UiT
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Determinants of *Staphylococcus aureus* Colonization and Infection

"Exploring the role of Cell Wall Anchored Proteins in Adhesion and Immune Evasion"

—

Clement Olufemi Ajayi

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Determinants of *Staphylococcus aureus* Colonization and Infection

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ABBREVIATIONS

SdrD	Serine-aspartate repeats containing protein D
SasG	*S. aureus* surface protein G
CWA	Cell wall anchored
MRSA	Methicillin resistant *S. aureus*
MSSA	Methicillin sensitive *S. aureus*
PFGE	Pulsed-field gel electrophoresis
MLST	Multilocus sequence typing
SSTI	Skin and soft tissue infections
SSSS	Staphylococcal scalded skin syndrome
SCC*mec*	Staphylococcal cassette chromosome *mec*
PVL	Panton-Valentine leukocidin
spa	*Staphylococcus aureus* Protein A
Dsg	Desmoglein
Dsc	Desmocollin
MSCRAMM	Microbial surface components recognizing adhesive matrix molecule
SERAM	Secretable expanded repertoire adhesive molecules
ADAM	A disintegrin and metalloproteinase
PSM	Phenol soluble modulin
TSST	Toxic shock syndrome toxin
LTA	Lipoteichoic acid
PIA	Polysaccharide intracellular adhesion
WTA	Wall teichoic acid
Clf	Clumping factor
FnBP	Fibronectin binding protein
Isd	Iron regulated surface
AMPs	Antimicrobial peptides
TLR	Toll like receptor
ROS	Reactive oxygen species
PSGL-1	P-selectin glycoprotein ligand 1
ICAM 1	Intercellular adhesion molecule 1
SCIN	Staphyloccocal Complement Inhibitor
Y2H	Yeast two-hybrid

LIST OF PAPERS

Paper I

Fatemeh Askarian, Clement Ajayi, Anne-Merethe Hanssen, Nina M. van Sorge, Ingvild Pettersen, Dzung Bao Diep, Johanna U.E Sollid, Mona Johannessen. 2016.
The interaction between *Staphylococcus aureus* SdrD and desmoglein 1 is important for adhesion to host cells. Scientific Reports.6:22134.

Paper II

Clement Ajayi, Espen Åberg, Fatemeh Askarian, Johanna U.E Sollid, Mona Johannessen, Anne-Merethe Hanssen. 2018.
Genetic variability in the *sdrD* gene in *Staphylococcus aureus* from healthy nasal carriers. BMC Microbiology. 18:34.

Paper III

Clement Ajayi, Joan Geoghegan, Fatemeh Askarian, Mona Johannessen.
Expression and Virulence properties of *Staphylococcus aureus* MSSA476 Surface protein G (SasG). Manuscript

SUMMARY

Staphylococcus aureus is an efficient human colonizer and pathogen. However, the molecular mechanisms involved in the interaction of *S. aureus* with the host during colonization and infection is not fully understood. Increasing incidences of antibiotics resistance by *S. aureus* demand development of alternative strategies to combat *S. aureus* infections. However, this requires an adequate understanding of the determinants involved in *S. aureus* colonization and infection of its host. This thesis is aimed at understanding the role of two *S. aureus* cell wall anchored proteins, Serine-aspartate repeats containing protein D (SdrD) and *S. aureus* surface protein G (SasG) in the bacterial adhesion and immune evasion.

In **Paper I**, we identified desmoglein 1 (Dsg1) as the host ligand for SdrD using the yeast two-hybrid assay. The interaction between SdrD and Dsg1 is specific as shown by the concentration dependent binding of recombinant SdrD to immobilized recombinant Dsg1 in a solid phase ligand-binding assay. Furthermore, using *in vitro* cell adhesion assay, we showed that this interaction between SdrD and Dsg1 promotes adhesion of *S. aureus* to human keratinocytes.

In **Paper II**, using multiple sequence alignment and phylogeny analyses, we showed the genetic variability in the A region sequences of *sdrD* gene in *S. aureus* isolates from anterior nares of healthy adults. We classified these variations into seven *sdrD* variants. In addition, we showed that these genetic variations occurred within several regions of the SdrD protein. However, the variations are concentrated on the N2-N3-B1 subdomains and R domain of SdrD. In addition, the variations within the N2-N3-B1 subdomains were mostly surface associated. Functional analyses using *in vitro* cell adhesion assay showed a significant difference between two of the *sdrD* variants.

In **Paper III**, we showed that SasG expression promoted bacterial adhesion to human keratinocytes. Furthermore, we showed that expression of the *S. aureus sasG* gene was upregulated in human blood and that early expression of SasG in bacteriological medium is induced by the presence of serum components. However, SasG did not promote the bacterial survival in an *ex vivo* human blood model but promoted bacterial aggregation in the presence of serum components.

Taken together, findings in this thesis indicate the complexities of the mechanisms involved in *S. aureus* interaction with the host. *S. aureus* colonization and evasion of host immune defense mechanisms is essential for subsequent development of infections. Additional studies are required to further elucidate these *S. aureus* virulence factors

INTRODUCTION

The interaction between *S. aureus* and human has garnered lots of interest in recent times. *S. aureus* persistently colonizes the squamous epithelium of the anterior nares of approximately 20-30% of the healthy adult human population, but can also be found in other body sites[1,2]. Even though colonization is asymptomatic, *S. aureus* colonization is an important risk factor for infection[1,3,4].

S. aureus possesses a repertoire of virulence factors which aids its ability to survive and cause infections in humans[5,6]. Despite advances made in healthcare treatment, *S. aureus* remains a leading cause of nosocomial infections among hospital patients[7,8]. *S. aureus* is an opportunistic pathogen and causes infections ranging from mild skin infections to severe infections[9]. In addition, the development of antibiotic resistance by *S. aureus* has further compounded *S. aureus* infections[7,10].

Challenges posed by these and many more, necessitates the need for an adequate understanding of the mechanisms deployed by *S. aureus* to successfully colonize and infect its host. Improved understanding could lead to the development of alternative therapies to combat *S. aureus* infections.

This study will increase the understanding of the determinants involved in *S. aureus* colonization and infection of humans, with focus on the role of two specific *S. aureus* cell wall anchored proteins.

1 *STAPHYLOCOCCUS AUREUS*

S. aureus was first described by Sir Alexander Ogston in the 1880s. He observed a grape-like cluster of bacteria from slide preparations of pus from post-operative wounds and abscess patients[11,12]. In 1884, Rosenbach was able to successfully isolate and grow the bacteria on solid medium. He named the bacteria *Staphylococcus aureus* because of the characteristic yellowish pigmentation of their colonies[13]. The yellow pigmentation of the colonies is due to the production of carotenoids called staphyloxanthin[14].

S. aureus belongs to the phylum *Firmicutes*, class *Bacilli*, order *Bacillales*, family *Staphylococcaceae,* genus *Staphylococcus*. The genus comprises 53 species and 28 subspecies (http://www.bacterio.net/staphylococcus.html, accessed 23 July 2018). Aside from *S. aureus*, it has other staphylococci including *S. epidermidis, S. hemolyticus, S. saprophyticus, S lugdunensis*. The *S. aureus* coccus size is about 0.5-1.0 µm in diameter and appears in pairs, short chains or grape-like clusters microscopically[15]. *S. aureus* is a facultative anaerobe, Gram-positive, non-motile and non-spore forming microbe. Their cell wall is made up of peptidoglycan, teichoic acid and other surface associated protein[16–18]. Peptidoglycan forms the bulk of the cell wall and is composed of a matrix of disaccharide chains cross-linked to one another[19]. Peptidoglycan of actively dividing cell is susceptible to the endopeptidase lysostaphin[20]. Teichoic acid makes about 30-40% of *S. aureus* cell wall weight and is linked with the peptidoglycan[21]. In addition, some *S. aureus* strains are also coated with a polysaccharide layer called capsule, which envelops their cell surface[22]. *S. aureus* expresses coagulase, an extracellular protein that binds to prothrombin and converts fibrinogen to fibrin[15]. Furthermore, they are catalase-positive and cause haemolysis when grown on blood agar plates[15].

S. aureus is part of the normal microbial flora of humans. It can inhabit diverse ecological niches within the human body, where it can thrive as an innocuous microbe or cause infections[9,15,23]. The bacterium is commonly found in the anterior nares of healthy adults[1,2]. In addition, *S. aureus* has also been indicated in animals such as dog, cat and pigs etc.[24]. *S. aureus* also possesses the ability to grow in harsh conditions such as high salt (10 % NaCl) or low pH conditions (≈ 4.0)[15].

1.1 Clinical Significance

S. aureus is the most common human pathogen of the genus *Staphylococcus* and is the etiological agent for several human diseases[25]. Infections caused by *S. aureus* can be classified based on the site and mechanism of occurrence into (1) local infections, associated with skin and soft tissue infections (SSTIs), (2) systemic infections such as bacteraemia, sepsis, pneumonia etc., (3) invasive device entry infection associated with patients on dialysis, intravascular catheters etc., and (4) toxin associated diseases such as toxic shock syndrome and staphylococcal scalded skin syndrome (SSSS) etc.[9,26,27].

Therapeutic interventions to combat *S. aureus* infections have been further compounded by the development of resistance to most known antibiotics especially to methicillin and other beta-lactam antibiotics[10]. Epidemiological studies have shown that *S. aureus* strains including the methicillin-resistant *S. aureus* (MRSA) strains are responsible for about 30% of deaths in USA[28]. To further give credence to its clinical significance to public health, *S. aureus* was listed as one of the "ESKAPE pathogens"[8], which are fundamentally a list of pathogens recognized as leading causes of nosocomial infections and development of antimicrobial resistance[7,29].

MRSA was first identified as a nosocomial pathogen in the United Kingdom in 1961[30]. Initially considered confined to hospital settings, MRSA was identified within the community in the USA in 1980[31]. Since then, MRSA strains have been identified within the community and hospitals in other parts of the world (reviewed in[32,33]). Despite increased awareness about MRSA, the bacterium remains a main public health priority in most European countries[34]. Infections caused by MRSA strains are generally classified into two groups: hospital-associated MRSA (HA-MRSA) and community-associated MRSA (CA-MRSA). In order to group these infections, different guidelines have been adopted. These guidelines include evidence based on epidemiological data of the infection[35], pulsed-field gel electrophoresis (PFGE) profiles[36] and the antibiotics susceptibility profiles of the isolated strain[37]. Overall, these groupings are essential to determine the antibiotic regimen needed to combat MRSA infection[38]. HA-MRSA infections need a more extensive and broad-spectrum based antibiotics treatment compared with CA-MRSA infections[38]. It was originally thought that HA-MRSA strains and CA-MRSA strains are epidemiologically distinct from each other[39]. However, CA-MRSA strains such as USA300 has moved into the hospital and established itself as a hospital associated strain[40,41].

HA-MRSA strains cause invasive infections while CA-MRSA strains are largely responsible for skin and soft tissue infections[39,42]. However, CA-MRSA strains have also been indicated in more invasive infections[43,44]. *S. aureus* strains responsible for HA-MRSA and CA-MRSA infections have certain attributes which make them unique from each other. The increased susceptibility of CA-MRSA strains to antimicrobials other than β-lactam antibiotics is because these strains contain one staphylococcal cassette chromosomes *mec* (SCC*mec*) element (type IV)[45]. On the other hand, HA-MRSA strains contain type I, II and III

SCC*mec*[46,47]. In addition, CA-MRSA strains have genes encoding Panton-Valentine leukocidin (PVL), which is not found in HA-MRSA strains[39,48].

The success of *S. aureus* as an infectious microbe reflects its possession of an array of abilities. These abilities enable to survive long on inanimate objects, effectively colonize and exist as an asymptomatic microbe on its host. Furthermore, expression of virulence factors implies it is able to enhance its virulence while damping effects of the host defence system[5,49,50].

1.2 Molecular Typing of *S. aureus*

Characterization of *S. aureus* isolates is important to determine their genetic relatedness and develop intervention during investigation of epidemic spread especially for MRSA strains. A number of molecular typing methods have been developed over the years and some of these are expatiated below.

