.

3.3.4 Construction of recombinant His_6-tagged fusion proteins

Recombinant proteins corresponding to a single B-repeat and the full A-domain of Aap were constructed, expressed and purified as described previously (Banner *et al.*, 2007). The region of short repeats within the A-domain of Aap (nucleotides 157-857) was cloned, expressed and purified using the primers raap157-857F/R (Table 3.1) and the techniques described previously (Banner *et al.*, 2007).

3.3.5 Cloning of Aap into *L. lactis* MG1363

Routine cloning techniques were performed as described by Sambrook *et al.* (Sambrook, 1889). The *aap* gene of *S. epidermidis* NCTC 11047 was amplified

using high fidelity DNA polymerase (Roche). Primers aapFSalI and aapRBamHI (Table 3.1) were used to amplify a 7195 bp fragment of the *aap* gene from immediately downstream of the N-terminal signal sequence to the stop codon. The single PCR product was purified and digested with SalI and BamHI and ligated into the SalI/BamHI site of the lactococcal surface expression vector pUB1000 (Heddle *et al.*, 2003) to give pUB1000*aap*. pUB1000 contains a cell-wall-associated expression cassette comprising a constitutive lactococcal promoter fused to the N-terminal signal sequence of the cell-wall-associated protein *sspA* from *S. gordonii*. Cloning *aap* into the BamHI/SalI cloning site of pUB1000 generated a fusion between the signal sequence of *sspA* and the *aap* gene such that the fusion protein would be correctly directed for export through the Sec pathway. pUB1000*aap* plasmids were transformed into competent *S. gordonii* DL1 as described previously (Haisman and Jenkinson, 1991) and transformants were selected on BHY agar with erythromycin. Transformants were screened for Aap expression by SDS PAGE of cell wall proteins (see below). Plasmids were purified from clones expressing five different sizes of Aap using a miniprep kit (Qiagen) and these were electroporated into electrocompetent *L. lactis* MG1363 as described previously (Hartford *et al.*, 2001a; Wells *et al.*, 1993). The number of B-repeats in each *aap* gene was determined by PCR of the insert region using primers Aap53-608 r.c and aapR (Table 3.1), which anneal on either side of the B-repeat region. Primers pUB1000F/R (Table 3.1), which anneal either side of the BamHI/SalI cloning site, were used to confirm the sequence of the 5' and 3' ends of the *aap* gene.

pUB1000*aap6highT*, a truncated derivative of pUB1000*aap6high* with the entire A-domain removed was generated using primers aapTFSalI and aapTRSalI (Table 3.1) and pUB1000*aap6high* as template DNA with expand high fidelity long template DNA polymerase (Roche). The forward primer annealed at the start of the B-repeat region and the reverse annealed immediately upstream of the A-domain. The PCR product, comprising the full pUB1000 backbone and the B-region of *aap* with a SalI cut site at either end (8.8 kb), was digested with SalI

and self-ligated. It was then electroporated directly into *L. lactis* MG1363 as described above. Primers pUB1000F and pUB1000R (Table 3.1) were used to confirm the correct insert sequence.

3.3.6 Western Blot of cell wall proteins

Cells wall proteins were extracted from stationary phase cultures (20 ml, OD_{600} 2.5) using mutanolysin (Sigma) as described previously (Demuth *et al.*, 1996). Stationary phase cultures (20 ml) were washed once in PBS and resuspended in spheroplasting buffer (50 µl; 20 mM Tris-HCl pH 6.8, 10 mM $MgCl_2$, 26 % (w/v) raffinose.$5H_2O$). Mutanolysin (final concentration of 500 Uml^{-1}; Sigma) was added along with 1 mM phenylmethylsulphonyl fluoride (Sigma). Proteins were separated by SDS PAGE (8 % gel) and blotted onto a PVDF membrane. The membrane was then blocked with 6 % (w/v) skimmed milk (Oxoid) in PBS containing Tween 20 (0.05 %; Sigma) overnight at 4 °C. Rabbit anti-Aap A-domain antiserum at a dilution of 1:5000 or anti-Aap B-repeat antiserum at a dilution of 1:1000 (Banner *et al.*, 2007) and a horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:35000; Sigma) were used to probe the blot. Proteins were visualised using Western Lightning chemiluminescence reagent (PerkinElmer, UK).

3.3.7 Transmission electron microscopy

Cell surface structures were analysed by negative staining with 2 % methylamine tungstate (Agar Scientific, UK) at pH 6.5 in the TEM as described previously (Banner *et al.*, 2007; Handley *et al.*, 1985).Immunogold negative staining was performed with anti-Aap A-domain antiserum and a secondary anti-rabbit immunoglobulin G conjugated to 10 nm gold particles (Agar Scientific, UK) (Banner *et al.*, 2007; McNab *et al.*, 1999). Cells were visualised in an FEI Tecnai 12 electron microscope (FEI Co., Eindhoven, The Netherlands) at 100KV.

3.3.8 Whole-cell immunoblotting

The method of Corrigan *et al.* (Corrigan *et al.*, 2007) was used. Stationary phase cells were washed and resuspended to an OD_{600} of 2 in PBS. Doubling dilutions of bacterial suspensions (10 µl) were then spotted on to a PVDF membrane (Bio-rad). The membrane was blocked, probed with anti-Aap A-domain antiserum and developed as for Western blots (see above).

3.3.9 Statistical analysis

Statistical analysis was performed using SPSS software (version 11.5). One-way ANOVA with Tukey post hoc test was used to determine statistical differences at the 0.05 level.

3.4 Results

3.4.1 *S. epidermidis* NCTC 11047 stationary phase cultures comprise two subpopulations defined on the basis of Aap expression

Our previous finding that the *S. epidermidis* NCTC 11047 WT population is comprised of two sub-populations (3) was confirmed using flow cytometry (Fig. 3.1). NCTC 11047 WT cells were labelled with anti-Aap A-domain antiserum and a secondary fluorescent antibody. Cells were found to have a low, background level of fluorescence (Fig. 3.1 left peak) with the same low intensity shown with pre-immune antiserum, or a higher level of fluorescence (Fig. 3.1 right peak) that indicated the presence of Aap. These results indicated that NCTC 11047 WT cells comprised 72 % Aap-expressing cells and 28 % Aap negative cells. The presence of these two subpopulations was also confirmed using anti-Aap B-repeat antiserum (data not shown) and the same ratio of the two cell types was detected.

To determine whether other Aap-expressing *S. epidermidis* strains also contained similar Aap negative sub-populations, RP62A and 13 other *S. epidermidis* strains isolated from catheters, hip-joints, anterior nares and the

skin (Table 3.1), were analysed by flow cytometry with anti-Aap A-domain antiserum. Only two strains (JBN3 and JBC7) contained Aap negative sub-populations and the remaining strains all comprised a single Aap expressing population (data not shown). In contrast to NCTC 11047, the majority of cells in stationary phase cultures of JBN3 and JBC7 did not express Aap with cultures containing 90 % and 75 % Aap negative cells respectively.

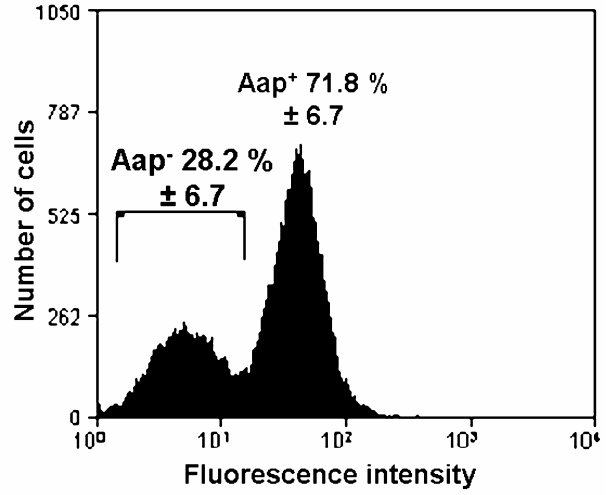

Figure. 3.1. Histogram showing flow cytometry results from one batch of NCTC 11047 WT cells labelled with anti-Aap A-domain antiserum and phycoerythrin conjugated secondary antibody. The mean percentages (± SD) of Aap negative Fib$^-$ (left peak) and Aap positive Fib$^+$ cells (right peak) are shown for three batches of NCTC 11047 WT cells.

3.4.2 The A-domain of Aap contributes to the adhesion of *S. epidermidis* 11047 WT/Fib$^+$ cells to corneocytes

The two sub-populations of NCTC 11047 have previously been found to have different cell surface properties, with the Fib$^+$ cells being more adherent to polystyrene and having higher cell surface hydrophobicity than the Fib- cells (Banner *et al.*, 2007). To compare the ability of the two sub-populations to colonize skin, their respective affinities for corneocytes were determined (Fig. 3.2a).

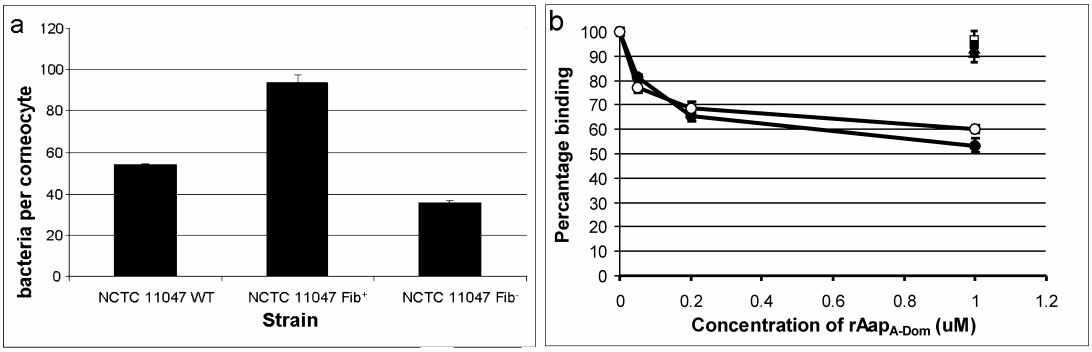

Figure. 3.2. Comparison of corneocyte binding of WT, Fib$^+$ and Fib$^-$ cells and the contribution of Aap A-domain in adhesion of NCTC 11047 to corneocytes. (a) Mean number of NCTC 11047 WT, Fib$^+$ and Fib$^-$ cells attached to corneocytes after 2h incubation (b) Blocking of adhesion of NCTC 11047 WT (filled symbols) and Fib$^+$ (hollow symbols) to corneocytes using specific rAap domains. Corneocytes were pre-incubated with increasing concentrations of rAap$_{A\text{-}Dom}$ (circles), 1 μM rAap$_{B\text{-}rep}$ (squares) or 1 μM rAap$_{A\text{-}reps}$ (triangles) and the adhesion of NCTC 11047 WT and Fib$^+$ cells, as a percentage of original binding, is shown. Results represent the mean and standard error of at least two experiments.

The Aap expressing Fib$^+$ subpopulation showed almost a two fold enhancement in adhesion compared to WT cells, whereas adhesion of the Aap negative Fib$^-$ sub-population was two thirds of the WT level. This suggests that Aap may be a contributory factor in adhesion to corneocytes. However, other factors must also mediate adhesion, as Fib$^-$ cells did attach to corneocytes. To determine whether the A-domain of Aap contributes to corneocyte adhesion the ability of rAap$_{A\text{-}dom}$ to block adhesion was tested (Fig. 3.2b). rAap$_{A\text{-}dom}$ inhibited adhesion of NCTC 11047 WT and Fib$^+$ cells to corneocytes in a concentration dependent manner whereas rAap$_{B\text{-}rep}$ and rAap$_{A\text{-}reps}$ were unable to block adhesion. In addition, adhesion of NCTC 11047 Fib$^-$ cells could not be significantly blocked by 1 μM rAap$_{A\text{-}dom}$ (91% ± 4 % binding, mean and standard deviation of 2 experiments). Therefore, the non-repetitive region of the A-domain of Aap specifically contributes to the adhesion of NCTC 11047 WT and Fib$^+$ to corneocytes.

However, it is not clear from these results whether Aap merely enhances adhesion or whether it can promote adhesion to corneocytes independently of other adhesive factors.

3.4.3 Aap is expressed on the surface of *Lactococcus lactis* MG1363 pUB1000*aap6high* as sparse peritrichous fibrils

To determine the ability of Aap to mediate adhesion of bacteria to corneocytes independently of other adhesins, Aap was expressed on the surface of *L. lactis* MG1363, a bacterium with very low affinity for epithelial cells (Roche *et al.*, 2003b). The *aap* gene was amplified from *S. epidermidis* NCTC 11047 genomic DNA and inserted into the lactococcal surface expression vector pUB1000. The number of B-repeats resulting from this procedure was reduced from the WT number of 12.2 (3), to 6, and the recombinant Aap was expressed in *L. lactis* MG1363 (termed MG1363 pUB1000*aap6high*, Table 3.1). Surface expression of Aap was demonstrated by Western blotting and by negative staining (Fig. 3.3).