1.2.1 Multilocus Sequence Typing (MLST)

MLST is a molecular typing method based on assessing the genomic variation within housekeeping genes. For *S. aureus* isolates characterization, MLST is based on the sequencing of about 450-500 bp internal fragments of seven housekeeping genes, that is carbamate kinase (*arcC*), shikimate dehydrogenase *(aroE*), glycerol kinase (*glpF*), guanylate kinase (*gmk*), phosphatase acetyltransferase (*pta*), triosesphonate isomerase (*tpi*) and acetyl coenzyme A acetyltransferase (*yqiL*)[51]. These sequences are submitted to the online *S. aureus* MLST database (http://saureus.mlst.net). Based on the variations within each gene sequence, an allelic identification number is assigned. The combination of allelic numbers for the seven genes gives a unique allelic profile called the sequence type (ST) for each *S. aureus* isolate. Further analysis to group related STs and assignment to MLST clonal complexes (MLST-CC) is performed using eBURST (http://saureus.mlst.net/eburst)[52].

1.2.2 *Staphylococcus* aureus Protein A (*spa*) Typing

spa typing is a molecular typing method based on variations within the *spa* gene of *S. aureus* isolates[53]. Using *spa* typing, as a molecular typing method is quite attractive because of its simplicity as it relies on amplification of sequences of a single gene. Furthermore, it is inexpensive and less laborious compared to MLST. The *spa* gene encodes Staphylococcal

Protein A, made up of a signal sequence, IgG binding domains and polymorphic X region. The X region consists of tandem repeats usually 24bp in length. Differences within these repeats can be attributed to deletions, duplications and point mutations[54]. These genetic differences generate unique *spa* profiles, which are used to characterize *S. aureus* isolates[53]. Genetic relatedness of the *spa*-types is inferred by using the 'based upon repeat pattern' (BURP) algorithm, which clusters the *spa* types into *spa*-clonal complexes (spa-CCs)[55].

2 *S. AUREUS* COLONIZATION

2.1 Significance of Colonization

Humans are constantly exposed to *S. aureus* in their environment and our body provides a range of ecological niches for the *S. aureus* and other microbes to thrive[56]. However, not every exposure to *S. aureus* will lead to successful colonization. *S. aureus* colonization of its host involves a complex interplay of factors from the bacterium and its host[57]. Longitudinal studies have shown that 20-30% of the healthy adult population is persistently colonized by the *S. aureus* in their anterior nares[1,2,58,59]. *S. aureus* colonization requires that the bacterium is able to adhere to the receptors present at the ecological niches. Furthermore, it must also be able to thrive and not be eradicated by the host defence mechanisms or resident microbiota[60,61].

S. aureus colonization is an important and essential risk factor for subsequent development of *S. aureus* infection and hospital acquired infections[1,4,62,63]. Danbolt established the first correlation between nasal carriage and furunculosis skin infection in 1932 (reviewed in[23]). In addition, correlation between *S. aureus* nasal carriage and infections such as continuous peritoneal dialysis (CPD) related infections[64], HIV[65], post-operative infections[66,67] and foot ulcer[68] have been observed. Studies have shown that the infecting strains are *S. aureus* strain, which had colonized its carriers' nares[62,69,70]. In addition, patients colonized with MRSA before hospital admission, have a higher risk of developing MRSA infections[71–73] and serve as depots for transmission to other patients[74].

2.2 Sites of *S. aureus* Colonization

The primary ecological niche for *S. aureus* colonization in human is the nose[63,75]. However, *S. aureus* have been reported to colonize other sites within the human body including skin[75], perineum[76], vagina[77], axillae[78], pharynx[75,77], gastrointestinal tract[75,79,80], urinary tract and throat[81,82]. Exclusive *S. aureus* throat, intestinal and pharynx colonization without nasal carriage have been reported[79,81–83]. In addition, some studies show higher incidences of *S. aureus* prevalence in the throat and pharynx compared to the nasal carriage[81,84]. The ability of *S. aureus* to survive in different ecological niches of the human body shows its versatility and diversity in colonizing its host.

2.3 Nasal Colonization by *S. aureus*

S. aureus nasal carriage influences the bacterial colonization of other parts of the human body[85]. This implies that *S. aureus* nasal carriage most likely serve as a repository for the dispersal of *S. aureus* into environment or colonization of other body parts[86]. Habits such as nose picking could be an avenue for transfer of *S. aureus* carried in the nose to other areas of the human body[87]. In addition, patients and healthcare workers nasally colonized by *S. aureus* can also spread the bacterium to non-colonized persons in hospital settings[74,88].

Based on the risk posed by *S. aureus* nasal carriage, calls to develop effective nasal decolonization strategies have increased[89,90]. Decolonization of *S. aureus* in the anterior nares following courses of intranasal application of the antibiotics mupirocin has been reported[91,92]. In addition, nasal decolonization treatment also eliminated *S. aureus* from the hands of health workers[93]. Eradication of *S. aureus* in the anterior nares in the patients reduced the occurrence of *S. aureus* infections[94–96]. Application of mupirocin has also been used to eradicate MRSA carriage[97]. These observations further strengthen the notion that the nasal environment provides a very viable environment for the colonization and subsequent propagation of *S. aureus*. However, despite the success of mupirocin in eradicating *S. aureus* nasal colonization, *S. aureus* has developed resistance to the antibiotic[98].

In human nose, the main ecological niche of *S. aureus* is the moist squamous epithelium of the anterior nares of healthy adults in a general population[2,63,99]. This has been further supported by *in vitro* cell studies, which showed an increased adherence of *S. aureus* to desquamated epithelial cells isolated from the anterior nares[100,101]. However, *S. aureus* also

colonize other regions nose as well, from mid region nares to the deeper regions of the nose[102]. Interestingly, Kaspar et al., observed within the sampling population of their study that the posterior region of the nose was consistently colonized compared to the anterior nares[103]. The surface of the anterior nares is lined with a skin-like epithelium while the middle and posterior region of the nose is lined with pseudostratified columnar ciliated epithelium[102,104,105]. The role of these different surface cellular constituents on nasal microbiota has been suggested[102]. However, in another study where the human nasal microbiome evaluated, they concluded that the epithelium constituent does not affect the nasal microbial diversity[103], but a large proportion of the participants in the study had chronic nasal inflammation.

The nasal cavity poses some obstacles which could make *S. aureus* nasal colonization challenging. As an entrance into the olfactory and respiratory system, the nose serves as a filter for air coming into the system[105]. Its production of mucus traps particulate molecules including bacteria in its mucus blanket[106]. In addition, cells of the nasal epithelium are constantly being shed, which further removes microbes from the nose. Aside from this, the nasal environment contains antimicrobial compounds such as lysozyme, lactoferrin and secretory IgA[107]. Interaction of the resident nasal microflora also influences *S. aureus* nasal colonization and persistence[102,108]. Bacteria such as *Corynebacterium pseudodiphtheriticum, S. epidermidis* and *S. lugdunensis* adversely influence *S. aureus* colonization while *C. accolens* promotes *S. aureus* growth[102,109,110].

For *S. aureus* to successfully colonize human nasal cavity, the bacteria should be able to multiply and overcome the defence mechanisms encountered in the nose[104,111]. *S. aureus* binds to the mucus components both *in vitro* and *in vivo* and probably could influence its effective clearance from the nasal cavity[112,113]. Although, nasal secretions from *S. aureus* carriers contain a higher concentration of α-defensins and β-defensins[114], *S. aureus* survives better in nasal fluids of *S. aureus* nasal carriers compared to non carriers[99]. In addition, haemoglobin found in nasal secretions from *S. aureus* carriers promote surfaces colonization by the bacterium[115]. Furthermore, there are increasing evidences of *S. aureus* being able to persist within the cells of the nasal tissue[116–118]. Recurrent *S. aureus* infections such as rhinosinusitis are due to the intracellular localization of *S. aureus*[119,120].

2.3.1 Patterns of Nasal Colonization

Nasal colonization by *S. aureus* involves a complex array of factors (reviewed in[23,104,121]), which are not fully understood yet. *S. aureus* nasal carriers within the healthy adult human population have been classified into two categories; persistent carriers and non-persistent carriers[2]. This classification replaced the traditional *S. aureus* nasal carriers types, which were persistent carriers, intermittent carriers and non-carriers[75]. There have been questions regarding the best definition for and/or criteria to use to classify a person as a persistent carrier of *S. aureus*. However, a international guideline has been adopted and is based on the "culture rule"[59]. Persistent carriers are defined as those who have at least two positive culture from nasal sample taken one week apart while non-persistent carriers have one positive *S. aureus* culture[59].

Persistent carriers are observed to have higher loads of *S. aureus*[2,102,122,123] and are more prone to *S. aureus* infection[62,64]. In addition, persistent carriers can serve as reservoir for the subsequent transmission of *S. aureus* to other members of the population[124]. This might be due to the ability of *S. aureus* to survive longer in persistent nasal carriers compared to the non-persistent carriers[2,58,125]. Persistent carriers are mainly colonized by a single strain of *S. aureus* over a period of time while non-persistent carriers can be colonized by different *S. aureus* strains throughout their life[1,2,126,]. Furthermore, antibody profile responses between persistent carriers and non-persistent carriers also differs[2]. *S. aureus* carriers are reported to have higher immunoglobulin G (IgG) titers and IgA against the bacteria compared to non-persistent carriers[127]. It is also thought that the continuous presence of *S. aureus* in persistent carriers provide a protective advantage for them[4,128,129]. This is logical since the infecting strains are usually the endogenous strains carried by the host[4,62]. Interestingly, when persistent *S. aureus* nasal carriers were artificially inoculated with mix of *S. aureus* inoculum, they reacquired their endogenous strain from the mix[2,125].

2.3.2 Structure and Components of Anterior Nares

The anterior nares surface is covered with stratified squamous epithelium continuous with of the external skin[118,130]. The surface is made up of two layers, which are the epidermis, outer layer and dermis, the inner layer (Figure 1). Interspacing these two layers are structures including sweat glands, hair follicles and sebaceous gland[131]. The epidermis is a multilayered structure resting on the basement membrane, which separates it from the dermis. The

epidermis is divided into four strata including the stratum basale, stratum spinosum, stratum granulosum and stratum corneum from bottom to top. Overall, the different stratum work together to make the nasal epithelium impenetrable for microbes and also withstand environmental onslaught[132].

The delineation of the epidermis into the respective strata begins at the basal layer via a maturation process referred to as epidermal differentiation. Keratinocytes at the basal layer are undifferentiated, attached to the basement membrane and continuously dividing[131]. As the epidermis is continuously desquamated, the basal layer provides a continuous supply of new cells to keep the maturation process and renewal of the skin ongoing[133]. At a point, keratinocytes at the basal layer undergo transformation, detach from basement membrane, stops dividing and start to differentiate. Thereafter, they migrate outwards, undergoing a maturation process that gives rise to the distinct layers of the epidermis[131,134].

Due to stratification of the epidermis, cells within each stratum have their own characteristic cellular features and expressed proteins. Keratinocytes at the basal layer highly express keratins 5, 14 and 15[135,136]. However, keratin 1 (K1) and 10 (K10) replaces these proteins as the cells migrate through the spinosum[136]. Cells at the granular layer contain lamella bodies (LBs). LBs contain lipids such as phospholipids, glucosylceramides, sphingomyelin, and cholesterol[137]. During the transition of the cells to stratum corneum, LBs fuse with the plasma membrane and release their content into the intercellular space[133,137]. At the stratum corneum, the cytoplasmic membrane of cells is replaced by cornified envelope (CE). Proteins such as filaggrin, involucrin, loricrin together with K1 and K10 make up the CE[138]. Lipids formed from the contents of LBs become covalently attached to the cornified envelope, giving these cells their characteristic features. Cells at the corneum layer are flattened, devoid of organelles and tightly packed together[132–134]. This enables the stratum corneum to serve the physical barrier functions of the skin[132].

Aside from the keratinocytes present in the epidermis, other cells present are the Langerhans cells and the melanocytes, which are involved in immune and ultraviolet protection respectively (reviewed in[139]). The dermis is made up of connective tissue and other molecules including elastin fibers and collagen. The dermis also provides residence for immune cells including macrophage, dendritic cells and T helper cells[139].

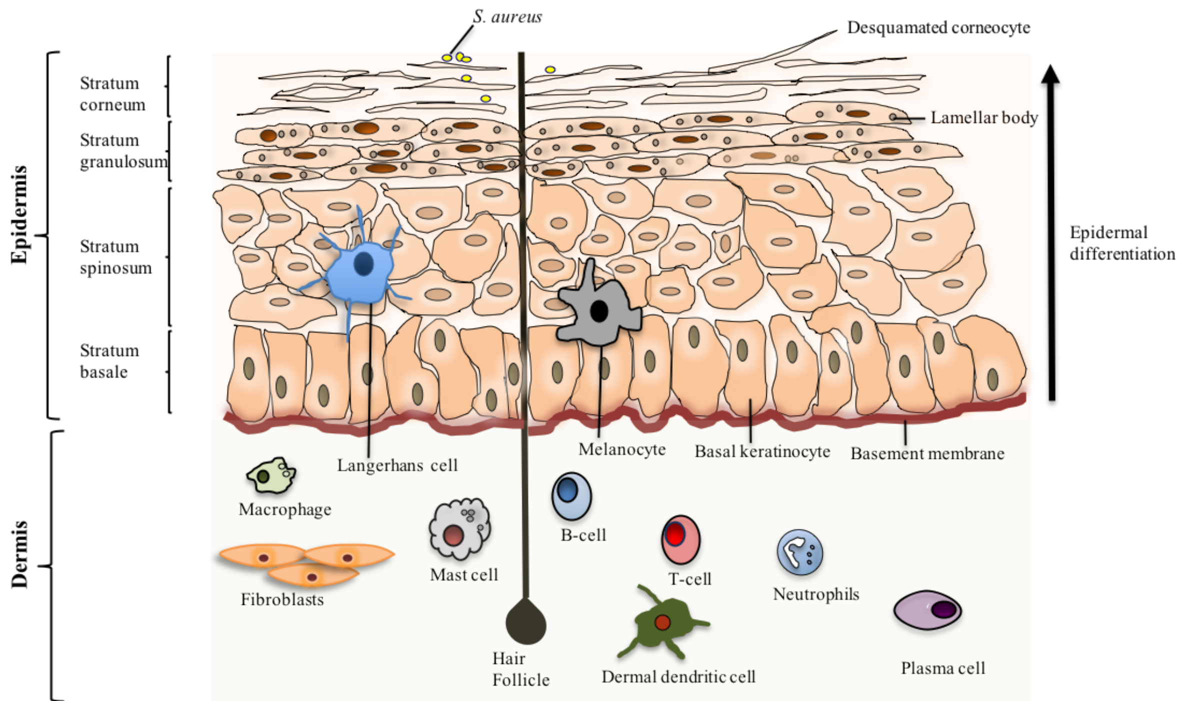

Figure 1. Structural components of the skin in the nasal anterior nares. The epidermis is composed of keratinocytes in different stages of differentiation. The epidermis is divided into strata including corneum, granulosum, spinosum and basale. The barrier function of the skin is provided by the stratum corneum. Cells at the stratum granulosum contain lamellar bodies, which releases its lipids content into the extracellular space to further strengthen the barrier. A layer of extracellular matrix called the basement membrane separates the dermis from the epidermis. Structures such as the hair follicles span the different layers of the skin. Immune cells such as the Langerhans cells are found in the epidermis while immune cells such as the mast cells, neutrophils, B cell, T cell and macrophage are found in the dermis. In addition, at the epidermis are the melanocytes, which are responsible for melanin production and ultraviolet protection. Based on[139,140].

2.4 Intercellular Junctions of the Epidermis

Intercellular junctions including the adherens junctions and desmosomes facilitate cell-to-cell adhesion within the epidermis, thus enabling it to serve as an effective physical barrier. Other junctions include the tight and gap junctions (Figure 2). In addition, hemidesmosomes facilitate adherence of cells within the basal layer to the basement membrane. Adherens junction is associated with actin cytoskeleton while the desmosomes are associated with keratin intermediate filament cytoskeleton. The intercellular junctions link the cytoskeleton to the cell's plasma membrane within a cell to that of the adjacent cell, creating a mesh network that gives structure and integrity to the epidermis[134,141].

Aside from the cells of the stratified epidermis, desmosomes are also found in tissues that experience intense mechanical stress such as myocardium, hepatocytes and gastrointestinal mucosa (reviewed in[142–144]). The corresponding effect of their mutations and other

autoimmune diseases that affect them on tissue integrity reflects desmosomes' importance in cell-to-cell adhesion[145–147]. Desmosomes are composed of two desmosomal cadherin proteins: desmocollins (Dsc) and desmogleins (Dsg), which form the extracellular transmembrane region of desmosomes. In addition, desmosomes cytoplasmic constituents compose of armadillo proteins (plakoglobins and plakophilins) and plakins (desmoplakins) (reviewed in[143,148]) (Figure 2).

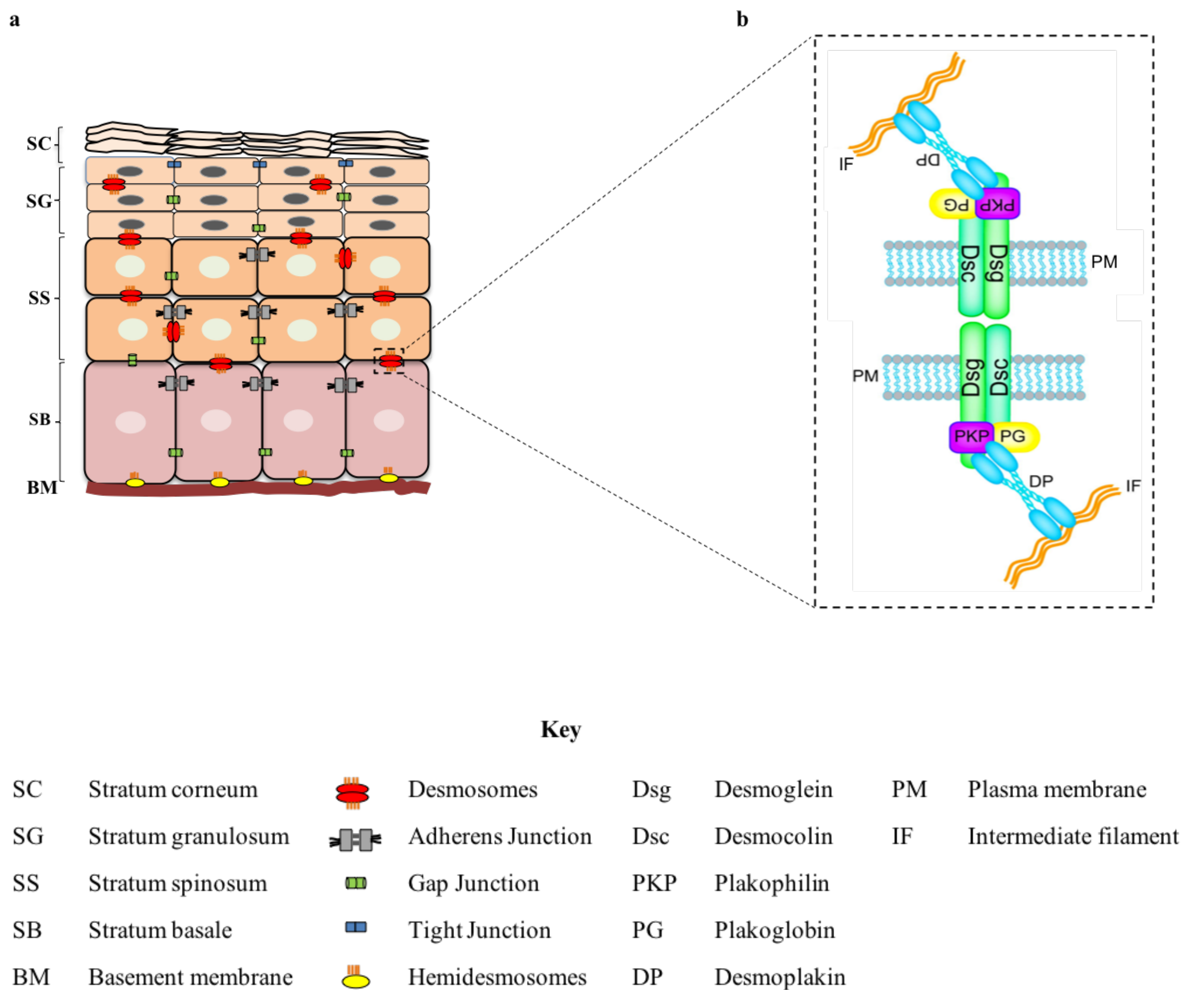

Figure 2. Intercellular junctions of the epidermis. **a.** Cell to cell adhesion junctions of the skin's epidermis are shown. Epidermal intercellular junctions include tight junctions, desmosomes, adherens junctions and gap junctions. Adhesion between keratinocytes at the stratum basale and the basement membrane is facilitated by the hemidesmosomes. Based on[131,134]. **b.** Proteins of the desmosome structure are shown. Desmogleins and desmocollins extend from the extracellular space across the plasma membrane into the intracellular space of the cell. Desmosomes' intracellular components composed of the desmoplakins, plakophilins, and plakoglobins. Desmoplakins bind to the intermediate filament within the cell´s cytoplasm. Cell to cell adhesion is facilitated via interaction of Dsg or Dsc on neighbouring cells Adapted with permission from[149].

In human, there are different isoforms of the desmosomal cadherin proteins: three Dsc (Dsc1-3) and four desmogleins (Dsg1- 4)[150,151]. Dsc and Dsg share similar structural features (Figure 3). Their structure comprises of an extracellular cadherin domain (EC1- 4), extracellular anchor (EA) followed by a single pass transmembrane region and an intracellular anchor at the cytoplasmic side. However, Dsgs have additional motifs on their intracellular region. The cadherin repeats are interspaced with calcium binding motifs and it has been shown that calcium plays an important role in the structural integrity of desmosomes during adhesion[152]. To facilitate adhesion, the desmosomal cadherin proteins can engage in a homotypic or heterotypic interaction with each other[148,153].

Desmoglein isoforms have varied expression patterns within the stratified epidermis[154]. This differential expression is essential for epidermal maturation process and maintenance of tissue homeostasis. Alterations in the expression patterns of the different isoforms result in abnormal epidermal differentiation, reduction in barrier function and compromise in cell-to-cell adhesion (reviewed in[144,148]). Within the epidermis, Dsg2 is expressed at the basal layer while Dsg3 is expressed at basal and spinosum layers. Dsg1 is concentrated in the suprabasal layers and Dsg4 expression is confined to the corneum and upper granular layers[134] (Figure 3).

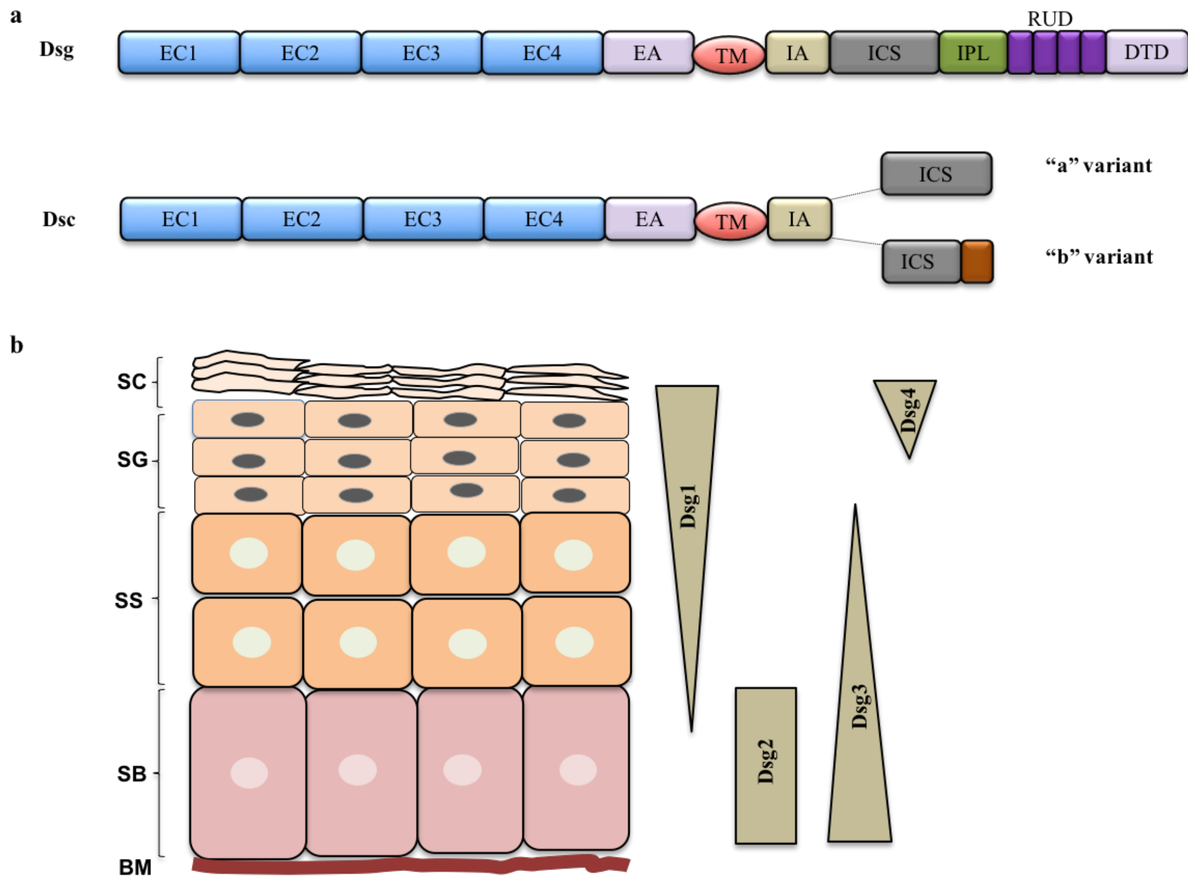

Figure 3. Structure and expression of the desmosomal cadherin proteins. **a**. Schematic representation of the different regions of desmogleins (Dsg) and desmocollins are shown. The desmosomal cadherins proteins contain four extracellular (EC) domains, an extracellular anchor (EA), transmembrane (TM) region, intracellular anchor (IA) and intracellular cadherin segment (ICS). Desmogleins contain additional regions including the intracellular proline rich linker (IPL), repeat unit domain (RUD) and desmoglein-specific terminal domain (DTD). Desmocollins isoforms have two splice variants "a" and "b". Based on[155]. **b**. Dsg protein isoforms expression patterns within the epidermis. SC-Stratum corneum, SG-Stratum granulosum, SS-Stratum spinosum, SB-Stratum basale, BM-Basement membrane) Based on[134].