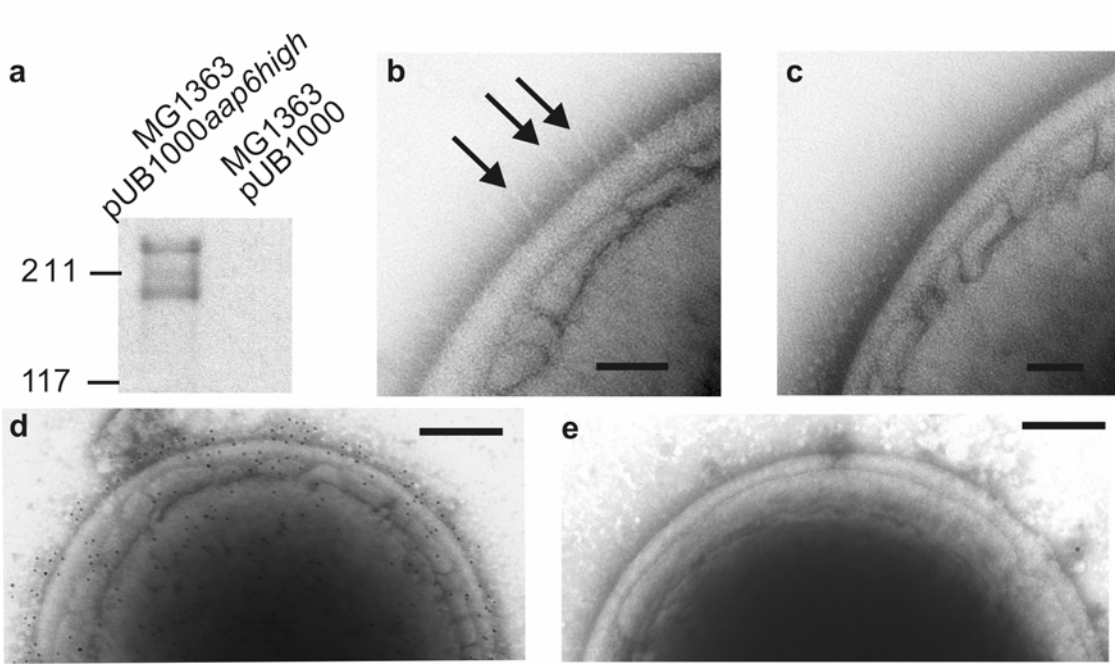

Figure. 3.3. Surface expression of fibrillar Aap on *L. lactis* MG1363 pUB1000*aap6high*. (a) Western blot of cell wall proteins probed with anti-Aap A-domain antiserum. (b-c) TEM of *L. lactis* MG1363 strains negatively stained with 2 % methylamine tungstate. (d-e) TEM of *L. lactis* MG1363 strains negatively stained and immunogold-labelled with anti-Aap A-domain antiserum and 10 nm gold-conjugated secondary antibody. (b) MG1363 pUB1000*aap6high* showing fibrils projecting from the cell wall. (c) MG1363 pUB1000 showing a smooth cell surface. (d) MG1363 pUB1000*aap6high* showing gold labelling evenly distributed over the cell. (e) MG1363 pUB1000 showing only a small amount of non-specific gold labelling.

A western blot of cell wall proteins showed two anti-Aap-A-Domain antiserum-reactive bands at ~ 270 and 200 kDa whereas no antibody reactive bands were seen for MG1363 pUB1000 indicating Aap was present in the cell wall of MG1363 pUB1000*aap6high* (Fig. 3.3a). Negative staining showed sparse peritrichous fibrils on the surface of MG1363 pUB1000*aap6high* in contrast to the smooth cell wall of MG1363 pUB1000 (Figs. 3.3 b & c). Finally, immunogold labelling with anti-Aap A-domain antiserum also showed gold particles sparsely distributed over the surface of MG1363 pUB1000*aap6high* but not on MG1363 pUB1000 (Figs. 3.3 d & e) proving that Aap is exposed on the cell surface when pUB1000*aap6high* is present. Together these results show the fibrillar structure of Aap was maintained in a *L. lactis* background but Aap fibrils were peritrichous rather than localised in a tuft as on NCTC 11047 WT and Fib^+ cells (Banner *et al.*, 2007).

3.4.4 The A-domain of Aap independently mediates adhesion of *L. lactis* MG1363 pUB1000*aap6high* to corneocytes

The ability of Aap to mediate adhesion to corneocytes independently of other adhesins was investigated by measuring adhesion of *L. lactis* MG1363 pUB1000*aap6high*. In order to prove the role of the A-domain in adhesion, MG1363 expressing truncated Aap with 6 B-repeats but no A-domain (MG1363 pUB1000*aap6highT*, Table 3.1) was generated. Surface expression of truncated Aap was proven by western blotting of cell wall proteins using anti-Aap B-repeat antiserum (Fig. 3.4a) and the presence of Aap fibrils was confirmed by negative staining in the TEM (data not shown). An intact A-domain was found to be required for Aap mediated adhesion to corneocytes (Fig. 3.4b). Adhesion of MG1363 pUB1000*aap6high* to corneocytes was over 30 times that of MG1363 pUB1000 showing that Aap can mediate adhesion to corneocytes independently of other adhesins. MG1363 pUB1000*aap6highT*, expressing the same amount of Aap as MG1363 pUB1000*aap6high* (Fig 3.4a) but lacking the A-domain, adhered at the same very low level as the MG1363 pUB1000 control (Fig. 3.4b). Furthermore, $rAap_{A\text{-}Dom}$ blocked adhesion of MG1363 pUB1000*aap6high* to

corneocytes in a concentration-dependent manner, with almost complete inhibition of adhesion at 1 µM $rAap_{A\text{-}Dom}$ (Fig. 3.4c). In contrast, addition of $rAap_{A\text{-}reps}$ and $rAap_{B\text{-}rep}$ to the adhesion assay did not significantly reduce adhesion, even at a concentration of 1 µM. These results prove that only the A-domain of Aap adheres to a ligand on the surface of human corneocytes.

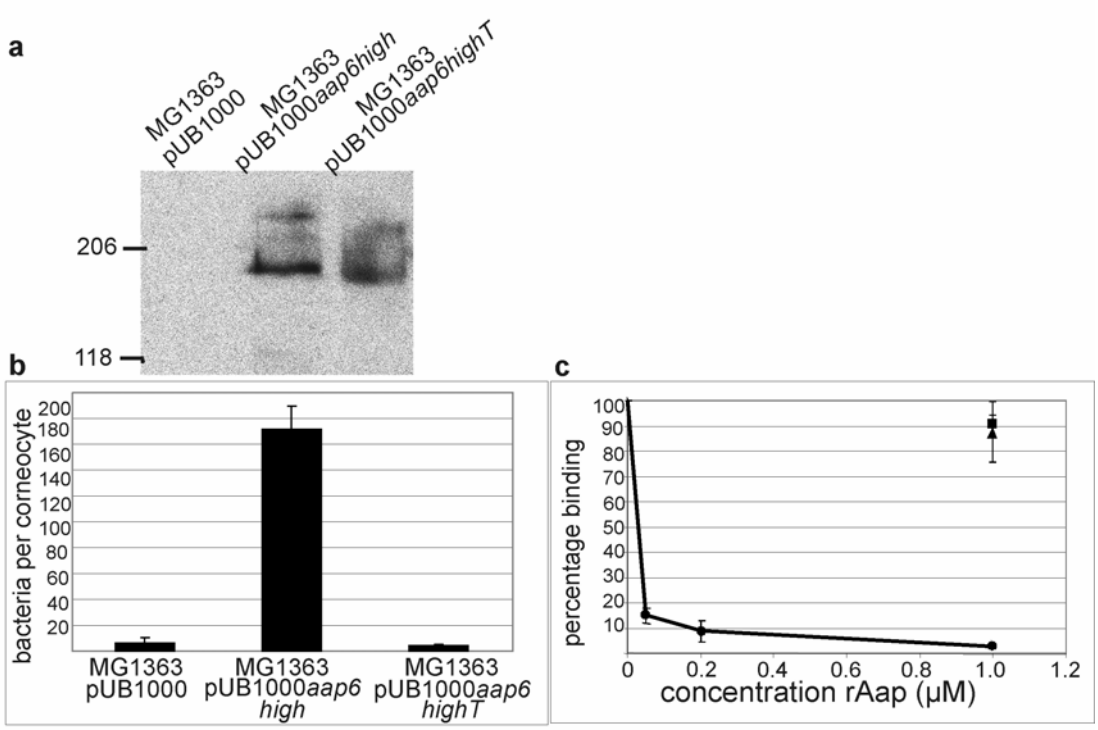

Figure. 3.4. Role of the A-domain of Aap in adhesion to corneocytes. (a) Western Blot of cell wall proteins from MG1363 containing pUB1000, pUB1000*aap6high* and pUB1000*aap6highT* and probed with anti-Aap B-repeat antiserum (b) Mean number of *L. lactis* MG1363 pUB1000*aap6high*, pUB1000*aap6highT* and pUB1000 control cells that adhered to corneocytes. (c) Corneocytes were pre-incubated with increasing concentrations of $rAap_{A\text{-}Dom}$ (circles), 1 µM $rAap_{A\text{-}reps}$ (triangles) or 1 µM $rAap_{B\text{-}rep}$ (squares) and the mean number of MG1363 pUB1000*aap6high* cells that adhered to corneocytes is shown. The results represent the mean and standard error of three experiments.

3.4.5 The A-domain of Aap mediates adhesion of *S. epidermidis* clinical isolates to corneocytes

The role of the Aap A-domain in adhesion to corneocytes was investigated for different *S. epidermidis* strains isolated from intravenous catheters, hip-joint

infections, anterior nares and the skin of different individuals (Table 3.1). For each isolate, between 40 and 114 bacteria were attached per corneocyte and there was no apparent correlation between numbers attached and isolate origin (data not shown). In order to determine whether the Aap A-domain mediated attachment of the strains, $rAap_{A\text{-}Dom}$ was used to block the adhesion of all the strains. The percentage reduction in numbers of bacteria attaching after blocking with $rAap_{A\text{-}Dom}$ was calculated (Fig. 3.5). The percentage reduction in attachment after blocking with $rAap_{A\text{-}Dom}$ varied between strains, with some strains being blocked almost completely (JBS4, 5 and 14) indicating Aap may be the major means of adhesion in these strains. However, other strains (JBN9, JBJ4 and JBC7) showed only a small reduction in adhesion after blocking with $rAap_{A\text{-}Dom}$ indicating these strains were not heavily dependent on Aap for attachment to corneocytes.

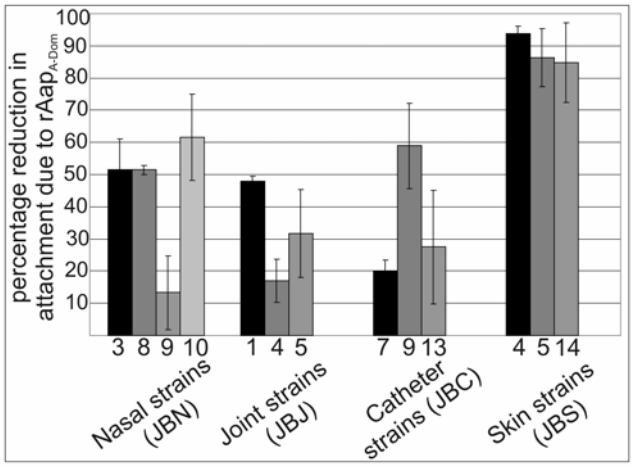

Figure. 3.5. Contribution of the A-domain of Aap to corneocyte adhesion for clinical isolates of *S. epidermidis*. Corneocyte adhesion of *S. epidermidis* isolates was blocked by pre-treating corneocytes with 1 μM $rAap_{A\text{-}Dom}$. The percentage reduction in number of attached bacteria due to blocking is shown. Results are the mean and standard error of 2 experiments.

Although only 10 % of JBN3 cells expressed Aap, as shown by flow cytometry (data not shown), blocking with $rAap_{A\text{-}Dom}$ reduced adhesion by 50 % (Fig 3.5). This suggests that ~50 % of cells that did attach to unblocked corneocytes were cells expressing Aap and their attachment was dependent on the Aap A-domain.

Therefore the sub-population (90%) that did not express Aap may have attached in fewer numbers due to relatively weak attachment via an as yet unidentified adhesin and this attachment would not have been affected by $rAap_{A\text{-}Dom}$. Detailed interpretation of the role of Aap in adhesion of each of these fresh strains is complex, however,the blocking experiment (Fig. 3.5) showed that reliance on Aap as a means of attachment to corneocytes varies form strain to strain and some strains are likely to express other molecules to promote corneocyte adhesion. It should be noted that the strains shown in Table 3.1 were selected for this study due to their ability to express Aap on the cell surface and not all *S. epidermidis* strains isolated from these sites in this study contained the *aap* gene.

3.4.6 The number of Aap B-repeats influences the level of adhesion of *L. lactis* MG1363 expressing Aap to corneocytes

Although the B-repeat region of Aap is not directly involved in corneocyte binding, the number of B-repeats in *S. epidermidis* strains varies from 3 up to 17 (Rohde *et al.*, 2007). Therefore, the influence of the length of the B-repeat region on corneocyte adhesion was investigated. Aap proteins with 2, 4, 5, 6 and 7 B-repeats were expressed on the surface of *L. lactis* MG1363 clones using the pUB1000 expression vector (Fig. 3.6 a & b). These strains (MG1363 pUB1000*aap2*, *4*, *5*, *6* and *7*, respectively) all expressed Aap at a lower level than the original MG1363 pUB1000*aap6high* (Fig 3.6b).

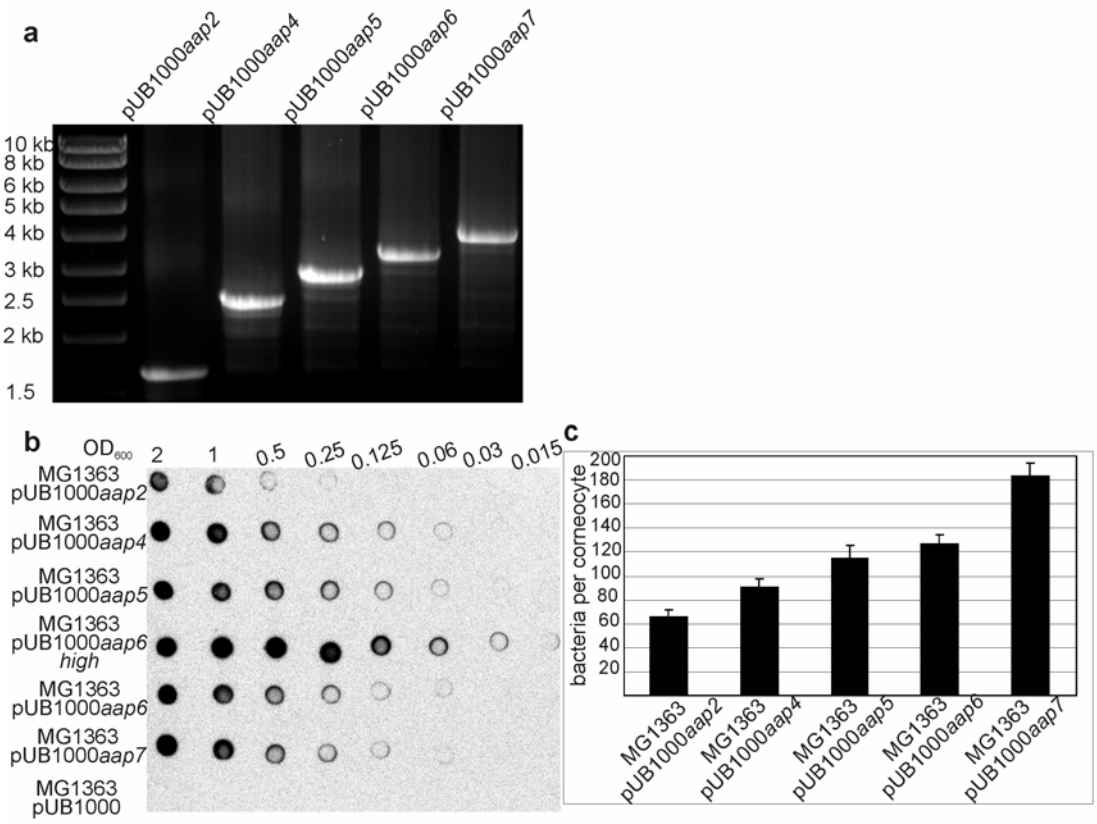

Figure. 3.6. Influence of numbers of B-repeats in Aap on adhesion to corneocytes. (a) Agarose gel showing size of *aap* in pUB1000*aap2*, *4*, *5*, *6* and *7*. The expected sizes of PCR products for pUB1000*aap* with 2, 4, 5, 6 and 7 B-repeats are 1652, 2420, 2804, 3188 and 3572 bp respectively. PCR was performed using primers Aap53-608rc and aapR (Table 3.1). (b) Whole-cell dot immunoblot of MG1363 pUB1000 expressing different lengths of Aap showing surface expression levels of Aap. Serial dilutions of cells were applied to a PVDF membrane and probed with anti Aap A-Domain antiserum. (c) Mean number of MG1363 pUB1000*aap* cells expressing different lengths of Aap that adhered to corneocytes.

Aap expression on the surface of MG1363 pUB1000*aap4, 5, 6* and *7* was approximately 25 % of that of the original MG1363 pUB1000*aap6high* (Fig. 3.6b). Expression of Aap with 2 B-repeats was reduced by a further 4 fold and a MG1363 pUB1000*aap2* transformant with greater Aap expression could not be identified. The reason for the relatively lower expression of Aap in these transformants is not clear but the promoter sequences, N-terminal signal sequences and LPXTG cell-wall-anchoring motifs were all found to be identical to that of pUB1000*aap6high* (data not shown).

Adhesion to corneocytes was tested using MG1363 pUB1000*aap* variants that expressed Aap at similar levels. A clear correlation was observed between the number of B-repeats and the number of bacteria adhering to corneocytes (Fig. 3.6c), with three times more *aap7* cells attached per corneocyte compared with *aap2* cells. MG1363 pUB1000*aap2* gave the lowest adhesion to corneocytes although it expressed Aap at 4-fold lower levels than MG1363 pUB1000*aap4*. However, it would appear that the length of Aap is more important than cell surface density because, in the case of MG1363 expressing low and high levels of Aap with 6 B-repeats, a 4-fold difference in Aap expression gave only a 1.4 fold reduction in adhesion (Figs. 3.4a & 3.6). These results demonstrate that longer fibrils, with a higher number of B-repeats, resulted in higher numbers of bacteria attaching to the corneocytes

3.5 Discussion

Banner *et al* (Banner *et al.*, 2007) used negative staining to show that the ratio of Aap expressing Fib^+ cells to Aap negative Fib^- cells in a NCTC 11047 WT population was 25 % Fib^+ to 75 % Fib^- cells(Banner *et al.*, 2007). However, flow cytometry with fluorescent antibodies has given a much more accurate assessment of the relative numbers of the two subpopulations, revealing that NCTC 11047 WT is comprised of 72 % cells that express Aap and 28 % that do not. The previous counting method resulted in an underestimate of Fib^+ cells due to difficulties in detecting fibrillar tufts by negative staining in the TEM. An as yet unknown mechanism must exist to control the ratio of the two sub-populations in stationary phase cultures as the ratio is consistent from batch to batch. New cultures of 11047 obtained from the NCTC collection always produced the same stable ratio after the first batch culture grown from the ampoules (data not presented).This consistently stable ratio of Fib^+ to Fib^- cells in different batches of 11047 WT cells cannot be explained by loss of Aap

expression resulting from a mutation. A random mutation occurring during cell division would not give reproducible sub-population ratios. The mechanism of control of the sub-population ratios is currently under investigation as is the mechanism by which stable subpopulations were previously generated by repeated hexadecane enrichment (Banner *et al.*, 2007).
Analysis of 13 other Aap expressing *S. epidermidis* isolates showed that two subpopulations were not unique to strain NCTC 11047 and in the strains we tested 15 % (2 of 13) contained a sub-population that did not express Aap. The presence, within a WT population, of a sub-population of cells expressing a surface protein is not commonly reported in the literature but *S. aureus* cells, in early exponential phase, are known to contain a sub-population of cells expressing the fibronectin binding protein FnBP (Mohamed *et al.*, 2000). However, the number of cells in this sub-population was found to decrease to zero in late exponential phase.

Aap positive cells of NCTC 11047 have a greater affinity for corneocytes compared to the Aap negative cells implying distinct roles for the two sub-populations in skin colonisation. It is thought that commensal strains can translocate from the skin and other sites to cause infection (Costa *et al.*, 2004). Weaker attachment of the Fib^- subpopulations (if present) could aid detachment from the skin leading to re-attachment on another surface (a catheter for example) via a different surface adhesin.

Heterologous expression of surface proteins on *L. lactis* is a common technique used to determine the function of a wide variety of surface proteins (Avall-Jaaskelainen *et al.*, 2003; Hartford *et al.*, 2001b; Hirt *et al.*, 2000). Aap fibrils were observed over the whole cell surface of MG1363 pUB1000*aap6high* and there was no localisation of fibrils, in direct contrast to the native asymmetrical tuft distribution observed in *S. epidermidis* NCTC 11047 WT cells (Banner *et al.*, 2007). In staphylococci the targeting of surface proteins to specific sites in the cell wall is at least partially dependent on the N-terminal signal sequence

(DeDent *et al.*, 2007). The *aap* gene in the pUB1000 constructs had the N-terminal signal sequence of the *S. gordonii* protein SspA in place of the native Aap signal sequence. However, as the Aap protein was expressed in a heterologous background this study suggests only that the tuft phenotype observed in NCTC 11047 is not intrinsic to the mature Aap fibril nor to the LPXTG sortase recognition sequence.

Measurements of lengths of cell surface fibrils from TEM images with negative staining is possible and tufts of Aap fibrils on *S. epidermidis* NCTC 11047 were 122.2 ± 10.8 nm from cell surface to tip (Banner *et al.*, 2007). Measurements of 28 individual fibrils on 7 different MG1363 pUB1000*aap6high* cells gave a mean fibril length of 42.5 ± 6.7 nm (data not shown) suggesting that these fibrils are shorter than those of NCTC 11047 which comprise 12.2 B-repeats. Although the value of 42.5 nm is likely to be an underestimate due to lack of resolution towards the end of the fibrils, it is consistent with the idea that fewer B-repeats give shorter fibrils.

The adhesion of NCTC 11047 and all other Aap expressing *S. epidermidis* strains tested in this study was at least partially dependent on the A-domain of Aap. Previous studies have shown the *aap* gene to be present in between 77 and 89 % of isolates (Rohde *et al.*, 2007; Yao *et al.*, 2005b). Therefore, it is likely that Aap is widely used to mediate attachment to the skin in addition to other unidentified adhesins. Expression of a range of different adhesins would ensure that bacteria could adhere to any given host regardless of possible variations in expression of host receptors. The *S. epidermidis* RP62A genome contains several surface adhesins (Bowden *et al.*, 2005), in addition to Aap, that could potentially promote adhesion to corneocytes such as members of the serine aspartate repeat (Srd) family, which have homologs in *S. aureus*. Three Sdr proteins in *S. aureus* (ClfB, SdrC and SdrD) as well as the Aap homologue SasG are known to contribute to adhesion to nasal epithelial cells (Corrigan *et al.*, 2009; Roche *et al.*, 2003b) raising the possibility that the Sdr proteins from

S. epidermidis (SdrF, H and Fbe) may have a role in adhesion to corneocytes. Also, in *S. aureus*, cell wall teichoic acids were found to have a role in colonisation of cotton rat nares and adhesion to nasal epithelial cells (Weidenmaier *et al.*, 2004). However, integral components of the cell wall such as teichoic acids are unlikely to have major roles in adhesion of *S. epidermidis* to corneocytes as $rAap_{A\text{-}Dom}$ almost completely blocked adhesion of the three WT *S. epidermidis* strains JBS4, 5 and 14.

The $rAap_{B\text{-}rep}$ protein was unable to inhibit Aap-dependent adhesion to corneocytes. A recent study suggested that recombinant B-repeats of Aap are only fully folded when capped at the C-terminus by an additional half repeat (Conrady *et al.*, 2008). For this reason it is unlikely that $rAap_{B\text{-}rep}$ used in this study is completely folded. However, Fig 3.4c demonstrated that the linear a.a. sequence of a single B-repeat was unable to adhere to corneocytes. In addition, MG1363 pUB1000*aap6highT* which contained the C-terminal half B-repeat, expressed Aap fibrils lacking the A-domain that were visible by negative staining in the TEM (Fig 3.4a & data not shown), strongly suggesting that the B-repeats are correctly folded when expressed on *L.lactis* MG1363. In conclusion the inability of the fibrillar, truncated Aap to mediate corneocyte adhesion proves that the B-repeats have no ligand-binding function.

Increasing numbers of B-repeats in the Aap molecule promoted increasingly enhanced adhesion of MG1363 pUB1000*aap* to corneocytes. As the B-repeats have no innate receptor-binding function, the length of Aap fibril must influence adhesion. This demonstrates a function for the B-region in projecting the terminal ligand-binding A-domain away from the cell to allow enhanced attachment of bacteria to corneocytes. MG1363 does not produce a capsule (Gasson, 1983b; Germond *et al.*, 2001) and no surface proteins could be seen on MG1363 pUB1000 by TEM suggesting that masking of shorter Aap fibrils by other bacterial surface components does not occur in this strain background. Longer Aap fibrils may allow bacteria to reduce electrostatic repulsive forces

between the bacterial cell surface and the surface of the corneocytes. Alternatively, longer fibrils would be expected to be more flexible, giving the A-domain a larger range of movement which may allow more A-domain-host ligand interactions to occur simultaneously.

The host receptor for Aap on corneocytes is currently unknown. The cornified cell envelope comprises a cross-linked network of proteins, the main constituent of which is loricrin (Kalinin *et al.*, 2001; Steinert and Marekov, 1995; Steinert *et al.*, 1998) and beyond this is a layer of lipids (Candi *et al.*, 2005). The precise molecular arrangement of these proteins and lipids, and their respective accessibilities to bacteria, are not known but *S. aureus* adhesin ClfB promotes adhesion to nasal epithelial cells via the envelope protein cytokeratin-10 (O'Brien *et al.*, 2002; Walsh *et al.*, 2004) as does the *S. aureus* protein IsdA which also binds to loricrin and involucrin (Clarke *et al.*, 2009). As these inner components of the cornified envelope are accessible to bacteria, other components of the protein envelope as well as components of the lipid layer may also be possible receptors for Aap. Aap and SasG of *S. aureus* share an as yet unknown corneocyte ligand and SasG was shown not to adhere to fibrinogen, fibronectin, human epidermal keratin, collagen, von Willebrand factor, laminin, heparin sulphate nor sub maxillary mucin (Roche *et al.*, 2003b). The A-domains of Aap and SasG share a 212 a.a. region that is 59% identical in terms of a.a. sequence (Roche *et al.*, 2003a) and it is likely that this region contains a binding site for a corneocyte ligand. However, there is the possibility that Aap may adhere to more than one ligand, as in the case of IsdA (Clarke *et al.*, 2009). The Aap and SasG A-domains contain unique regions (Roche *et al.*, 2003a) that could contain additional, as yet unidentified, ligand binding sites.