3 *S. AUREUS* DETERMINANTS OF COLONIZATION AND/OR INFECTION

S. aureus can exist as a commensal or a pathogenic microbe within its human host. This requires achieving a proper balance between efficient attachment at the colonized site and withstanding the mechanical forces that aim to dislodge it from those niches. Furthermore, as a pathogen, the bacteria should be able to survive and establish itself once the host defence mechanisms are breached[49]. In addition, it should be able to cause tissue damage and spread to other sites within the host body to establish infection.

S. aureus expresses a barrage of virulence factors that facilitate its ability to interact with host tissue and the extracellular matrix components. Broadly, *S. aureus* virulence factors can be classified into secreted factors and cell surface factors (Figure 4). Together, these factors function to (1) adhere to the host cell surface and components, (2) spread bacteria through the host, (3) evade host immune defence, and (4) produce toxins and other products, which can cause damage to the host's cells. Coupled with these factors, *S. aureus* also possesses regulatory components and mechanisms, which ensures that the bacterium expresses these factors only when needed (reviewed in[5,6,156,157]).

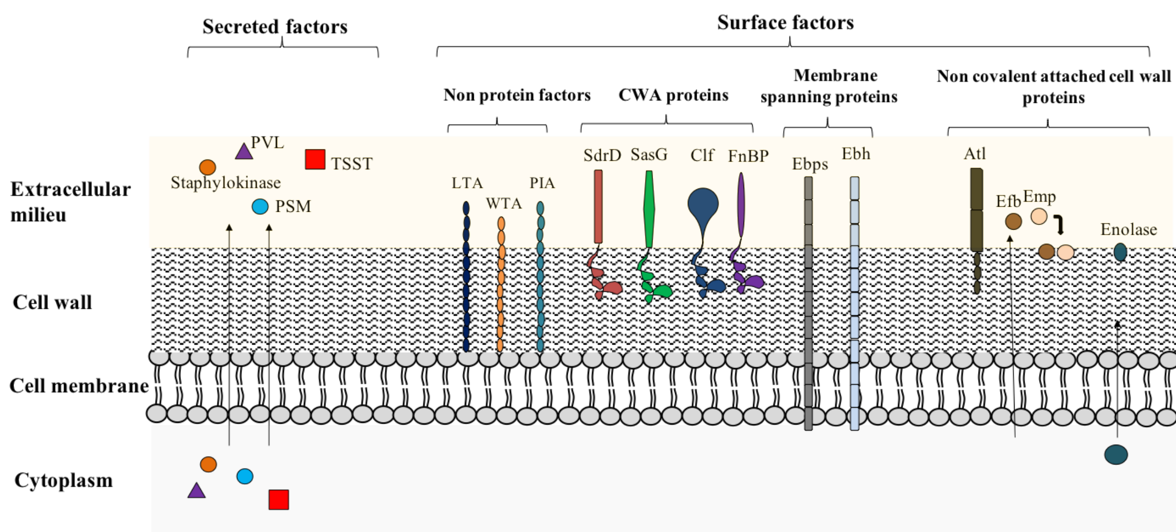

Figure 4. Schematic representation of localization of selected *S. aureus* virulence factors. *S. aureus* produces many factors which contribute to its colonization and/or infections. Examples of secreted factors: include Panton-Valentine leukocidin (PVL), phenol-soluble modulins (PSMs) toxic shock syndrome toxin (TSST) and Staphylokinase. Examples of cell surface factors include lipoteichoic acid (LTA), wall teichoic acid (WTA), polysaccharide intracellular adhesin (PIA), serine- aspartate repeat containing protein D (SdrD), surface protein G (SasG), clumping factor (Clf), fibronectin binding protein (FnBP), autolysin (Atl), extracellular matrix-binding protein homologue (Ebh), elastin binding proteins (Ebps), extracellular fibrinogen binding protein (Efb), extracellular matrix protein (Emp) and enolase. Based on [5,6,18,156,158,159].

3.1 *S. aureus* Secreted Factors

S. aureus produces many factors that are secreted into the extracellular milieu. These secreted factors include enzymes, superantigens and membrane damaging toxins[159,160]. Superantigen factors such as toxic shock syndrome toxin (TSST) activate the host's T cells leading to their excessive proliferation and production of cytokines, overall causing the fatal Staphylococcal toxic shock syndrome[159]. Membrane damaging toxins bore into the cytoplasmic membrane of

the host cells leading to their lysis and escape of their intracellular contents[159]. Membrane damaging toxins include proteins such as Hemolysin-α (α- toxin), Panton-Valentine leukocidin (PVL), Phenol-soluble modulins (PSMs) and gamma-toxin (gamma-hemolysin, HlgA, HlgB, HlgC)[159,161]. These proteins have different mechanisms of action. For example, PVL binds to C5aR and C5L2 receptors on neutrophils[162] while the effect on PSMs on host cells is thought to be receptor independent[159]. In addition, α- toxin binds with A disintegrin and metalloproteinase 10 (ADAM10)[163]. The interaction disrupts focal adhesion and degrades E-cadherins, subsequently leading to loss of epithelial integrity[163,164]. *S. aureus* also secretes enzymes such as Staphylokinase, Staphylocoagulase and Von Willebrand factor (vWF), which further influence the bacterial virulence (reviewed in[159]). In addition, *S. aureus* Exfoliative toxin (ET), has been indicated in the pathogenesis of staphylococcal scalded skin syndrome (SSSS)[165].

3.2 *S. aureus* Cell Surface Factors

The *S. aureus* cell surface is decorated with proteinaceous and non-proteinaceous molecules[18,156]. The proteinaceous cell surface molecules include: (1) Cell wall anchored proteins (CWA) which are covalently linked to the bacterial cell wall[166], (2) Non covalently attached cell wall associated proteins including proteins with specific cell wall-binding domains e.g. autolysin (Atl), 'secretable expanded repertoire adhesive molecules' (SERAMs) and cytoplasmic wall binding proteins, and (3) Membrane spanning proteins such as extracellular matrix-binding protein homologue (Ebh) and elastin binding proteins (Ebps)[18,156]. The non-proteinaceous *S. aureus* cell surface molecules include the Wall teichoic acid (WTA), Lipoteichoic acid (LTA), Polysaccharide Intracellular adhesin (PIA) and other polysaccharides[18,156].

Although, there are ongoing investigations to further understand the contributions of these cell surface factors in *S. aureus* colonization and/or virulence, the functions of some of these cell surface factors has been described (reviewed in[5,6,156,158]). For example, WTA plays an important role in the early stages of *S. aureus* nasal colonization[167] and interacts with human nasal epithelial cells via a type F scavenger receptor called SREC 1[168]. PIA and LTA are involved in *S. aureus* biofilm formation[169]. Ebps binds elastin, a major component of the extracellular matrix[170]. SERAMs proteins including extracellular adherence protein (Eap) and extracellular matrix binding protein (Emp) bind to extracellular matrix molecules including fibronectin, fibrinogen, collagen (reviewed in[171]).

3.2.1 *S. aureus* Cell Wall Anchored Proteins

CWA proteins are the main group of *S. aureus* cell surface factors. They meditate adhesion of *S. aureus* to the host's extracellular matrix and receptor(s) present on the host's cell surface (reviewed in[5,6,154,164]). They are involved in colonization, immune evasion, biofilm function and other functions that contribute to *S. aureus* virulence (reviewed in[5,6]).

CWA proteins contain a signal sequence peptide at their amino terminal and a sorting signal at their carboxyl terminal[5]. The signal sequence directs the translated product to sites within the bacterial peptidoglycan cell wall[172]. The LPXTG motif in sorting signal at the carboxyl terminal, facilitates the covalent anchorage of CWA proteins to the dividing peptidoglycan of *S. aureus* cell wall[173]. The anchorage is facilitated via the action of the transpeptidase enzyme called sortase A (SrtA)[166]. Interspacing the two terminals are different regions with diverse functionality. Based on their structure and function, CWA proteins have been classified into four groups (reviewed in[5]). These are (1) Microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) which include clumping factor A (ClfA) and ClfB, serine-aspartate repeat containing protein (Sdr) C, D and E, bone sialo binding protein (Bbp), collagen adhesion (CNA) and Fibronectin-binding protein A (FnBPA) and FnBPB, (2) Neat motif family e.g. Iron-regulated surface (Isd) proteins, (3) Three helical bundle family e.g. Protein A and (4) G5-E repeat family e.g. *S. aureus* surface protein G (SasG). Recently, a review suggested two additional groups based on functional motifs without structural details and the location of biological functions in a disordered region (reviewed in[6]). These are (1) The legume lectin domain e.g. serine-rich adhesin of platelets (SraP) and (2) fibronectin binding by tandem β-Zipper[6].

Studies have shown the molecular mechanism behind CWA proteins involvement in *S. aureus* virulence and their interaction with some host components[5,6,156]. CWA proteins are involved in nasal colonization. For example, *in vitro* studies have shown that ClfB promotes *S. aureus* binding to cytokeratin 10[174] and loricrin[101]. The importance of ClfB-loricrin interaction nasal colonization was emphasized by the reduced adherence of *S. aureus* in loricrin deficient mouse[101]. Furthermore, ClfB promotes *S. aureus* nasal colonization and persistence in humans artificially inoculated with ClfB expressing *S. aureus*[175]. In addition, ClfB has been shown to bind to cytokeratin 8[176]. Other CWA proteins including as SasX, SdrC and IsdA also promote adherence of *S. aureus* to human nasal epithelial cells[100,177]. Deciphering CWA proteins functions are often complicated because *S. aureus* CWA proteins

are multifunctional and the proteins sometimes have redundant or complementary functions. For example, CWA protein including FnBPA, FnBPB, ClfA, ClfB and IsdA all bind to fibrinogen while IsdA, IsdB and IsdH bind to the haemoglobin component called haem[5,6].

3.2.1.1 Serine-Aspartate Repeat Containing Protein D (SdrD)

S. aureus SdrD belongs to the MSCRAMMs group of CWA proteins. The *sdrD* open reading frame (ORF) is encoded at the *sdr* locus in tandem with the ORFs of *sdrC* and *sdrE*[178]. The prevalence of the *sdr*D gene within the genome of *S. aureus* strains varies[179–181] and Trad et al., observed a correlation between the presence of *sdrD* gene and bone infections[182].

SdrD shares some structural similarities with *S. aureus* virulence factors ClfA and ClfB[178] (Figure 5). Its structure comprises of a signal sequence and a sorting signal at its amino (N) and carboxyl (C) terminus respectively. The N-terminal signal sequence is followed by the A region, the B repeat and R domain (reviewed in[5]). SdrD A region is subdivided into N1, N2 and N3 domains and is responsible for ligand binding via a dock-lock- latch mechanism[183]. SdrD B repeats compose of B1- B5 subdomains are composed of 110-113 amino acid residues and functions as a spacer, extending the ligand binding A region further from the cell wall[121]. The B1- B5 subdomains contains EF motifs, which bind calcium in a sequential manner[184,185]. Furthermore, SdrD R domain is made up of serine aspartate repeats[5,178].

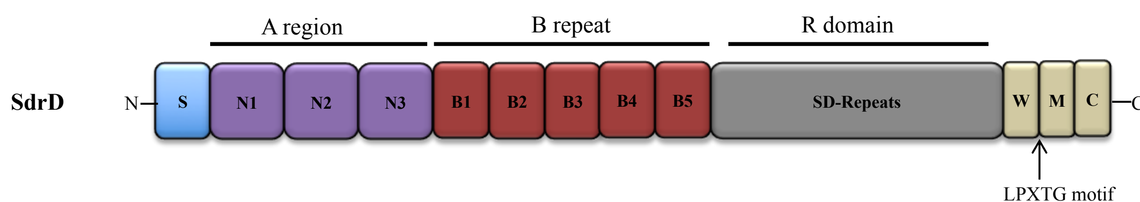

Figure 5. Schematic representation of *S. aureus* Serine Aspartate repeats containing protein D (SdrD). The location of the S: Signal sequence, N1, N2, N3 subdomains of the SdrD A region, B1-B5 subdomains of the SdrD B repeat, SD-Repeats: Serine-Aspartate repeats of the SdrD R domain, W: wall spanning domain, M: membrane spanning domain, C: cytoplasmic domain, LPXTG: cell wall sorting signal are indicated. Based on[5,186].

The function and molecular mechanism of SdrD in *S. aureus* virulence is still being investigated. The *sdrD* gene expression is upregulated during nasal colonization[187,188] and

SdrD promote increased *S. aureus* adhesion to desquamated nasal epithelial cells[100]. However, SdrD may also have a role during *S. aureus* infection, because its expression is increased in human blood[189] and it promotes *S. aureus* survival in human blood *ex vivo*[190]. In addition, there is an increased level of Immunoglobulin G (IgG) against SdrD in serum of *S. aureus* infected patients[191]. Moreover, SdrD is crucial in abscess formation following invasive *S. aureus* infection[192]. Furthermore, mice immunized with a vaccine preparation composed of SdrD, SdrE, IsdA and IsdB, showed an increased level of protection against *S. aureus* infection[193]. These findings suggest that the SdrD protein could be important in *S. aureus* colonization and infection of its host.

3.2.1.2 *S.aureus* Surface Protein G (SasG)

The SasG protein belongs to the G5E group of *S. aureus* CWA proteins. The protein has some structural organization and sequence similarity with the Plasmin sensitive proteins (Pls) and the Accumulation associated protein (Aap) of *S. aureus* and *S. epidermidis* respectively[194]. The SasG protein consists of an A region and B repeat made up of tandem repeats of G5 and E[5,194] (Figure 6).

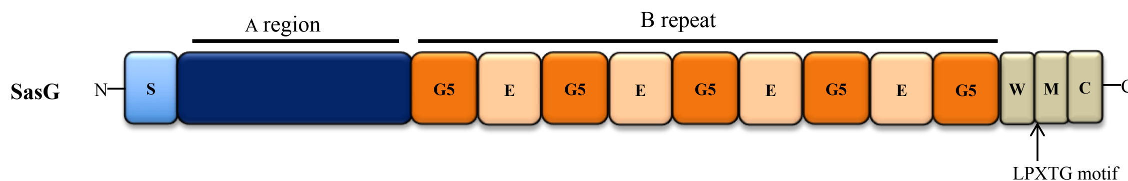

Figure 5. Schematic representation of *S. aureus* Surface protein G (SasG). S: Signal sequence, ligand binding A region, G5-E repeats of the SasG B repeat, W: wall spanning domain, M: membrane spanning domain, C: cytoplasmic domain, LPXTG: cell wall sorting signal are indicated. Based on[194,195]

The *sasG* gene is highly prevalent in clinical isolates compared to carriage isolates[194]. SasG is involved in intercellular aggregation of SasG expressing *S. aureus*[196,197]. SasG also promotes biofilm formation[198] and Geoghegan et al. showed that the biofilm formation process is mediated by the intercellular dimerization B repeat of neighbouring SasG expressing cells[199]. In addition, it was shown that the intercellular dimerization of SasG B repeats occurs in a zinc dependent manner[197,199]. Furthermore, SasG promotes adhesion of *S. aureus* to

desquamated nasal cells[195,198] and the adhesion is mediated by the SasG A region[195]. However, SasG does not promote adhesion to buccal epithelial cells or keratinocytes[195]. The *sasG* gene is highly expressed in nasal samples from *S.aureus* nasal carriers[188] and also high levels of IgG against SasG have been observed in sera of infected patients[194]. This suggests that SasG is relevant for *S. aureus* virulence. However, expression of SasG reduces adherence of *S. aureus* to fibronectin and fibrinogen[195]. This was hypothesized to be the effects of SasG masking other adhesins on S. aureus due to its B repeat extension from the cell surface[198].

3.3 Expression variation in genes encoding *S. aureus* cell surface molecules

The expression patterns of *S. aureus* virulence genes could suggest how and when the expressed virulence factors are important during *S. aureus* colonization and/or infection. Some studies have tried to delineate which *S. aureus* virulence factors are expressed during nasal colonization[200,201]. For example, analysis of nasal samples from persistent *S. aureus* carriers revealed an early upregulation of the WTA biosynthesis genes, *tagO* and *tarK,* during the initial stages of nasal colonization[200]. Other CWA genes such as *clf*B, *fnb*A and *isd*A are upregulated much later during colonization[200]. This suggests that WTA is important for prompt *S. aureus* nasal colonization.

In *S. aureus*, about 24 different CWA proteins can be expressed[5]. However, the CWA proteins expressed depends on strain[202], the growth phase and conditions[187,189,203]. For example, CWA genes such as *isdA* are highly expressed in iron-limiting conditions[204] , others such as *clfB* and *spa* are expressed predominantly during the exponential growth phase[205,206] while *clfA* is expressed in the stationary growth phase[207,208]. In addition, expression of CWA genes *sasD* and *sdrH* were highly upregulated in persistent *S. aureus* nasal carriers compared to non-persistent carriers[209]. These differences are a result of the regulatory factors in *S. aureus* including the accessory gene regulator (*agr*) locus, the staphylococcal accessory regulator A (*sarA*), which direct expression of these factors in response to cues within its environment such as bacterial density, available nutrients etc.[157,208,210].

3.4 Genetic diversity in *S. aureus* Cell Surface Molecules

Studies have also revealed genetic diversity within the sequences and region of genes encoding virulence factors between *S. aureus* from diverse background[202,211]. Genetic variations range from sequence variations within an individual gene[212] to the absence or presence of genes within the genome of different *S. aureus* strains[202,213]. For example, the A domain of *S. aureus* virulence factor FnBPA exists as different isotypes[214,215]. These variations were mainly concentrated in the N2-N3 subdomains of the A domain[117]. Though variations within FnBPA A domain isotypes did not affect their ligand binding activity, it affected their antigenicity[214,215]. This suggests that sequence variations within virulence genes could have important implications on the virulence functions. Indeed, single nucleotide polymorphism in *fnbp* genes have been shown to be associated with increased cardiovascular devices infection[216,217]. Furthermore, sequence variations have been reported within other *S.aureus* CWA genes such as *fnbp*[218,219] and *sdrD*[211] of *S. aureus* isolates from different host origins.

A correlation between the presence of *sdrD* gene and bone infections have been observed[179,182]. McCarthy and Lindsay reported that CWA genes such as *fnbpA, isdA* and *isdH* were present in all the 58 *S. aureus* isolates studied while genes such as *sdrC*, *sdrD* and *sasG* were absent from some of these isolates[202]. They also observed that the collagen adhesion gene, *cna* was absent from the genome of the majority of these isolates[202]. Sabat et al. found that the prevalence of *sdrD* gene was significantly higher in MRSA strains while *sdrC* gene was limited to MSSA strains[179]. Furthermore, *fnbp*B gene was found to be more prevalent among invasive isolates compared to carriage isolates[219,220].

Overall, genetic and expression variation within virulence genes between *S.aureus* isolates further indicate the complexity of identifying specific factors that account for how *S. aureus* could be an effective colonizer or cause a wide range of diseases. What this implies is that the dynamics of *S. aureus* interaction with humans cannot just be explained based on a single bacterial virulence determinant.

4 *S. AUREUS* AND HOST INTERACTION: HOST IMMUNITY

The host immune system can recognize, resist and eliminate *S. aureus* (reviewed in [221,222]). It is divided into the innate immune system and adaptive immune system. The innate immune responses are the first line of defences that are initiated immediately upon contact with pathogens.. Innate immune responses are fast, non-specific but are able to discriminate invading pathogens from self and other beneficial commensal flora. The adaptive immunity is a delayed, specific response and is stimulated by components of the innate immune system. The adaptive immune system develops immunological memory, which enables rapid response to subsequent reinfection by the same pathogen. Adaptive immunity against *S. aureus* infection begins later during the time course of infection. Responses by the adaptive immunity lead to the activation of B and T cells, production of antibodies and also release of cytokines. This can further modulate and/or amplify the initial response mounted by the innate immunity[222]. Phagocytosis by the neutrophils is believed to be one of the main clearance mechanisms for *S. aureus* infection[221,224].

4.1 Innate Immunity

Innate immune system can be broadly grouped into anatomical barriers, toll-like receptors, complement system and phagocytes[223] (Figure 7)

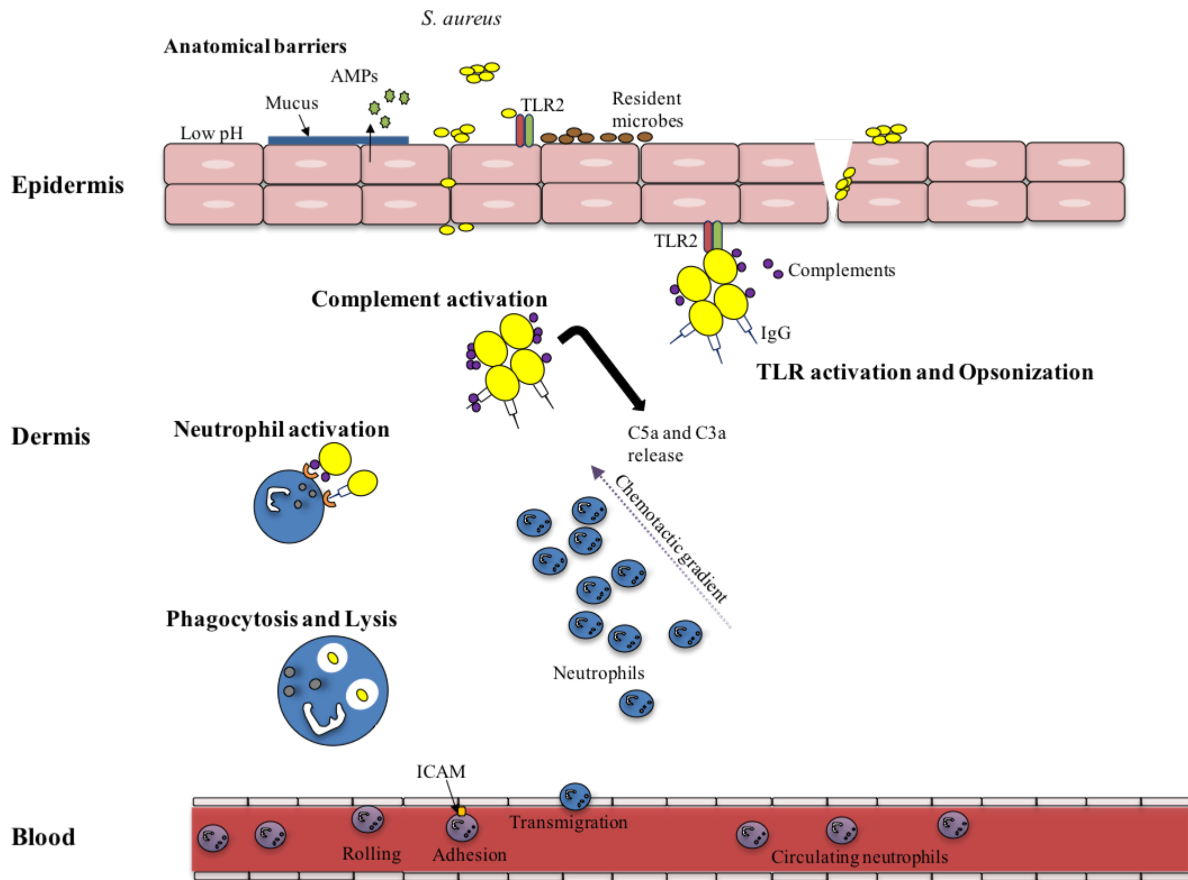

Figure 7. Host Immune responses to *S. aureus* colonization and/or infection. *S. aureus* colonization of the anterior nares and skin surface is inhibited by the host immune defence mechanisms including antimicrobial peptides (AMPs) release, Toll-like receptor 2 (TLR2) recognition of conserved motifs on the bacterial surface, mucus production, the presence of resident microbes and low pH. Upon breaching the epidermis, components of the host immune defence including complement factors and Immunoglobulins (Ig) detect the bacteria. These components opsonize the bacteria surface leading to the activation of the complement cascade. This leads to production of complement factors C5a and C3a.These products initiate recruitment of circulating neutrophils from the blood. In addition, TLR activation induces chemokine production, which together with C5a and C3a form a chemotactic gradient that directs and guides the neutrophils to the infection site. Neutrophils recognize the opsonized bacteria via their Fc and complement receptors. Consequently, the bacteria are phagocytosed and killed by the neutrophils. Based on[225,226].