This study has confirmed that Aap is a fibrillar adhesin and has shown that the terminal A-domain directly mediates adhesion to corneocytes implying a role for Aap in colonisation of the skin. Aap is also known to promote biofilm formation

making this cell-wall-anchored protein a bifunctional molecule important for both the commensal and pathogenic lifestyles of *S. epidermidis*.

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Chapter 4:

Heterologous expression of the accumulation associated protein (Aap) of *Staphylococcus epidermidis* predicts a role for the A-domain in adhesion and early biofilm formation

4.1 Abstract

Staphylococcus epidermidis is a primary cause of nosocomial infections due to its ability to attach to medical implants and accumulate in a secreted polymeric biofilm matrix. The cell-wall-anchored fibrillar protein, Aap, mediates adhesion to corneocytes, promotes biofilm accumulation and comprises a stalk-like B-region containing tandem repeats and a terminal A-domain. Proteolytically cleaved Aap, comprising the B-region only, promotes accumulation possibly by binding a putative *S. epidermidis* surface ligand. To further investigate the function of specific domains of Aap in initial attachment and accumulation, Aap fibrils were expressed with and without the A-domain on the surrogate hosts *Lactococcus lactis* MG1363 and *Staphylococcus aureus* RN4220. The A-domain and B-region promoted initial attachment to unmodified polystyrene. However, only the A-domain promoted adhesion to tissue culture treated polystyrene and adhesion was enhanced by proteolytic removal of an N-terminal portion of the A-domain. Biofilms were cultivated in a flow-cell system and confocal microscopy was used to measure the volume of microcolonies that form during the initial stage of accumulation. Aap B-repeats did not promote microcolony formation in these surrogate hosts possibly due to the lack of a putative ligand. Aap, with the A-domain, enhanced microcolony formation, suggesting a potential role for the A-domain in accumulation in the early stage of biofilm formation. The A-domain of Aap is therefore multifunctional because, in addition to mediating adhesion to corneocytes it can promote initial attachment to polystyrene and functions in the early accumulation stage of biofilm formation.

4.2 Introduction

The opportunistic pathogen *Staphylococcus epidermidis* is an increasingly prevalent cause of infections associated with medical devices such as central venous catheters, prosthetic joints and heart valves which can lead to bacteraemia or endocarditis (Arber *et al.*, 1994; Rupp and Archer, 1994; Uckay *et al.*, 2009). *S. epidermidis* is able to colonize medical devices through biofilm formation and in most cases infection cannot be cleared without removal of the implant; at great cost to the health services and with consequential increased morbidity and mortality (Bengtson, 1993). *S. epidermidis* is a permanent commensal resident on human skin (Kloos and Musselwhite, 1975) providing a potential source for contamination of implants (Haas *et al.*, 2005; Otto, 2008). It is therefore essential to fully understand the processes and molecules that mediate adhesion and biofilm formation.

Biofilm formation by *S. epidermidis* on medical devices is traditionally described as occurring in two distinct phases; initial attachment and accumulation. Each of these phases is likely to involve multiple molecular mechanisms and an additional stage, dispersal, may also occur (Otto, 2008). Initial attachment of *S. epidermidis* to the abiotic surfaces of implants may provide the first stage in colonization and three cell surface proteins have been reported to mediate attachment to polystyrene namely SSP1 & 2 (the products of one unidentified gene giving two SDS PAGE bands) (Timmerman *et al.*, 1991; Veenstra *et al.*, 1996) and the bifunctional autolysins AtlE and Aae (Heilmann *et al.*, 1997; Heilmann *et al.*, 2003). However, very little is known about the *S. epidermidis* cell surface molecules that are used to attach to abiotic surfaces.

In order to remain attached to an implanted device *S. epidermidis* must also adhere to the host matrix proteins that coat the device surface (Francois *et al.*, 1998). This is mediated by a class of proteinacious adhesins termed MSCRAMMs (microbial surface components recognizing adhesive matrix

molecules) (Patti *et al.*, 1994a). Many of these are anchored covalently to the cell wall by a sortase enzyme that recognizes a C-terminal LPXTG motif (Schneewind *et al.*, 1995). Adhesion to fibronectin, fibrinogen and collagen is mediated by the cell-bound MSCRAMMs Embp (Williams *et al.*, 2002), SdrG (Fbe) (Nilsson *et al.*, 1998) and SdrF (Arrecubieta *et al.*, 2007) respectively and the bi-functional autolysins, AtlE and Aae, provide less specific attachment to vitronectin, fibronectin and fibrinogen (Heilmann *et al.*, 1997; Heilmann *et al.*, 2003).

The second phase of biofilm formation in *S. epidermidis*, the accumulation phase, involves the formation of microcolonies that expand in size and coalesce as the biofilm matures (Otto, 2009). This process is promoted by several cell wall anchored and secreted polymers which allow intercellular adhesion. These include polysaccharides, proteins and extracellular DNA. The β-1-6-linked N-acetylglucosamine polysaccharide (polysaccharide intercellular adhesin, PIA) (Mack *et al.*, 1996), encoded by the *icaADBC* operon (Gerke *et al.*, 1998; Heilmann *et al.*, 1996b), has the most well-established role in *S. epidermidis* biofilm accumulation and is commonly found in clinical isolates (Galdbart *et al.*, 2000; Otto, 2009). However, not all clinically significant strains carry the *ica* operon and in some of these strains it is suggested that the LPXTG-anchored accumulation associated protein (Aap), plays a major role in biofilm formation (Rohde *et al.*, 2005; Rohde *et al.*, 2007). More recently another cell surface protein, Embp, has also been shown to promote accumulation (Christner *et al.*, 2010). In addition, cell wall anchored proteins Bhp (Cucarella *et al.*, 2001; Tormo *et al.*, 2005a) and SesC (Shahrooei *et al.*, 2009) have been implicated in biofilm formation. Extracellular DNA, released though the action of the autolysin AtlE, may also play a role in biofilm development by making up part of the extracellular matrix (Qin *et al.*, 2007). There are a wide variety of factors that may promote biofilm formation although not all are present on every *S. epidermidis* strain (Otto, 2009). In order to disrupt *S. epidermidis* biofilm formation on medical devices, further understanding of the functions of these

factors is required. Given the varied contribution of several polymers to biofilm formation, examination of the roles of individual polymers in heterologous expression systems will show more clearly some of their potential functions.

Here, the role of Aap in the initial adhesion and accumulation stages of biofilm formation in two very distinct surrogate hosts was investigated. *Lactococcus lactis* MG1363 was selected as a non-staphylococcal surrogate host to identify functions of Aap that have not been detected in *S. epidermidis* strains due to functional redundancy among surface molecules. Aap function was also analysed in *Staphylococcus aureus* RN4220, a weak biofilm forming strain (Cramton *et al.*, 1999; McKenney *et al.*, 1998), to analyze the contribution of Aap to the process of biofilm formation in a staphylococcal host that was not *S. epidermidis*.

Aap is known to be a bi-functional molecule as it has a role in accumulation (Hussain *et al.*, 1997; Rohde *et al.*, 2005) as well as mediating attachment to human cornified epithelial cells (Macintosh *et al.*, 2009). Aap has a modular domain architecture and proximal to the N-terminal export sequence is a ~ 60 kDa A-domain that mediates the adhesion to corneocytes (Macintosh *et al.*, 2009). Within the N-terminal portion of the A-domain are 10 repeats of 16 amino acids (A-repeats). However, the majority of the Aap molecule, the B-region, comprises larger repeats of 128 amino acids, the number of which varies from 5 to 17 depending on the strain (Rohde *et al.*, 2007), and this region provides the stalk of the Aap fibril projecting the A-domain away from the cell. The length of the B-region affects the adhesive function of the A-domain as longer B-regions promoted greater adhesion to corneocytes (Macintosh *et al.*, 2009). B-region length is also important in the Aap homologue, SasG of *S. aureus*. SasG shares the same domain architecture (although it lacks the A-repeats) and functions as Aap (Roche *et al.*, 2003b) and more than 4 B-repeats are required for SasG-dependant biofilm formation (Corrigan *et al.*, 2007).

It is thought that the B-repeat region of Aap, rather than the A-domain mediates biofilm accumulation as only a proteolytically truncated form of Aap from *S. epidermidis* 5179 lacking the A-domain promoted biofilm formation in 96-well plates (Rohde *et al.*, 2005). Also, it has been proposed that intercellular adhesion in strain RP62A occurs mainly through Zn^{2+} dependant homo-dimerisation of B-regions (Conrady *et al.*, 2008). However, this finding is at odds with other work suggesting that PIA is the main determinant of biofilm formation in the same strain (Chaignon *et al.*, 2007). Discrepancies such as this and strong evidence suggesting SasG promotes biofilm formation when the A-domain is present (Corrigan *et al.*, 2007) highlight the need for further studies into the role of specific Aap domains in biofilm formation. A recent review of staphylococcal biofilms (Otto, 2008) suggested that the commonly used 96-well plate biofilm assay inadequately recreates *in vivo* biofilm conditions and that more complex *in vitro* methods such as flow-cells are required to better assess the function of molecules such as Aap involved with biofilm formation.

Previously we have shown that Aap is expressed as cell surface fibrils that form lateral tufts on a sub population of cells in *S. epidermidis* NCTC 11047 (Banner *et al.*, 2007) and also that the A-domain of Aap mediates adhesion to human corneocytes (Macintosh *et al.*, 2009). Here we show that Aap complete with the A-domain has the additional dual functions of promoting adhesion to polystyrene and facilitating the early stage of biofilm accumulation when expressed on two surrogate hosts. The implications of these observations for the attachment of *S. epidermidis* to medical implants and in biofilm formation are discussed.

4.3 Experimental procedures

4.3.1 Bacterial strains and plasmids

The strains used in this study were *Lactococcus lactis* MG1363 (Gasson, 1983a) and *Staphylococcus aureus* RN4220 (Kreiswirth *et al.*, 1983), which is a restriction deficient mutant of strain 8325-4 that can stably maintain plasmids. The pUB1000 plasmid (Heddle *et al.*, 2003) was used here to express Aap on the surface of MG1363 and RN4220. The pUB1000 constructs used in this study were previously referred to as pUB1000aap6high or pUB1000aap6highT (Macintosh *et al.*, 2009) but here they are referred to simply as pUB1000aap6 and pUB1000aap6T. MG1363 cultures were incubated statically at 30 °C for 18 h in M17 broth (Oxoid) supplemented with 0.5 % glucose (GM17). RN4220 cultures were grown statically at 37 °C for 18 h in tryptic soy broth (TSB, Oxoid). Erythromycin (5 µg/ml) was added to media when strains containing pUB1000 were cultured. Cloning and expression of $rAap_{6.5B\text{-}rep}$ (see below) was performed in *E. coli* M15 pREP4 pQE30Xa cultured in Luria-Bertani (LB) broth at 37 °C with shaking (200 r.p.m).

4.3.2 Negative staining in the TEM

Cell surface fibrillar structures were detected by negative staining with 2% methylamine tungstate (Agar Scientific) at pH 6.5 by TEM as described previously (Handley *et al.*, 1985; Macintosh *et al.*, 2009).

4.3.3 Quantification of adhesion to tissue culture treated 96-well plates

A previously published method (Banner *et al.*, 2007) was used to quantify adhesion to tissue culture treated polystyrene with some modifications. Briefly, stationary phase bacterial cultures were washed three times in phosphate

buffered saline (PBS, Sigma) and resuspended to an OD_{490} of 1.0. Cell suspensions (200 µl) were then added to flat bottomed tissue culture treated 96-well plates (Corning) and incubated at 37 °C for 2 h. Wells were then washed three times with PBS (200 µl), dried at 42 °C for at least 3 h and stained with 4 % (v/v) crystal violet (CV) for 10 min. Excess CV was washed out of the wells with distilled water and ethanol was used to solubilise the remaining stain. OD_{530} of the well was then measured.

Trypsin treatment of *L. lactis* MG1363 pUB1000aap6 was carried out as described previously (Veenstra *et al.*, 1996). Trypsin (10 µ/ml, Sigma) was added to stationary phase cultures and the cells were incubated at 37 °C for 10 min. Cells were then washed 3 times in PBS at 4 °C and the adhesion procedure described above was followed. For inhibition of adhesion, $rAap_{A\text{-}dom}$ and $rAap_{sh\text{-}A\text{-}reps}$ (Banner *et al.*, 2007; Macintosh *et al.*, 2009) were added to wells and incubated for 2 h at 37 °C. Wells were then washed 3 times in PBS to remove unbound rAap and bacteria were added as described above.