4.1.1 Anatomical barriers: Immune properties of the Skin

The skin is the first barrier, which protects against onslaught of microbes present in the environment[139]. The skin's immune protection is ensured by tightly packed keratinocytes and also the continuous desquamation of the epidermal cells[132]. In addition, filaggrin components breakdown at the stratum corneum leads to the production of acidic components such as urocanic acid (UCA) and pyrrolidone carboxylic acid (PCA)[227]. These components contribute to the skin surface's low pH and also inhibit expression of *S. aureus* CWA proteins ClfB, FnbpA and protein A[227]. Commensal microbes of the skin also ensure

protection against *S. aureus*. For example, PSM and serine protease Esp produced by *S. epidermidis* on the skin inhibits colonization by *S. aureus*[109,228].

Furthermore, antimicrobial peptides such as β-defensins, RNase7, and cathelicidin expressed by epidermal keratinocytes show inhibitory activity against *S. aureus*, thus preventing successful colonization[132,229]. Human β-defensins are highly potent against *S. aureus*[230]. Cathelicidins disrupt the *S. aureus* cell membrane by forming pores in them[231] and has been shown to be highly effective in killing extracellular and intracellular *S. aureus*[232]. In the skin, RNase7 was found at the stratum corneum and inhibited colonization of skin explants by *S. aureus*[233,234]. The production of these antimicrobial peptides can be induced by the presence of *S. aureus* or components such as LTA[235,236]. Aside from these, antimicrobial peptides can also induce cytokine release and recruitment of immune cells such as macrophages, dendritic cells to the infection site[237,238].

4.1.2 Toll-like Receptors

Cells within the nasal cavity, skin and other *S. aureus* colonization sites possess receptors called pathogen recognition receptors (PRRs). These PRRs recognize conserved microbial components referred to as pathogen associated molecular patterns (PAMPs) in *S. aureus* and other pathogenic microbes[239]. *S. aureus* PAMPs include LTA, lipoproteins (LPP), teichoic acid and other surface associated components (reviewed in[240]). An important group of PRRs are the Toll-like receptors (TLRs). The TLRs are transmembrane proteins composed of an extracellular domain, a transmembrane region and cytosolic Toll/IL-1 receptor (TIR) domain[240].

The important TLR responsible for recognition of *S. aureus* and its microbial component is TLR2. Its importance in mitigating *S. aureus* infections has been demonstrated in mouse lacking TLR2[241,242]. Furthermore, diminished TLR2 stimulation in atopic dermatitis patients have been suggested to contribute in *S. aureus* skin infection[243]. To become functionally activated, TLR2 forms heterodimer complex with either TLR6 or TLR1, via which it interacts with LTAs and lipoproteins expressed on the surface of *S. aureus*[240,244]. Interaction of TLR2 with its ligands, resulting in activation of intracellular signalling cascade that leads to the activation of transcription factor nuclear factor- κB (NF-κB) which consequently leads to the production of pro-inflammatory products such as chemokines and cytokines[245,246]. NF-κB also promotes the expression of adhesion molecules such as E-selectin, Intercellular

adhesion molecule 1 (ICAM1) and vascular cell adhesion molecule 1 (VCAM1)[247]. These adhesion molecules recruit circulating immune cells such as neutrophils from the blood. Furthermore, TLR2 activation promote epidermal tight junction formation thus enhancing skin's barrier function[248]. In addition, activation of TLR2 by the skin's commensal microbes also enhances the production of antimicrobial peptides, which can inhibit *S. aureus* colonization and infection[249].

4.1.3 The Complement System

The complement system is made up of more than 30 protein found in blood and tissues. Complement proteins are inactive until they are cleaved. After activation, they react with each other, generating a sequence of events that helps to combat the pathogen. Complement system can be activated via three different pathways, which are the classical pathway (CP), the alternative pathway (AP) and the lectin pathway (LP). These pathways differ in the molecules that can activate them. The classical pathway is activated either by direct binding of C1q to the bacterial surface or C1q binding to antibody complexes (IgM or IgG) present on bacterial surface. In contrast, binding of the spontaneously generated C3b on bacteria activates alternative pathway. The lectin pathway is activated by mannose binding lectin or ficolin to the mannose containing carbohydrates on the bacterial surface. Complement activation irrespective of the pathways results in the production of C3 convertases (reviewed in[240,250,251]).

Complement activation serves three purposes. First, the activated complement factors bind the pathogen surfaces, opsonizing them thus making phagocytosis of the pathogen highly efficient. Second, the effector proteins such as C5a and C3a generated during complement activation, serve as chemoattractants for the recruitment of immune cells (phagocytes) from circulation. Furthermore, activation of complement can also lead to the generation of membrane attack complex (MAC) that lyse the pathogen's membrane especially for Gram-negative bacteria (reviewed in[240,250,251]). The importance of complements in combating *S. aureus* has been demonstrated by the increased death observed in complement depleted mouse after *S. aureus* bacteraemia[252]. Furthermore, it has also been shown that activation of complements on *S. aureus* surfaces reduced their adherence to endothelial cell surfaces[253].

4.1.4 Neutrophils

Neutrophils are the first set of phagocytes to migrate to the site of *S. aureus* infection (reviewed in[254]). Their importance in combating *S. aureus* infection is demonstrated by the increased predisposition of individuals with defective neutrophil functions to *S. aureus* infections[254,255]. The primary role of neutrophils in combating infection is phagocytosis of the pathogens recognized by the PRRs. They are also play an important role in abscess formation upon *S. aureus* infection[256].

Recruitment of circulating neutrophils to the infection site is facilitated by a gradient of chemotactic signals including Interleukin-8 (IL-8), complement factors C3a and C5a[254,257]. The recruitment process can be divided into four stages, which are rolling adhesion, integrin activation, firm adhesion and transmigration[258]. Capturing of circulating neutrophils is initiated by their attachment to adhesion molecules such as E- selectin, P-selectin, Intracellular adhesion molecule (ICAM) etc. present on the endothelial cells[259]. Attachment to these adhesion molecules is facilitated by receptors such as P-selectin glycoprotein ligand 1(PSGL-1) expressed by neutrophils[260]. Subsequently, they leave blood circulation and transmigrate across the endothelial walls towards the infected tissue site[261].

Efficient phagocytosis by neutrophils is enhanced by the presence of opsonins such as complement factors and immunoglobulins on the pathogen's surface[254]. Present on neutrophils cell surface are receptors such as Fc and complement receptors, which interact with these opsonins (reviewed in[250,258,262]). However, neutrophils mediated phagocytosis of pathogens have also been observed to occur at a slower rate in absence of opsonization[263]. These interaction leads to the phagocytosis of the pathogen and subsequently formation of phagosomes. Phagosomes undergo series of maturation process, which eventually lead to bacterial killing (reviewed in[264]). Reactive oxygen species (ROS), proteinases and AMPs etc. produced by neutrophils ensure bacterial killing[265]. Furthermore, neutrophils can trap and kill *S. aureus* via its neutrophil extracellular traps (NETs) covered with antimicrobials[266].

4.2 *S. aureus* Immune Evasive Strategies

S. aureus has evolved evasion mechanisms or strategies that help it to counteract host immune responses against them (Figure 8). These strategies are facilitated by secreted or surface bound virulence, which help *S. aureus* to disrupt normal host defences functionality, thus creating an environment for the bacteria to thrive and survive better. *S. aureus* immune evasion strategies are exhibited in different ways. The most prominent of these evasion strategies is *S. aureus* ability to circumvent neutrophils' phagocytic and intracellular killing function[49,257]. Aside from these, *S. aureus* possesses ability to reduce antimicrobial peptides effectiveness, inhibit TLR signalling and complement activation and opsonization (reviewed in[49,50,267]).

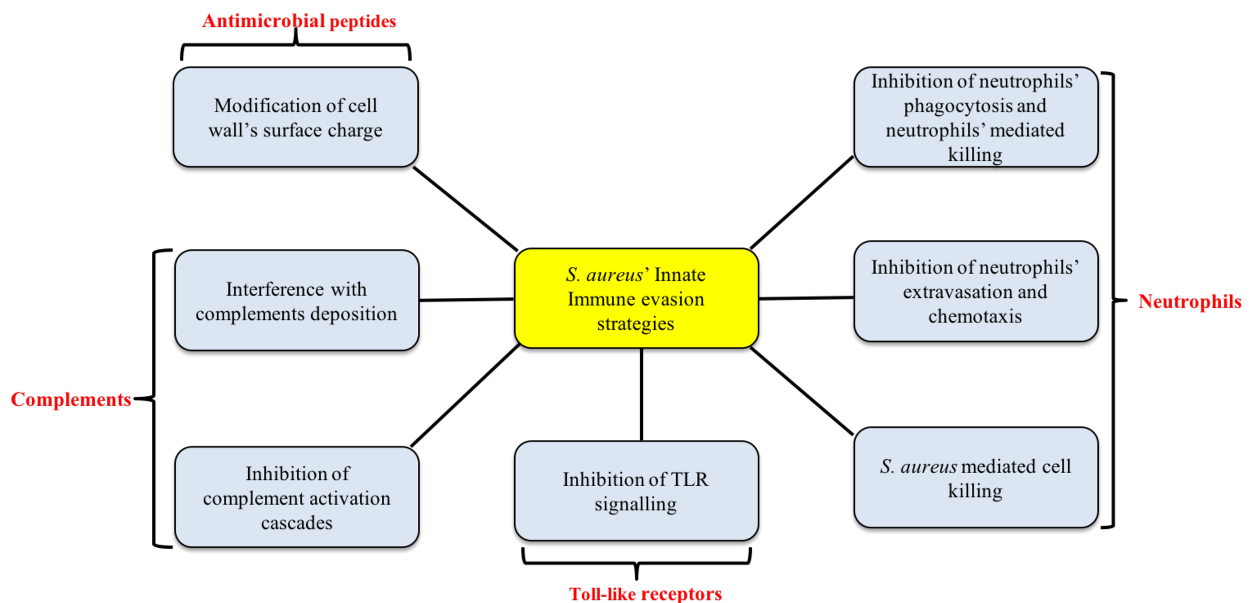

Figure 8. *S. aureus* immune evasion strategies to host innate immune defence mechanisms. Host factors are written in red while the mechanisms behind the immune evasion are listed in blue boxes.

4.2.1 Inhibition of Phagocytes

S. aureus concerted strategies to inhibit phagocytosis are targeted at phagocytes recruitment and subsequent ingestion of the microbe. Recruitment of circulating neutrophils is inhibited by *S. aureus* chemotaxis inhibiting proteins (CHIPS), which binds to C5a and formyl peptide receptors on the neutrophils' cell surface[268,269]. This prevents the neutrophils from following the chemotactic gradient necessary to get to the infection site. In addition, binding of *S. aureus* surface proteins such as Second binding protein of immunoglobulins (Sbi)[270] and

Staphyloccocal protein A (SpA) to the Fc of IgG, reorients antibodies in the wrong direction preventing opsonization and phagocytosis[49,258]. Masking of bacterial surface receptors or epitope by *S. aureus* capsule polysaccharide also inhibits phagocytosis[271]. Complement mediated opsonization of the bacterial surface is affected by proteins such as extracellular adherence proteins (Eap), Staphyloccocal Complement Inhibitor (SCIN) and extracellular fibrinogen binding protein (Efb) [49,258]. CWA proteins such as ClfA and IsdH also inhibit the phagocytosis[272,273].

4.2.2 *S. aureus* Resistance to Killing

When *S. aureus* is ingested, it can still survive within the phagocytes by inhibiting the cytotoxic processes leading to bacterial degradation. *S. aureus* products such as staphyloxanthin and superoxide dismutases protect the bacteria from effects of the reactive oxygen species of neutrophils' phagosomes[49,274] .