4.3.4 Quantification of adhesion to unmodified polystyrene

Adhesion to unmodified polystyrene was quantified by a method described previously (Heilmann *et al.*, 1996a). Stationary phase bacterial cultures were washed 3 times in PBS and resuspended to an OD_{490} of 0.2. Aliquots (10ml) were then added to unmodified polystyrene petri dishes (Sterilin Ltd, UK) and incubated at 37 °C for 2 h. Dishes were then washed 3 times in PBS and dried for at least 3 h. Dishes were then stained with CV for 10 min and rinsed with distilled water to remove excess stain. Photographs of the bacteria were then taken at random positions on the dish (3 photographs for each dish) using a light microscope with x100 objective lens and digital camera. To calculate the number of bacteria per microscope view a grid was placed over the photographs and the number of bacteria in a random sample of ten squares were counted to calculate the number of bacteria per microscope view.

4.3.5 SDS PAGE and Western blotting of cell-wall proteins

Cell-wall proteins of *L. lactis* MG1363 were extracted and analysed by Western blot as described previously (Banner *et al.*, 2007; Macintosh *et al.*, 2009). Trypsin treatment was performed as described above. Cell-wall proteins were extracted from stationary phase cultures of *S. aureus* RN4220 (30 ml). Cells were washed once in PBS then resuspended in 50 µl spheroplasting buffer (20 mM Tris-HCl [pH 6.8], 10 mM $MgCl_2$, 26% [wt/vol] raffinose · $5H_2O$) containing lysostaphin (0.2 mg/ml), lysozyme (0.4 mg/ml) and phenylmethylsulfonyl fluoride (1 mM, Sigma) and incubated at 37 °C for 25 min to release cell-wall proteins without lysing the cells. Cells were then centrifuged in a bench-top microfuge (13,000 rpm for 5 min) and the supernatant containing cell-wall proteins was analysed using SDS PAGE (8% acrylamide gel).

4.3.6 Mass spectrometry of SDS PAGE bands

Aap SDS PAGE bands were excised and digested with trypsin as described previously (Rosenfeld *et al.*, 1992). Digested samples were analysed by LC-MS/MS using a NanoAcquity LC (Waters, Manchester, UK) coupled to a LTQ Velos (Thermo Fisher Scientific, Waltham, MA). Peptide data were analysed using Scaffold 2 software (Proteome software Inc, USA) and aligned to the sequence of Aap from *S. epidermidis* NCTC 11047 (accession number HM587132) to determine the section of Aap that had been cleaved.

4.3.7 Biofilms cultured under flow conditions

Biofilms were cultivated in a flow-chamber with channel dimensions of 1 x 4 x 40 mm. The flow-chamber system was assembled as described previously (Christensen *et al.*, 1999). A 24 x 50 mm glass coverslip was used as substratum for biofilm growth. The flow-chamber was sterilized with hypochlorite (0.5 %) as described previously (Lappann *et al.*, 2006). Inocula were prepared

as follows: *L. lactis* MG1363 stationary phase cultures were diluted in PBS to an OD_{490} of 1.0 whereas *S. aureus* RN4220 stationary phase cultures were diluted 1:100 into TSB diluted (1:20) in AB medium (Clark and Maaløe, 1967) in accordance with a previously published method for culturing staphylococcal biofilms under flow conditions (Qin *et al.*, 2007). Aliquots of the inoculum (200 µl) were then injected into channels of the flow-chamber. After inoculation flow chambers were incubated statically for 1 h to allow bacteria to attach to the cover-slip and medium (GM17 for *L. lactis* MG1363 and TSB diluted in AB medium for *S. aureus* RN4220) was then pumped through the channels (0.2 mm/s) using a Watson-Marlow 205S peristaltic pump. All biofilms were grown at 37 °C.

4.3.8 Biofilm Image acquisition and analysis

All images were taken using a Leica SP5 upright CLSM (Leica) using a x63/1.4 oil emersion objective lens. Bacteria were stained using the green fluorescent dye SYTO 9 (8.7 µM, Invitrogen) and images were acquired at random positions roughly half way down the centre of the flow-chamber channels (9 images per channel). Imaris (Bitplane) was used to create top down and sagittal views. The freely available COMSTAT computer program (Heydorn *et al.*, 2000) was used to calculate the average microcolony volume.

4.3.9 Biofilms cultured in 96-well plates

Biofilms were cultured and quantified in 96-well plates by a previously published method (Christensen *et al.*, 1985). Stationary phase *S. aureus* RN4220 cells were diluted 1:100 in fresh heart infusion medium (Oxoid) supplemented with glucose (0.5 % w/v) and aliquots (200 µl) were added to wells and incubated for 24 h at 37 °C. Wells were then washed 3 times with PBS (200 µl), dried at 42 °C for at least 3 h and stained with 4 % (v/v) CV for 10 min. Excess CV was washed out of the wells with distilled water and ethanol was used to solubilise the remaining stain. OD_{530} of the well was then measured. For inhibition of

biofilm formation rAap and BSA (Sigma) were added to the wells containing bacteria 2 h after the start of incubation to ensure primary adhesion to the wells was not affected.

4.3.10 Cloning, expression and purification of rAap in E. coli

To express and purify $rAap_{6.5\ B\text{-}rep}$ the B-region of *aap* including the 3' half repeat was PCR amplified from the genomic DNA of *S. epidermidis* strain RP62A, which carries an *aap* gene with 12.5 B repeats (accession number, NC 002976) and ligated into pQE30Xa (Qiagen) to create a N-terminal His_6 tag fusion. The forward primer (5'–CCGGGATCCACATTGACATACACTCCTAAAGCA-3') and reverse primer (3'–CGGAAGCTTGGACCATATTTTGTCACATC-3') incorporated a 5' BamHI and a 3' HindIII restriction enzyme site (restriction sites underlined), respectively. This PCR gave a single DNA band on an agarose gel at the expected size of ~ 4800 base pairs. The PCR product was cloned into the BamHI and HindIII sites of pQE30Xa and transformed into *E. coli* M15 $pREP_4$ (Qiagen).

All the resulting transformants that were tested contained the B-region with fewer than 12.5 B-repeats. The transformant containing pQE30Xa with the greatest number of B-repeats (6.5) was selected and the B-region insert was confirmed by sequencing. $rAap_{6.5B\text{-}rep}$ containing 6.5 repeats was purified by Ni^{2+} ion affinity chromatography using a HisTrap HP column (Amersham) with a 40 mM imidazol wash step and elution was performed in 250 mM imidazol. After dialysis into PBS, protein concentration was determined by the Bradford assay using a protein concentration kit (Biorad) with BSA standards according to manufacturers instructions. Integrity and purity of the protein was confirmed by SDS PAGE. $rAap_{A\text{-}dom}$ and $rAap_{sh\text{-}A\text{-}reps}$ were purified as described previously (Banner *et al.*, 2007; Macintosh *et al.*, 2009).

4.4 Results

4.4.1 Aap mediates adhesion to polystyrene surfaces

L. lactis MG1363 and *S. aureus* RN4220 were used as heterologous expression models to investigate the relative importance of the A-domain and the B-repeat region of Aap in mediating attachment to polystyrene. These surrogate hosts were selected in order to analyze the function of Aap in the context of a relatively non-adhesive (MG1363) and a more adhesive (RN4220) bacterial surface (Fig. 4.1A).

Previously we cloned the *aap* gene of *S. epidermidis* NCTC 11047 (accession number HM587132) into the surface expression vector pUB1000 and Aap was expressed on the surface of *L. lactis* MG1363 (Macintosh *et al.*, 2009). One pUB1000 construct comprised the B-region with 6.5 repeats and the A-domain of Aap (pUB1000aap6) whereas the other comprised the B-region only, also with 6.5 B repeats (pUB1000aap6T). Negative staining in the TEM of *L. lactis* MG1363 containing pUB1000aap6 (Macintosh *et al.*, 2009) and pUB1000aap6T (Fig. 4.1A) showed both constructs gave rise to cell-surface-anchored fibrils proving both Aap constructs are correctly folded on the cell surface. In addition, in this study, the pUB1000 constructs were electroporated into *S. aureus* RN4220 and correct targeting of Aap to the cell wall was proved by SDS PAGE of cell wall proteins (Fig. 4.1B). RN4220 pUB1000aap6 gave two Aap bands (arrows) at ~ 200 and 270 kDa and pUB1000aap6T gave one band (arrow) at ~ 190 kDa just as seen for MG1363 expressing the same constructs (Macintosh *et al.*, 2009). The pUB1000aap6T band was smeared as reported previously (Macintosh *et al.*, 2009; Rohde *et al.*, 2005).

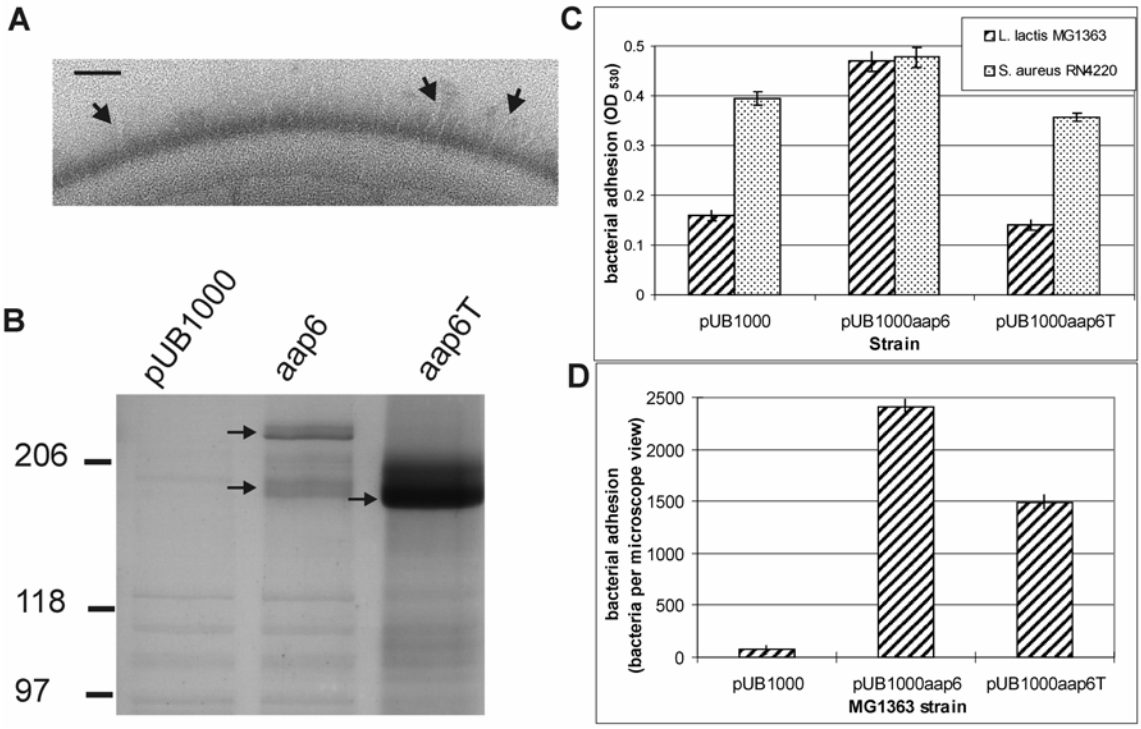

Figure. 4.1. Surface expression of Aap and Aap-dependant polystyrene adhesion. (A) *L. lactis* MG1363 pUB1000aap6T negatively stained with 2% methylamine tungstate showing the peritrichous cell surface Aap fibrils (arrows). Scale bar is 50 nm. (B) Coomassie stained SDS PAGE of cell wall proteins of *S. aureus* RN4220 pUB1000, pUB1000aap6 and pUB1000aap6T showing Aap bands (arrows). Molecular weight markers are shown in kDa. (C) Adhesion to polystyrene of *L. lactis* MG1363 and *S. aureus* RN4220 containing pUB1000, pUB1000aap6 and pUB1000aap6T. Cells were allowed to adhere to 96-well plates for 2 h and bound cells were stained before the OD_{530} of the well was recorded. (D) Adhesion to unmodified polystyrene of *L. lactis* MG1363 containing pUB1000 constructs. Cells were allowed to adhere for 2 h and were stained before being counted. Results represent the means and standard deviations from at least two independent experiments.

L. lactis MG1363 and *S. aureus* RN4220 containing pUB1000aap6 and pUB1000aap6T were tested for their ability to adhere to tissue culture treated polystyrene 96-well plates. Expression of Aap complete with the A-domain was found to promote attachment to 96-well plates as MG1363 pUB1000aap6 gave an approximately three-fold increase in adherence over the MG1363 pUB1000 vector control (Fig. 4.1C). Adhesion of RN4220 pUB1000aap6 was also greater than the RN4220 pUB1000 control but to a lesser extent (~ 20 %) due to a higher background level of polystyrene adhesion of this strain. The Aap B-

region, however, was unable to promote adhesion in either MG1363 or RN4220 strain backgrounds (Fig. 4.1C). Expression of Aap B-region was found to decrease the adhesion of RN4220 by ~ 10 % possibly due to masking of other native surface adhesins responsible for adhesion to polystyrene. These data show that only the A-domain of Aap can promote adhesion to tissue culture treated polystyrene 96-well plates.

In contrast, both the B-region and A-domain promoted adhesion to unmodified polystyrene as MG1363 pUB1000aap6 and pUB1000aap6T gave 33-fold and 20-fold increases in adhesion respectively compared to the pUB1000 control (Fig. 4.1D). Taken together, the results of these two adhesion assays show that Aap mediates adhesion to both model abiotic polystyrene surfaces. However, whereas the A-domain attaches to both surfaces the B-region only attaches to unmodified polystyrene.