S. aureus have also developed strategies to combat the effects of AMPs. *S. aureus* secreted protein aureolysin degrades the LL37, a potent bactericidal agent[275]. It can also modify its surface via the action of *dtl* operon, thus preventing the binding of AMPs[276]. Staphylokinase also binds inhibits the activity of defensins on *S. aureus* by binding with them[277]. In addition, *S aureus* produces toxins such as phenol soluble modulins (PSMs) which form pores on the phagocytes and thus facilitating the escape of the ingested *S. aureus*[278,279]. CWA proteins such as ClfA mediate survival of bacteria by promoting abscess formation. Others such as SdrD, SdrE and SpA also contribute to *S. aureus* survival in blood [190,280,281].

Some of the molecules expressed by *S. aureus* to circumvent host immune responses are listed in Table 1

Table 1: Examples of molecules used by *S. aureus* to evade or alter the host immune responses

Immune Response	Evasion factor	Abbreviation	Function	Effect	Reference
Neutrophils	Staphyloccocal superantigen protein 5	SSL5	Binds P-selectin glycoprotein ligand-1	Disrupts neutrophils chemotaxis	[282]
	Staphylococcal superantigen protein 11	SSL11			[283]
	Chemotaxis inhibitory protein of *S. aureus*	CHIPS	Binds to C5a receptors and formyl peptide receptor like-1	Disrupts neutrophils chemotaxis	[268,269]
	Formly peptide receptor like-1 inhibitory protein	FLIPr			[284]
	Extracellular adherence protein	Eap	Binds to ICAM1	Blocks neutrophils adhesion to endothelial lining	[285,286]
	Staphylococcal binder of Immunoglobin	Sbi	Binds IgG	Blocks antibody mediated opsonization and phagocytosis	[270,287]
	S. aureus Protein A	Spa			Reviewed in[33,49,50]
	Staphyloxanthin		Carotenoid biosynthesis	Protection against Reactive oxygen species (ROS) effects	[288]
	Catalase and Superoxide dismutase		Eliminate/ Inactivate ROS		[289]
	Phenol Soluble modulin	PSM	Bore pores in membrane of cells	Destroys neutrophils and other host immune cells	Reviewed in[278]
	Panton- Valentine leukocidin	PVL			Reviewed in[33,49,50]
	Leukocidin GH	LukGH			
TLR	Staphylococcal superantigen 3	SSL3	Binds TLR2 ligand binding site	Blocks TLR2 immune recognition	[290]
	TIR containing protein	TIRS	Binds TLR2´s TIR domain	Blocks TLR2 mediated NF-κB activation	[291]
Antimicrobial peptide	*dlt* operon	WTA	Modification of cell wall components	Reduced antimicrobial peptide activity	[276]
	Staphylokinase	Sak	Binds alpha defensins		[277]
Complement	Staphylococcal Complement Inhibitor	SCIN	Binds complement factor C3 and C3 convertases	Disrupts complement mediated opsonization of *S. aureus* and phagocytosis	[292]
	Staphylococcal binder of Immunoglobin	Sbi			[293]
	Extracellular fibrinogen binding protein	Efb			[294,295]
	Staphylokinase	Sak	Converts *S. aureus* surface bound plasminogen into plasmin	Removes opsonins on the microbial cell surface	[277,296]
	Serine aspartate repeat containing protein E	SdrE	Binds Factor H		[280]

5 OBJECTIVES

The main objective of this study was to investigate the molecular mechanisms underlying the determinants involved in *S. aureus* colonization and/or infection of its host. We aimed to provide increased knowledge regarding the role of the *S. aureus* CWA proteins SdrD and SasG in bacterial adhesion to host cells and/or evasion of the host immune defence mechanisms.

Our specific research questions were:

Paper I

S. aureus SdrD has been shown to promote bacterial adhesion to desquamated nasal epithelial cells.

> **Does *S. aureus* SdrD interact with specific host ligand(s) in the epithelium? What could be the role of such ligand interaction?**

Paper II

Sequence diversity has been observed within *S. aureus* CWAs genes such as *fnbp* and *clfA*.

> **How conserved is the *sdrD* gene within *S. aureus* isolates from healthy individuals? Could variation within the *sdrD* gene influence SdrD function?**

Paper III

S. aureus SasG is a surface protein known to promote bacterial adhesion to desquamated nasal epithelial cells.

> **Does *S. aureus* SasG contribute to host cells adhesion to human keratinocytes? Under what conditions is SasG expressed? Does *S. aureus* SasG have immune evasion properties?**

6 METHODOLOGY

A detailed description of the experimental procedures performed in this thesis is listed and explained in the articles and manuscript herein. An overview of some of the experimental procedures performed is explained below.

6.1 Host Protein-Pathogen Protein Interaction

Protein-protein interaction detection assays have been employed to determine the interaction between host proteins and *S. aureus* virulence factors. Elucidating these interactions is particularly helpful in deciphering some of the molecular mechanism engaged by *S. aureus* in colonizing humans. It has been suggested that this might form the basis for the development of some anti-colonization strategies in future[297]. In **Paper I**, a yeast two-hybrid (Y2H) assay[298] was ordered from Hybrigenics to find the potential host partners for *S. aureus* SdrD protein. Basically, Y2H assays are performed in yeast strains containing two proteins. One of the proteins referred to as "bait" is fused with a DNA binding domain (DBD) while the other protein referred to as "prey" is fused with an Activation domain (AD). Physical interaction of the two proteins brings these two domains in close proximity and thus forming a functional transcription factor. The transcription factor then activates the reporter gene, which can be assayed by growing the yeast strain on a selective medium or observing for colorimetric changes. In **Paper I**, the SdrD A region fused with GAL4DBD was the **bait protein** and this was used to screen the human reconstituted skin libraries fused with GAL4AD (the "**prey**"). We selected Y2H assay because it allowed for screening of large proteins libraries for SdrD putative host partners. It is important to note that Y2H assay has some limitations, one of which is the high false positive rates of potential candidates[299]. However, **in Paper I**, we have employed complementary methods to validate the putative partners as indicated by the Y2H.

6.2 Solid Phase Ligand Binding Assay

Solid phase ligand binding assay allows for further characterization and validation of protein-protein interactions *in vitro*. The principle underlying this method is based on the enzyme linked immunosorbent assay (ELISA). For this assay, the first protein (protein A) is immobilized onto a solid phase such as wells of microtiter plate. Subsequently, the free in solution second protein (protein B) is added to the wells containing immobilized protein A.

After washing, the binding of the two proteins is determined via colorimetric quantification. It is expected that the colour intensity should be directly proportional to the amount of the bound proteins. In **Paper I**, variants of this assay were adopted to confirm the interaction between *S. aureus* SdrD and Dsg1, the Y2H assay detected putative interaction partner. For example, purified recombinant SdrD A region protein were coated onto wells of microtiter plates. Subsequently, recombinant Dsg1 protein was added. If there were any interaction, it would be expected that the added Dsg1 would bind to the SdrD A region. This was assayed by adding antibodies against Dsg1, followed by the secondary antibodies, followed by quantification of binding. In addition, in **Paper I**, we also immobilized Dsg1 on wells of microtiter plates. Wells coated with IgG_1 were used as a control. Thereafter, we added *S. aureus* NCTC 8325-4 or *S. aureus* NCTC 8325-4Δ*sdrD* to the coated wells of microtiter plates. We hypothesized that if SdrD binds with Dsg1, expression of SdrD in *S. aureus* NCTC 8325-4 should increase binding to immobilized Dsg1. Subsequently, the plates were washed, fixed and stained with crystal violet. The stained well were subsequently dissolved with acetic acid. Semi quantitative measurement of the interaction was achieved by measuring absorbance of the wells.

6.3 Genetic manipulation of *S. aureus*

Studying the biological functions of *S. aureus* virulence factors often involves a deletion or heterologous expression of genes encoding these factors. Manipulations of *S. aureus* DNA have been performed in this thesis.

In **Paper I** and **III**, isogenic mutants of *S. aureus* were generated using the allelic replacement method[300,301]. This method relies on the replacement of DNA with a mutant allele of the DNA via homologous recombination. To create a markless deletion of the target gene, DNA sequences flanking up and down of the target gene are cloned into a *S. aureus* shuttle plasmid such as pKOR1[300] and pIMAY[302]. The replication origin for these plasmids is temperature sensitive i.e. they can only replicate autonomously at certain permissive temperature. Furthermore, the plasmid contains a selective marker that allows selection of successful transformants. Thus, electroporation of the cloned plasmid into *S. aureus* and growing the bacteria at the selective temperature allows for plasmid replication. Subsequently, temperature shift is used to facilitate integration and excision of plasmid in and out of the *S. aureus* genome. However, it is important that caution is taken during genetic manipulations to ensure and verify that no other undesired modifications are introduced in

S. aureus genome. Thus, it is important to verify that other biological functions (such as growth rate, hemolytic effect etc.) in the wild type and its isogenic mutant are comparable.

Genetic modifications of certain *S. aureus* strains are difficult due to the *S. aureus* restriction-modification (RM) systems, which attacks foreign DNA (reviewed in[303]). *S. aureus* RM systems methylate its DNA in specialized pattern, which are subsequently read and protected from degradation. However, foreign DNA without the recognizable methylation pattern is degraded when transformed into *S. aureus*. Type I and type IV RM systems are mainly responsible for this in *S. aureus*. To bypass this, in **Paper I,** the cloned plasmid was transformed into *E. coli* DC10B[302,304]. *E.coli* DC10B is deficient in Dcm methylase, which methylates cytosine residues. Thus, plasmids transformed in it are not methylated on their cytosine residues, thus bypassing the type IV RM system challenge. In **Paper III**, the cloned plasmid was transformed in *E. coli* IM01B or SA08B[301]. Aside from being *dcm* deficient, these *E. coli* strains have been engineered to methylate the adenine residues on the transformed plasmid based on the methylation pattern of particular *S. aureus* clonal complexes, this allows for bypass of Type I and type IV RM systems challenges in *S. aureus*.

Falkow explained Koch´s postulates as it relates to microbial pathogenicity investigation[305]. He stipulated that it is important that expression of the gene in its isogenic mutant (complementation) or expression in another model strain (heterologous expression) should restore or confer virulence capacity in order to confirm that the gene is actually involved in its pathogenicity. Therefore in **Paper I**, heterologous expression of SdrD was achieved by cloning *sdrD* gene into pMG36e plasmid[306] and which was then subsequently transformed into *Lactobacillus lactis.* In **Paper II**, *S. aureus* NCTC 8325-4Δ*sdrD* was complemented with pMG36e plasmid containing variants of *sdrD* gene. This ensured that the *sdrD* variants were expressed in a common genetic background, therefore eliminating contributory effects of other factors not within the scope of the study. In **Paper III**, we made heterologous expression and complementation constructs for SasG by transforming pALC2072::$SasG_{MSSA476}$ into *S. aureus* SH1000 and MSSA476Δ*sasG* respectively.

6.4 Bioinformatic analysis

Bioinformatic tools and analyses have taken a front seat in providing more understanding about *S. aureus* based on the sequences of their genes or genomes. In addition, relatedness of *S. aureus* strains can also be inferred by comparing the sequences to each other and also with

other reference isolates which are present in the database. This has helped in prompt resolution and typing of *S. aureus* responsible for infections.

In **Paper II**, genetic variation in *sdrD* gene between *S. aureus* isolates from healthy carriers was investigated. Multiple sequence alignment (MSA) was performed in order to facilitate comparison of the *sdrD* A region sequences from different *S. aureus* isolates. This will show the sequence diversity of *sdrD* gene within *S. aureus* strains from different human hosts. MSA can either be carried out either as global or local alignments. The major difference between them is that global alignment tries to align the whole sequence while local alignment aligns the sequence based on the region of highest similarity between the sequences. Multiple Alignment Fast Fourier Transform (MAFFT)[307] was used for the alignment. MAFFT has been suggested to provide accurate MSA data compared to some other alignment tool such CLUSTALW[308]. In addition, phylogenetic analyses were performed using Randomized Axelerated Maximum Likelihood (RaxML)[309] and Multilocus sequence typing (MLST)[51].

6.5 Host Model Systems to study functions of *S. aureus* Virulence Factors

There is need to develop or use an appropriate *in vitro* or *ex vivo* model to investigate the virulence effect of *S. aureus* proteins. Depending on the physiological conditions that are being monitored, different technical methods can be used. The model system of choice should mimic the conditions that the bacteria would normally be exposed to during colonization or infection. However, the limitation of a host model system is that it is not possible to include all human components and conditions that would normally be present during *S. aureus* colonization or infection.