4.4.2 Trypsin treatment of Aap cleaves the A-repeats of the A-domain

The expression of Aap on the cell surface of *L. lactis* MG1363 promotes adhesion to polystyrene and it yields two SDS PAGE bands similar to those seen for *S. epidermidis* strains expressing Aap (Banner *et al.*, 2007; Bowden *et al.*, 2005; Rohde *et al.*, 2005). The cause of the two bands and any functional significance is not yet clear.

To investigate whether Aap on the cell surface was sensitive to proteolysis resulting in two bands, stationary phase MG1363 pUB1000aap6 cells were treated with trypsin (10 µg/ml) for 15 min. The top Aap band disappeared and the bottom band became more intense (Fig. 4.2A, arrow and double arrow respectively). Both these bands were strongly reactive with antiserum specific to the A-domain of Aap (Fig. 4.2B). In contrast, truncated Aap of MG1363 pUB1000aap6T was only slightly degraded by trypsin as shown by a slight broadening of the SDS PAGE band (data not shown). This strongly suggests

that the A-domain of Aap contains a region that can be cleaved by trypsin causing a shift in Aap band migration on SDS PAGE. Stationary phase cultures of MG1363 pUB1000aap6 grown overnight in GM17 broth contained mainly the non-proteolytically processed form of Aap (Fig. 4.2A single arrow).

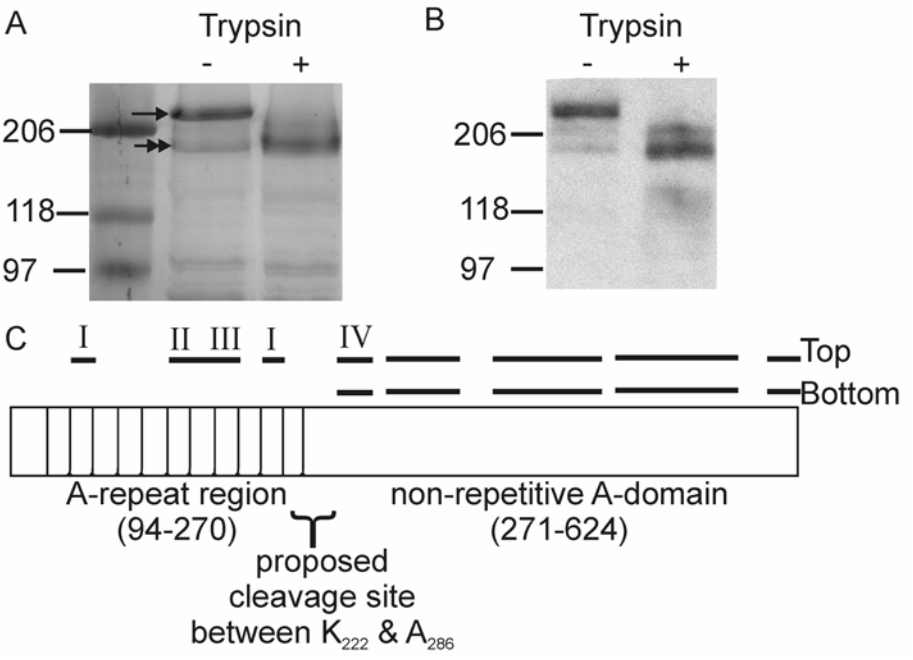

Figure. 4.2. Proteolytic cleavage of the A-repeats of Aap A-domain. (A) Coomassie stained SDS PAGE of cell wall proteins from *L. lactis* MG1363 pUB1000aap6 without (-) and with (+) trypsin treatment. The top and bottom Aap bands are marked with an arrow and a double arrow respectively. Molecular weight markers are shown in kDa. (B) Western blot of cell-wall proteins of *L. lactis* MG1363 pUB1000aap6 before and after trypsin treatment probed with antiserum specific to the A-domain of Aap. (C) Schematic diagram of the mature A-domain of Aap (after cleavage of the export sequence) and the position of tryptic peptides (bold lines) detected in the top or bottom pUB1000aap6 SDS PAGE bands. Roman numerals label the peptides referred to in the text. Peptide I is shown twice as the sequence appears in two of the repeats. Amino acid numbers are shown and they refer to the sequence from *S. epidermidis* NCTC 11047 (accession number HM587132). N.B. the B-region is not shown.

The approximate cleavage point within the A-domain was mapped using mass spectrometry (Fig. 4.2C). The top and bottom SDS PAGE bands (Fig. 4.2A, arrows) were excised and subjected to trypsin digestion. Tryptic peptides were then detected by liquid chromatography mass spectrometry. Figure 4.2C shows

where the peptides from the top and bottom Aap bands aligned to the mature (after cleavage of the export sequence) A-domain sequence. The three peptides labelled I, II & III (A_{111}EEGGNAEAAQSEPTK$_{126}$, A_{175}EEGGNAEAAQSEPTKTEEGSNVK$_{198}$ and A_{199}AQSEPTKAEEGSNAEAPQSEPTK$_{222}$, respectively) from the A-repeats were present only in the top Aap band whereas peptide IV (A_{286}NSDNDTQTQFSEAPTR$_{303}$) and the other peptides from the non-repetitive region were present in both bands (Fig. 4.2C, bold lines). The presence and absence of specific peptides was confirmed by repeating the experiment with a second cell-wall protein preparation. These results show that the top Aap band represents complete Aap whereas the bottom band represents a truncated form of Aap lacking the A-repeats. The proteolytic cleavage point is located between K_{222} and A_{286} (Fig. 4.2C).

4.4.3 Trypsin treatment of the A-domain of Aap enhances adhesion to polystyrene

To determine whether or not proteolytic cleavage of the A-repeat region altered A-domain dependant adhesion the effect of trypsin treatment on *L. lactis* MG1363 pUB1000aap6 adhesion to polystyrene was tested.

Trypsin treated MG1363 pUB1000aap6 cells showed a significant increase of 35% ($P < 0.01$) in adhesion to 96-well plate polystyrene compared to untreated pUB1000aap6 cells (Fig. 4.3). In contrast, identical treatment of MG1363 pUB1000aap6T caused a decrease in adhesion of 54 % ($P < 0.01$) showing that proteolytic processing of cell surface components of MG1363 or the B-region of Aap reduced adhesion. Therefore, the increase in adhesion of cells expressing Aap encoded by pUB1000aap6 was due specifically to the proteolytic removal of the A-repeats. This shows that the non-repetitive region of the A-domain promotes adhesion to polystyrene and suggests that the A-repeats inhibit adhesion or have a reduced affinity for polystyrene compared to the non-repetitive region.

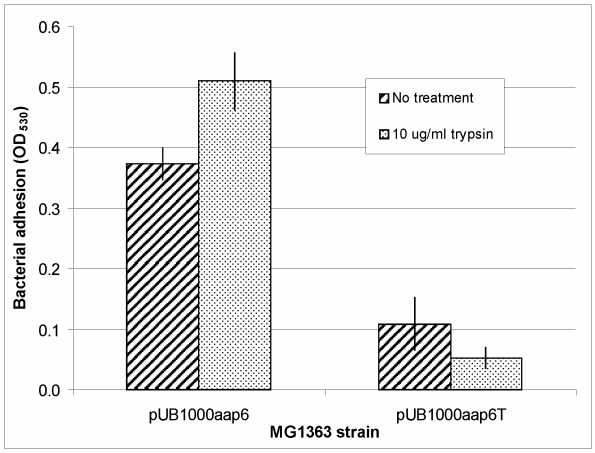

Figure. 4.3. Proteolytic cleavage of A-repeats enhances adhesion. Effect of trypsin treatment (10 µg/ml, 15 min) on *L. lactis* MG1363 pUB1000aap6 and pUB1000aap6T adhesion to polystyrene. Results represent mean and standard error of three independent experiments.

However, recombinant proteins corresponding to both the full A-domain ($rAap_{A\text{-}dom}$) and the A-repeats ($rAap_{sh\text{-}A\text{-}reps}$) could inhibit adhesion of MG1363 pUB100aap6 at far lower concentrations than BSA (Fig. 4.4) suggesting that the A-repeats, as well as the non-repetitive region, can adhere to polystyrene. Polystyrene wells were pre-incubated with $rAap_{sh\text{-}A\text{-}reps}$, $rAap_{A\text{-}dom}$ or BSA and then washed so that the amount of protein remaining bound to the wells was related to the affinity of that protein for polystyrene. At a concentration of 1 µM all three proteins abolished adhesion (data not shown) showing that the proteins had completely coated the wells and MG1363 pUB1000aap6 could not bind to protein coated wells. The $rAap_{A\text{-}dom}$ (comprising A-repeats and non-repetitive region) was more effective than $rAap_{sh\text{-}A\text{-}reps}$ alone at blocking adhesion at very low concentrations suggesting the non-repetitive A-domain had a greater affinity for polystyrene than the A-repeats.

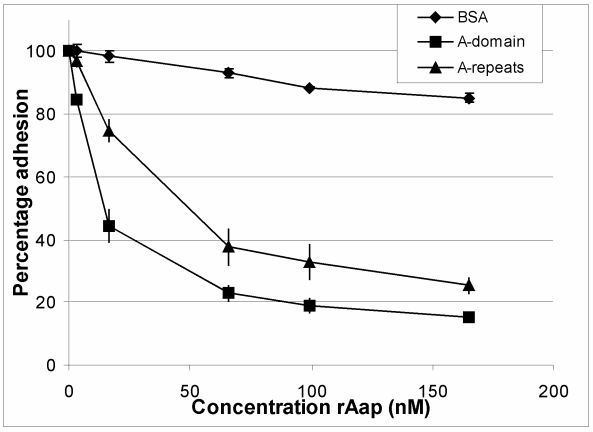

Figure. 4.4. The A-repeats and the full A-domain of Aap block adhesion to polystyrene. Wells were blocked with increasing concentrations of $rAap_{A\text{-}dom}$ and $rAap_{sh\text{-}A\text{-}reps}$ and BSA and then washed before addition of *L. lactis* MG1363 pUB1000aap6. Results represent the mean and standard error of three experiments.

Taken together these adhesion results prove that the A-domain of Aap is responsible for adhesion to tissue culture treated polystyrene. Furthermore, the ability of the A-domain to mediate adhesion can be increased *in vitro* by proteolytic removal of the A-repeats.

4.4.4 Aap complete with the A-domain enhances the formation of early biofilm microcolonies by *L. lactis* MG1363 in a flow-cell system

Rohde *et al.*, (Rohde *et al.*, 2005) reported that when the A-domain of Aap is completely removed by proteolysis, the B-region can promote intercellular adhesion in the biofilm accumulation phase and subsequently it was suggested that the B-regions may dimerise with other B-regions from neighboring cells to promote intercellular adhesion (Conrady *et al.*, 2008). To investigate the theory that the B-repeats alone can mediate biofilm accumulation, the ability of *L. lactis* MG1363 pUB1000aap6T to accumulate under flow conditions was tested in a flow-cell system.

Confocal laser scanning microscopy (CLSM) was used to view biofilms grown on a glass coverslip. The volume of individual microcolonies was a measure of

the ability of cells to accumulate. Bacteria in suspension were allowed to attach for one hour in static conditions to a glass microscope cover-slip in a flow-cell. Medium was then flowed though the flow-cell (0.2 mm/s) for 24 h to allow multiplication of attached bacteria. CLSM was used to view attached microcolonies of bacteria and the volume of each individual microcolony was calculated. No informative 96-well plate biofilm assay data are available for *L. lactis* MG1363 strains as cells could not adhere to wells that had been conditioned with GM17 medium unlike *S. aureus* RN4220 which could adhere to medium conditioned wells (data not shown).

L. lactis MG1363 pUB1000, pUB1000aap6 and pUB1000aap6T were compared in the flow-cell system and all developed into biofilms comprising small, distinct microcolonies after 24 h (Fig. 4.5). Longer incubation did not result in further development of the biofilm (data not shown). MG1363 pUB100aap6, with the A-domain, (Fig. 4.5A) produced microcolonies with the greatest volume (Fig. 4.5B) although microcolony size varied within single microscope views (Fig. 4.5A). Detailed analysis of 27 CLSM image files for each pUB1000 construct (nine images for three independent experiments) using the COMSTAT program showed that MG1363 pUB1000aap6 gave individual microcolonies that were, on average, 80 % larger than those of MG1363 pUB1000 and pUB1000aap6T (Fig. 4.5B). The average microcolony volumes of MG1363 pUB1000 and pUB1000aap6T were not significantly different showing that the B-region alone was not able to promote accumulation in this background. In contrast, pUB1000aap6 with the full A-domain enhanced microcolony formation showing that the A-domain is potentially involved in the early accumulation phase of biofilm formation.

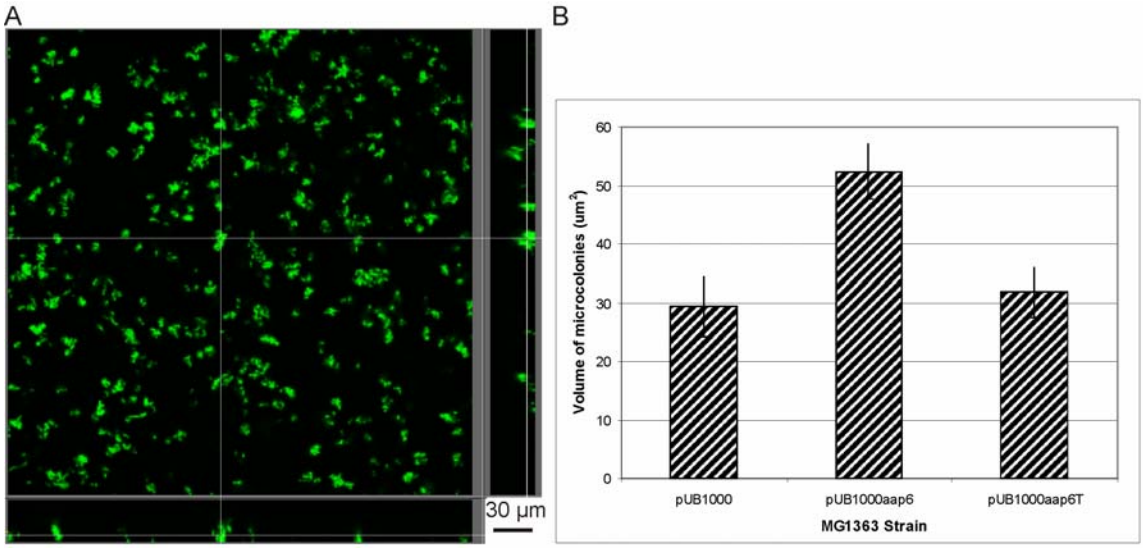

Figure. 4.5. Biofilms of *L. lactis* MG1363 cultured under flow conditions (0.2 mm/s) for 24 h. (A) MG1363 pUB1000aap6 biofilm showing top-down and sagittal views. (B) Average microcolony volumes of MG1363 biofilms calculated by COMSTAT. Results represent mean and standard error of three independent experiments with nine pictures taken of each experiment.

4.4.5 Full length Aap also promotes accumulation in *S. aureus* RN4220

The ability of full length Aap rather than only the B-repeats to promote accumulation was further investigated in a staphylococcal host using *S. aureus* RN4220 containing the pUB1000 constructs, pUB1000aap6 and pUB1000aap6T. In this way the influence of *S. aureus* cell surface molecules in accumulation could be assessed.

Biofilms were cultured under the same flow conditions that were used for the *L. lactis* MG1363 strains. All three *S. aureus* RN4220 strains; pUB1000, pUB1000aap6 and pUB1000aap6T produced small, sparsely distributed microcolonies after culturing for 24 h in diluted (5 %) TSB (Fig. 4.6A-B). However, *S. aureus* RN4220 pUB1000aap6 produced the largest microcolonies that were ~ 150 % greater in volume than those of RN4220 pUB1000 and pUB1000aap6T (Fig. 4.6C). *S. aureus* RN4220 pUB1000 and pUB1000aap6T

microcolonies were not significantly different in volume. Contrary to *L. lactis* MG1363, all *S. aureus* RN4220 biofilms continued to develop after 24 h resulting in fusion of microcolonies leading to a confluent biofilm (data not shown).

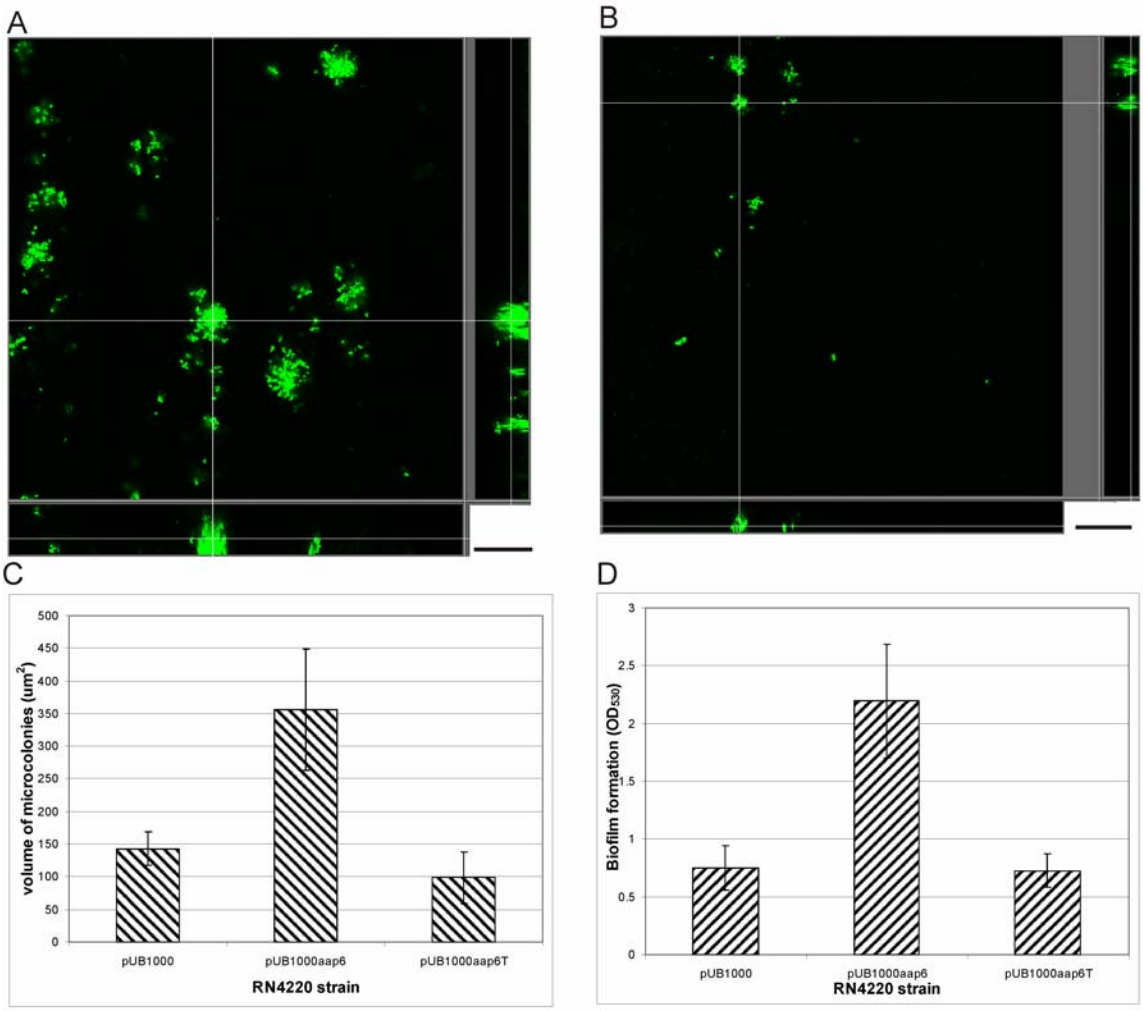

Figure. 4.6. Effect of Aap expression on biofilm formation by *S. aureus* RN4220 strains cultured under both flow and static conditions. (A & B) CLSM images of biofilms formed by RN4220 pUB1000aap6 (A) and RN4220 pUB1000aap6T (B) grown under flow conditions (0.2 mm/s) in diluted TSB for 24 h. Scale bars represent 30 µm. (C) Average microcolony volumes of biofilms of RN4220 cultured under the same flow conditions. Results were calculated by COMSTAT and represent the mean and standard error of three independent experiments with nine images taken of each experiment. (D) *S.aureus* RN4220 strains were grown in a 96-well plate biofilm assay for 24 h. Results represent mean and standard deviation for two independent experiments.

Furthermore, in a 96-well plate 24 h biofilm assay *S. aureus* RN4220 pUB1000aap6 gave a 190 % increase in OD_{530} compared to RN4220 pUB1000 and pUB1000aap6T (Fig. 4.6D). RN4220 pUB1000 and pUB1000aap6T did not differ significantly in biofilm forming ability confirming that Aap complete with the A-domain contributes to biofilm formation in this background. The B-repeats, however, had no influence on the ability of the *S. aureus* host to form biofilms under flow or in static conditions.

4.4.6 Biofilm formation is not blocked by recombinant Aap A-domain or B-region proteins

Since Rhode *et al.*, (Rohde *et al.*, 2005) reported that the accumulation phase of biofilm formation involving proteolytically cleaved Aap could be blocked by addition of recombinant Aap B-region protein, this was investigated in *S. aureus* RN4220 pUB1000aap6, in the presence of the A-domain. A rAap protein comprising 6.5 B-repeats ($rAap_{6.5B\text{-}rep}$) and $rAap_{A\text{-}dom}$ and BSA were added at a range of concentrations to a 96-well plate biofilm assay (Fig. 4.7).

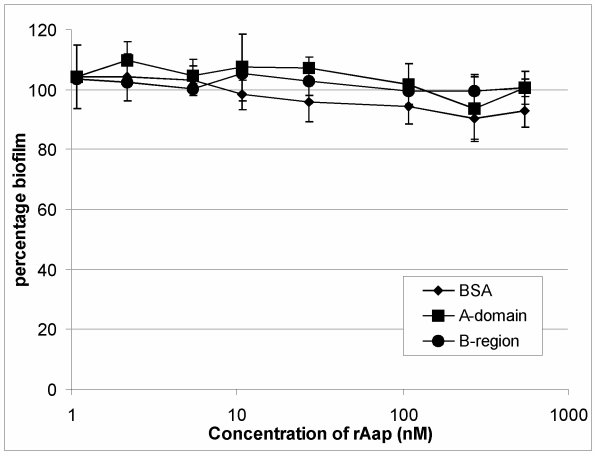

Figure. 4.7. The effect of adding rAap on biofilm formation of *S. aureus* RN4220 pUB1000aap6 in a 96 well plate biofilm assay. The level of biofilm formation in the presence of $rAap_{6.5B\text{-}rep}$, $rAap_{A\text{-}dom}$ and BSA is shown as a percentage of the untreated biofilm. Results represent mean and standard deviation of two independent experiments.

None of the three added proteins could significantly block biofilm formation by *S. aureus* RN4220 pUB1000aap6 even at 540 nM (Fig. 4.7). These data show that when the A-domain is present Aap can enhance biofilm formation via a mechanism that cannot be inhibited or blocked by the presence of either recombinant B-repeats or the A-domain.

4.5 Discussion

This paper describes two previously unreported functions of Aap, complete with the A-domain; in adhesion to polystyrene and in the early stage of biofilm development. A proteolytically processed form of Aap comprising only the B-region is already known to be involved in biofilm formation in *S. epidermidis* (Hussain *et al.*, 1997; Rohde *et al.*, 2005) but, until now, the A-domain has never been implicated in this process.

Aap mediated adhesion to two types of polystyrene surface (unmodified and tissue culture treated) which were used as representative abiotic substrata. Tissue culture treated polystyrene has additional polar chemical groups rendering the surface hydrophilic in contrast to untreated polystyrene which is hydrophobic. It is well known that bacterial cell surface hydrophobicity, increased by expression of generic bacterial cell surface proteins, mediates attachment to hydrophobic abiotic surfaces such as those used for some medical implants (Pascual *et al.*, 1986; Reifsteck *et al.*, 1987; Vacheethasanee *et al.*, 1998). However, Aap is an adhesin that promotes attachment to both hydrophobic and hydrophilic polystyrene surfaces and, as such, Aap may be important for initial attachment of *S. epidermidis* to plastic medical devices such as catheters.

Aap is widely reported to appear as two bands on SDS PAGE (Banner *et al.*, 2007; Bowden *et al.*, 2005; Rohde *et al.*, 2005) and here we report that this is due to proteolytic cleavage of the A-repeats. A previous study used a mass

spectrometry approach to show a single SasG SDS PAGE band could contain multiple N-termini suggesting successive N-terminal truncation of SasG on the surface of *S. aureus* (Suh *et al.*, 2010). The N-termini in a single SasG band differed by as many as 81 amino acids and were present at varying concentrations. N-terminal sequencing of such samples would merely yield information about any N-termini that were not blocked, a modification that has been reported for Aap (Rohde *et al.*, 2005). For these reasons a mass spectrometry approach was used here to determine the extent of proteolytic truncation of Aap. The cleavage site was mapped to a region between K_{222} an A_{286} meaning an N-terminal portion of the A-domain including the A-repeat region was cleaved.

Cleavage of the A-repeats was found to increase adhesion to tissue culture treated polystyrene. The A-repeats may sterically hinder the non-repetitive A-domain but, as the $rAap_{sh\text{-}A\text{-}reps}$ protein coated the polystyrene and blocked adhesion of MG1363 pUB000aap6 almost as efficiently as $rAap_{A\text{-}dom}$, an adhesive role for the A-repeat region as well as the non-repetitive region cannot be ruled out. There may be functional redundancy between the A-repeats and non-repetitive A-domain or the two regions may have moderately different affinities for different abiotic surfaces, such as the modified polystyrene used in this study.

The roles of the Aap B-region and A-domain in the accumulation stage of biofilm formation were investigated. Biofilms were grown in a flow-cell system to more accurately recreate *in vivo* conditions. Flow-cells have been widely used to analyze specific structural characteristics of biofilms (Klausen *et al.*, 2003a; Lappann *et al.*, 2006) and here a flow-cell was used to show that, in the surrogate hosts *L. lactis* MG1363 and *S. aureus* RN4220, only expression of pUB1000aap6 (with the A-domain) could enhance microcolony formation in the early stage of biofilm accumulation.

In the *L. lactis* MG1363 host Aap complete with the A-domain increased the volume of the microcolonies and so promoted early biofilm formation but it was insufficient for the development of a thick confluent biofilm. Cultivation of all MG1363 biofilms beyond 24 h did not result in further expansion of microcolony volume and some microcolonies were lost or became partially disintegrated. Conversely all *S. aureus* RN4220 biofilms showed continued expansion and coalescence of microcolonies for at least a further 24 h (the maximum time tested, data not shown). The reason for the senescence and even decline of *L. lactis* MG1363 biofilms is unknown but sheer forces from the flow conditions may have caused them to lose their structural integrity or to detach from the substratum. Other surface-bound molecules present on *S. aureus* RN4220 may have allowed further biofilm maturation by this surrogate host. Some studies report that RN4220 forms a weak biofilm in 96-well plates as it produced low OD values for the 96 well plate biofilm assay (Cramton *et al.*, 1999; McKenney *et al.*, 1998) whereas another (O'Neill *et al.*, 2007) reports that it forms a stronger biofilm and produces PIA. Here RN4220 formed a weak biofilm but biofilm formation was greatly increased by expression of Aap with the A-domain. Aap, expressed alone on the cell surface of *L. lactis* MG1363 pUB1000aap6, may be insufficient to promote development of fully mature biofilms due to the absence of other factors required for later stages of biofilm development. The flow cell system for biofilm formation has not been used before to investigate the role of Aap in biofilm formation and this new data on the function of Aap in surrogate hosts clearly predicts a previously undiscovered role for the Aap A-domain in the early stage of microcolony formation.

The B-region of Aap alone did not enhance accumulation on either surrogate host in the flow-cell system. One recent study suggested Zn^{2+}-dependant dimerisation of Aap B-regions only could promote biofilm formation (Conrady *et al.*, 2008). However, very high concentrations of Zn^{2+} were required to promote dimerisation of recombinant B-repeats suggesting dimerisation could be artefactual. Biofilm formation by *S. epidermidis* RP62A was inhibited by Zn^{2+}

depletion but this may have been due to the absolute requirement of AtlE for Zn^{2+} (Lutzner *et al.*, 2009; Zoll *et al.*, 2010) as the AtlE protein is required for *S. epidermidis* biofilm formation (Qin *et al.*, 2007). Therefore, Zn^{2+} dependent dimerisation of B-regions may not occur in biofilm formation *in vivo*.

Another study proposed that the B-region mediated accumulation by binding a putative surface ligand only after proteolytic cleavage of the entire A-domain (Rohde *et al.*, 2005). A biofilm-negative *S. epidermidis* strain (1585) gained the ability to form a biofilm in a 96-well plate assay when expressing surface-anchored recombinant Aap B-region alone but not when the A-domain was present. In addition biofilm formation of *S. epidermidis* 5179-R1 was blocked by addition of soluble recombinant B-region, strongly implicating the B-repeats of Aap in biofilm formation. A putative B-region ligand must be lacking on *L. lactis* MG1363 and *S. aureus* RN4220 as Aap B-regions could not enhance biofilm formation on either surrogate host and $rAap_{6.5B\text{-}rep}$ did not inhibit RN4220 pUB1000aap6 biofilm formation.

Further evidence for the involvement of the A-domain in biofilm formation, in addition to the results presented here, comes from work on *S. aureus* biofilms and a study of the Aap homologue SasG (Corrigan *et al.*, 2007). Expression of recombinant fibrillar SasG protein (with the A-domain) promoted biofilm formation (Corrigan *et al.*, 2007). Cleavage of the A-domain did not occur as immunogold staining with antibody to the A-domain labelled the SasG A-domains at the distal end of the fibrils. A high concentration of protease inhibitor prevented biofilm formation suggesting the involvement of proteolysis in biofilm formation but, as biofilm formation is a multifactorial process (Otto, 2009), the specific proteolytic target, or targets, are unknown. Current evidence suggests that SasG, complete with the A-domain promotes biofilm formation.

In addition to the role of Aap A-domain in adhesion to corneocytes from human skin (Macintosh *et al.*, 2009), we have shown here that Aap with the A-domain

can also promote adhesion to both treated and unmodified polystyrene as representative abiotic surfaces. Furthermore, Aap complete with the A-domain enhanced the early stage in biofilm accumulation in two different surrogate hosts under flow conditions. Therefore the A-domain has three independent roles, functioning on the skin and on artificial surfaces. These results highlight the potential significance of the multi-functional A-domain of Aap in medical device related infection as Aap is predicted to help *S. epidermidis* make the transition between the commensal and the pathogenic lifestyle of *S. epidermidis.*

4.6 Acknowledgements

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Chapter 5:
General Discussion

5 General Discussion

The incidence of medical device related infection caused by CoNS and also *S. aureus* has been on the rise for several decades due to the increased use of medical implants (Rupp and Archer, 1994). It is highly likely that implants such as catheters, heart valves and artificial joints will continue be used for the foreseeable future as a means to treat a variety of medical conditions. It is therefore essential to develop improved strategies to limit the risk of contamination of implants and to treat infections when contamination occurs. Development of such strategies will, in part, require a more complete understanding of bacterial factors involved in the process of infection. To address this lack of understanding an expanding field of molecular microbiology research is currently concerned with examining the function and regulation of bacterial surface polymers that may be involved in adhesion and biofilm formation. This thesis reports on the function of one such surface polymer, Aap, and has demonstrated that the A-domain of this protein is predicted to have multiple roles in the process of contamination of medical implants. The results presented here therefore contribute to an understanding of the way in which *S. epidermidis* (and other related species) cause nosocomial infections associated with indwelling medical devices.

Chapters 3 and 4 of this thesis contain detailed discussions of the specific results presented in those chapters. The purpose of this section is to place the overall findings of this thesis in the context of the present scientific understanding of the opportunistic pathogen *S. epidermidis*. Firstly however, the advantages and limitations of the heterologous expression approach, used throughout this thesis, as well as priorities for extending this present study of Aap will be discussed.

5.1 Heterologous expression as a tool to determine the function of bacterial surface proteins

Heterologous expression of surface proteins from *Lactobacillus brevis* (Avall-Jaaskelainen *et al.*, 2003), *Enterococcus faecalis* (Hirt *et al.*, 2000) and *S. epidermidis* (Hartford *et al.*, 2001b) on the surrogate host *L. lactis* has been used previously to demonstrate protein function. This approach is especially useful in analyzing the function of surface proteins of *S. epidermidis*, a bacterium that expresses a large number of cell surface polymers (Otto, 2009). Gene deletion in the native host is a common method to determine the function of proteins. However, such experiments performed in the native *S. epidermidis* background may fail to identify all the functions of a specific protein due to functional redundancy between surface molecules on the *S. epidermidis* cell surface. A high degree of functional redundancy has been reported for *S. aureus* adhesins mediating attachment to nasal epithelial cells (Corrigan *et al.*, 2009) and results from this thesis suggest functional redundancy also occurs between corneocyte adhesins of *S. epidermidis*. Heterologous expression on *L. lactis* is therefore useful due to the lack of other staphylococcal proteins on the cell surface.

The difficulty of functional redundancy is compounded by the diversity between *S. epidermidis* strains which is very high not just globally (Miragaia *et al.*, 2007) but also in strains isolated from a single country (Miragaia *et al.*, 2005) or even a single hospital (Dominguez *et al.*, 1996). Therefore, selection of the appropriate *S. epidermidis* strain to perform a gene deletion and functional studies is difficult. Functional redundancy of adhesins and biofilm linked polymers may occur on some strains but not others. A specific gene should be deleted from several strains with different compliments of other *S. epidermidis* cell surface molecules to ensure all functions of the deleted gene are detected.

Obviously there are also limitations to the approach of heterologous expression. The function of any protein that requires additional molecules, present on the

native host but not on the heterologous host, will not be detected. For example, in this study *L. lactis* and *S. aureus* are suggested to lack a putative Aap B-region receptor and, as a result, the predicted function of the B-region in accumulation (Rohde *et al.*, 2005) was not detected. In spite of this possible lack of a B-region receptor, the previously unknown function of the A-domain in early microcolony formation could be clearly observed. This thesis has highlighted the value of heterologous expression in a surrogate host such as *L. lactis* as a useful tool in discovering functions of Gram positive surface proteins.

There are several LPXTG proteins present in the genome sequence of *S. epidermidis* (Bowden *et al.*, 2005) and *S. aureus* (Roche *et al.*, 2003a) with no known function. A useful approach to determine the function of these potentially clinically significant proteins may be to express the proteins on *L. lactis*. The LPXTG proteins could then be screened for functions such as mediating adhesion to different host matrix proteins or cells without the problem of functional redundancy. After identification of a LPXTG protein function, for example in adhesion to a host matrix protein, it would be useful to asses the contribution of the LPXTG protein to adhesion of several *S. epidermidis* strains. This could be achieved by blocking a specific host receptor with soluble recombinant LPXTG proteins and testing the adhesion of different strains. Alternatively, gene deletions or heterologous expression of LPXTG proteins in several *S. epidermidis* strains would be the most conclusive method to asses the contribution of specific LPXTG proteins to adhesion.

5.1.1 Future Aap work and challenges for heterologous LPXTG protein expression on *S. epidermidis* strains

Results presented in chapter 4 of this thesis describe a potential function for the A-domain of Aap in the early stages of biofilm formation. However, in some strains of *S. epidermidis* the B-region alone promotes intercellular adhesion and biofilm formation (Rohde *et al.*, 2005). Future work on Aap should be focused on determining the role of Aap in biofilm formation by a diverse selection of *S.*

epidermidis strains. This would allow the contribution of other biofilm promoting molecules to be examined. For example some *S. epidermidis* strains may express a surface ligand for the B-region of Aap allowing B-region dependent biofilm formation. At the present time further improvement in methodology to genetically manipulate *S. epidermidis* is required as there are a very limited number of strains that can be readily transformed in the laboratory. The pUB1000Aap constructs used in this thesis could theoretically be electroporated into several different strains of *S. epidermidis* in order to determine the role of the A-domain and B-region in strains expressing different surface molecules. However, several attempts to electroporation pUB1000 into *S. epidermidis* strain Tu3298 failed. This may have been due to the pUB1000 vector as other plasmids have previously been electroporated into this strain. More competent strains of *S. epidermidis* must be identified and a different plasmid vector may be required in order to investigate the role of Aap in several different strain backgrounds.

5.2 Aap contributes to multiple aspects of the lifestyle of *S. epidermidis*

Results presented in chapter 3 report a function for the A-domain of Aap in adhesion to corneocytes indicating a role for Aap in colonization of the skin. Several WT *S. epidermidis* strains used the A-domain adhered to corneocytes but the fibrillar B-region was also important to project the A-domain away from the bacterial cell surface.

Chapter 4 presents a role for the A-domain of Aap in adhesion to abiotic surfaces (specifically polystyrene) and the early stage of biofilm formation indicating a possible role in early colonisation of medical devices. A-domain dependent adhesion to polystyrene was found to be enhanced by proteolysis of the A-repeat region. I suggest that the A-repeat region may be unstructured and therefore susceptible to stepwise proteolytic cleavage leading to degradation of

the entire A-repeat region. In this case the presence of extracellular proteases, for example in the host, may enhance adhesion of *S. epidermidis* to abiotic surfaces. The A-domain of Aap also increased the size of microcolonies formed in the early stage of biofilm formation by *S. aureus* RN4220. This strain contains the gene for the Aap homologue SasG but does not express SasG at a level detectable by SDS PAGE of cell wall proteins. The A-domain of Aap could therefore be important in the early stages of biofilm formation on medical devices. More extensive proteolytic cleavage of the entire A-domain, proposed by Rohde *et al* (2005), could then promote further biofilm development in some *S. epidermidis* strains via cell-cell interactions involving the B-region of Aap.

Together these findings suggest that Aap is multifunctional and plays an important role in colonisation of the two main niches of *S. epidermidis*, on the skin and on the surface of medical devices. However, Aap is not the only multifunctional surface protein in *S. epidermis*. The AtlE protein degrades peptidoglycan (Heilmann *et al.*, 1997), promotes adhesion to polystyrene and allows release of extracellular DNA (Qin *et al.*, 2007) implying roles in cell division and colonisation of medical devices. The fibronectin-binding MSCRAMM, EMBP (Williams *et al.*, 2002) was recently reported to have an additional role in biofilm formation and protection from opsonisation by macrophage (Christner *et al.*, 2010) indicating an important role in colonisation of devices in the host. The multifunctional proteins AtlE, EMBP and Aap all have roles that could be important in both the commensal lifestyle of *S. epidermidis* (cell division and adhesion to host tissue) and pathogenesis (adhesion to medical devices and biofilm formation) and this may give an insight into the emergence of *S. epidermidis* as nosocomial pathogen.

The first use of medical devices provided a new niche for *S. epidermidis*. The ability of *S. epidermidis* to colonise this new niche may have been coincidental and to due to the existence of proteins like Aap, AtlE and EMBP. The idea that surface proteins could have coincidental roles in colonising medical devices was

reported in a recent review (Otto, 2009) and the multifunctional role of Aap described in this thesis supports and adds to this view of the organism as an "accidental pathogen". The A-domain of Aap helps to mediate adhesion of *S. epidermidis* to the skin but can also promote adhesion and the early stages of biofilm formation on an abiotic surface suggesting that the A-domain of Aap may have promoted the transfer of *S. epidermidis* from the skin to the surface of medical devices.

Chapter 6:
References

6 References

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