In **Paper I, II** and **III**, cell adhesion assays were performed to evaluate the contribution of the studied virulence factor to *S. aureus* adhesion. As keratinocytes are the most abundant cells types found in the skin and the anterior nares of the nose[132,134], the model cell type chosen for our *in vitro* studies was the immortalized human keratinocytes cell line (HaCaT)[310] (**Paper I, II** and **III**). However, in **Paper I**, we also used human embryonic kidney cells 293 (HEK293)[311]. Basically, in cell adhesion assay, the cells are seeded into tissue culture treated wells and incubated to facilitate attachment to the well surface. Upon addition of *S. aureus* to the seeded cells, *S. aureus* surface protein should interact with host surface expressed proteins. As a control, a bacterium lacking the gene encoding the protein is used. The degree

of adherence can therefore be compared, in order to see if the protein contributes significantly to *S. aureus* adhesion to the host cells.

As discussed previously, *S. aureus* virulence factors also contribute to its ability to evade and survive host defence mechanisms. Human blood composes of components that aid to combat bacterial infections. The whole blood assay has been used to study the expression and immune evasion effect of *S. aureus* virulence genes[189,190,287,312]. Blood used for this assay is usually freshly drawn blood from healthy participants. The blood is taken in tubes containing hirudin, which serve as anticoagulant and also preserve the complement activity of the blood[313]. Therefore, this assay provides a more accurate reflection of *S. aureus* response to immune system components. In **Paper III**, the expression and contribution of *S. aureus* MSSA476 *sasG* gene to survival was assayed in whole human blood assay.

6.6 Recombinant protein expression

Advances in the molecular biology techniques have made it possible to express protein products of exogenous DNA within a heterologous expression system. Recombinant protein production has helped to overcome the challenges of getting high amounts of the desired protein. The recombinant protein process involves cloning the DNA sequences of the desired protein into an expression vector such as pGEX-4T-1 and pRSETB. The cloned vector is transformed into a heterologous expression vehicle such as bacteria, yeast or mammalian cells. Thereafter, expression of the protein is induced and subsequently the recombinant protein is isolated and purified.

In this study, we have expressed SdrD and SasG proteins and its subdomains using the *E. coli* expression system. We used this system because *E. coli* is easy to culture, easy to handle and has a fast growth rate. In addition, compared to other heterologous expression vehicles, *E. coli* genetics is better understood and this system produces a high yield of the recombinant protein. However, there are drawbacks associated with using *E. coli* as an expression vehicle including likelihood of protein not being expressed in its natural form, production of insoluble protein, and lack of post-translational modifications such as glycosylation. Strategies including use of fusion tag and optimization of expression conditions such as lowering of temperature and addition of glucose etc. have been suggested to overcome these challenges (discussed in[314,315]).

7 SUMMARY OF RESULTS

Paper I: The interaction between *Staphylococcus aureus* SdrD and desmoglein 1 is important for adhesion to host cells

- The expression of SdrD in *S. aureus* NCTC8325-4 promoted increased binding of the bacteria to HaCaT cells compared to its isogenic *sdrD* mutant *S. aureus* NCTC8325-4Δ*sdrD*. Furthermore, the heterologous expression of SdrD in *L. lactis* transformed with pMG36e::$SdrD_{NCTC8325-4}$ increased bacterial adhesion to HaCaT cells compared to *L. lactis* transformed with empty pMG36e.

- The Y2H assay identified Dsg1 as putative host interaction partner for *S. aureus* SdrD.

- *S. aureus* SdrD facilitated the binding of *S. aureus* to Dsg1. This was observed by the increased adherence of *S. aureus* NCTC8325-4 to immobilized recombinant Dsg1 compared to its isogenic *sdrD* mutant *S. aureus* NCTC8325-4Δ*sdrD*. The increased adherence of *S. aureus* NCTC8325-4 occurred in a dose dependent manner.

- Preincubation of *S. aureus* NCTC8325-4 with recombinant Dsg1 reduced the adherence of the bacteria to immobilized recombinant Dsg1. In addition, recombinant human Dsg1 binds to immobilized recombinant SdrD A region in a dose dependent manner.

- The interaction between *S. aureus* SdrD and Dsg1 is specific. Recombinant SdrD interacted with HaCaT cells expressing Dsg1 but not with neutrophils, which does not express Dsg1. Furthermore, transfection of HEK293 cells with plasmid expressing Dsg1 facilitated increased adhesion of *S. aureus* NCTC8325-4 compared to its isogenic *sdrD* mutant *S. aureus* NCTC8325-4Δ*sdrD*.

Paper II: Genetic variability in the *sdrD* gene in *Staphylococcus aureus* from healthy nasal carriers

- The study revealed that the prevalence of *sdrC*, *sdrD* and *sdrE* genes varied within *S. aureus* strains isolates from the anterior nares of healthy adult participants. The *sdrC* gene was almost always present in the isolates, while the *sdrD* gene was present in 29% of the isolates. None of the *S. aureus* isolates carried the *sdrD* gene as a single gene.

- Sequence alignment of the *sdrD* A region from 48 *S. aureus* isolates from healthy individuals together with those of 6 reference strains, showed diversity within this region across the isolates. The diversity within the *sdrD* A region of *S. aureus* strains analysed was classified into seven *sdrD* variants.

- The entire *sdrD* gene for the seven *S. aureus* isolates representing the seven *sdrD* variants also showed sequence variation. The size of the SdrD polypeptides also varied. The amino acid variations in SdrD were concentrated in the N2-N3 domains of the A region, B1 subdomain and R chain.

- Structural modelling revealed that the amino acid variations in the N2-N3-B1 domains were majorly concentrated in the N3 domain. Most of the amino acid variations were surface associated. In addition, there were amino acid variations within the groove formed between the N2-N3 domains.

- The cell adhesion assay revealed that the adhesion of the *S. aureus* expressing the *sdrD* variants to HaCaT cells were comparable. Only two of the *sdrD* variant*s* differed significantly when compared to each other.

- Overall, our findings showed genetic variability in the *sdrD* gene from *S. aureus* strains isolated from healthy adult individuals.

Paper III: Expression and Virulence properties of *Staphylococcus aureus* MSSA476 Surface protein G (SasG)

- The presence of $SasG_{MSSA476}$ promoted bacterial adhesion to HaCaT cells in a heterologous $SasG_{MSSA476}$ expression system compared to its empty vector control.

- Pairwise comparison of amino acid sequences of $SasG_{MSSA476}$ and $SasG_{NCTC8325}$ revealed variations within the A region and B repeats of the two proteins.

- Expression of *sasG* gene in MSSA476 is upregulated upon exposed to human blood. In addition, the presence of serum components induced early expression of SasG protein in MSSA476 grown in bacteriological medium.

- The presence of SasG did not contribute to bacterial survival in human blood *ex vivo*

- SasG mediated bacterial aggregation in the presence of FBS.

8 GENERAL DISCUSSION

Adequate understanding of the mechanisms of *S. aureus* colonization is vital in developing alternatives strategies to combat its ability to cause infections and diseases. Host colonization by *S. aureus* involves a complex interaction of diverse factors, which are the pathogen, host and the environment[23,316]. In this thesis, mechanisms of some of the bacterial determinants involved in *S. aureus* colonization and infection were investigated. Particularly, we focused on the role of the *S. aureus* CWA proteins SdrD and SasG in adhesion and immune evasion processes.

8.1 *S. aureus* CWAs proteins: Interaction with Epithelial Cells

Nasal colonization is an important risk for the subsequent development of *S. aureus* infection[4,72]. This is facilitated by several adhesins such as CWA proteins expressed by *S. aureus* strains[5,6,101]. In this study, we revealed the role of two *S. aureus* CWA proteins, SdrD (**Paper I** and **II)** and SasG (**Paper III)** in promoting *S. aureus* adherence to human keratinocytes. The importance of these proteins to *S. aureus* adherence was shown by the significant impairment in adherence of their isogenic mutant compared to the wildtype. Adhesion of the mutants to keratinocytes could not be totally abolished due to the functional redundancy that exists within *S. aureus* CWA proteins (reviewed in[5,156]). However, using complemented mutants and/or heterologous expression construct, we confirmed SdrD and SasG importance in promoting adherence to human keratinocytes (**Paper I**, **II** and **III**). Our findings further reinforce the previous understanding regarding the multiple mechanism used by *S. aureus* in its interaction with host cells and tissue[5,6,156,171].

Previous *in vitro* studies have shown that *S. aureus* CWA proteins SdrD, SasG, SdrC, SasX and ClfB promoted bacterial adherence to desquamated nasal epithelial cells[100,195,317]. Furthermore, *in vivo* studies have shown ClfB and IsdA involvement in nasal colonization[175,318]. The desquamated nasal epithelial cells are limited to the stratum corneum layer of the skin epidermis[133]. On the other hand, keratinocytes are abundant and present throughout the different layers of the skin epidermis and are the most abundant cells present in the skin epidermis[139]. Therefore, it may not be surprising that *S. aureus* expresses surface proteins that interact with the keratinocytes (**Pape**r **I**, **II** and **III**). The ability of *S. aureus* to interact with cells beyond the

superficial layers could provide an advantage in colonization and immune evasion. Although, in murine model, SdrD did not facilitate nasal colonization[319] but expression of *S. aureus sdrD* and *sasG* genes are increased during nasal colonization in human[187,188]. However, the involvement of SdrD and SasG proteins in establishing successful nasal colonization is still unknown and requires further studies.

Our result showing *S. aureus* SasG protein mediated bacterial adherence to keratinocytes (paper **III)** contradicted results of Roche et al[195]. However, this may be due to differences in strains or experimental procedures. For example, in **Paper III** the heterologous expression construct, *S. aureus* SH1000 transformed with pALC2073::$SasG_{MSSA476}$ was used while the previous study used *L. lactis* transformed with pKS80::sasG[195]. Furthermore, the SasG mediated adherence to keratinocytes could be influenced by or require the concerted and/or contributory effect of other *S. aureus* surface proteins. Interestingly, SasG mediated bacterial adhesion was only observed in FBS-deficient cell culture medium, the presence of FBS attenuated the bacterial adhesion to keratinocytes (**Paper III**). Differences in adherence based on media have been observed previously. The ability of ClfB expressing *S. aureus* to adhere to cytokeratin 10 differed depending on whether the bacteria were grown in either nutrient deficient or nutrient rich medium[175]. This further shows the complexity and multifactorial nature underlying the molecular mechanism of CWA proteins interaction with host cells[5]. SasG protein interaction with the host cells requires more studies to further delineate its mechanism of interaction.

The interaction of *S. aureus* CWA proteins with host molecules mediates adherence of *S. aureus* cells to host cells and it is also essential for nasal colonization[101]. Host molecules such as loricrin and cytokeratin 10 are important host ligands for *S. aureus* ClfB and IsdA[101,320]. In **Paper I**, we revealed that *S. aureus* SdrD interacts directly with cell adhesion molecule Dsg1. SdrD A-domain has previously been shown to be essential for the protein ligand binding function[5,183]. Here, we demonstrated that Dsg1 binds SdrD A-domain in dose dependent manner (**Paper I**). Furthermore, we showed that the interaction between *S. aureus* SdrD and Dsg1 promoted bacterial adhesion to human keratinocytes (**Paper I**). However, some interaction between the isogenic *sdrD* mutant and Dsg1 was seen, suggesting other surface proteins might interact with Dsg1. This is not surprising, taken into consideration the functional redundancy that

exist among *S. aureus* CWA proteins. For example CWA protein FnBPA, FnBPB and IsdA could all bind to fibronectin while ClfA, ClfB FnBPA and FnBPB bind to fibrinogen[5]. Therefore, further studies are required to investigate if there is other *S. aureus* surface proteins that interact with Dsg1.

8.1.1 Implication of SdrD-Dsg1 interaction in *S. aureus* colonization and/or infection

Dsg1 is a transmembrane desmosomal cadherins protein involved cell- cell adhesion and maintenance of integrity of the epidermis[134,148]. Dsg1 has an extracellular region which interacts with either Dsc1 or Dsg1 of its neighbouring cells, thus ensuring cell cohesion[143,148]. It is concentrated in all the layers of the epidermis except the stratum basale[134,148]. Our findings in **Paper I**, demonstrated *S. aureus* ability to use proteins involved in host's cell to cell adhesion as its receptor. One can speculate that SdrD-Dsg1 interaction could be a means for *S. aureus* to breach the epithelial barrier and disseminate further into the tissue. Initially thought to limited to the epidermal stratum corneum, *S. aureus* cells have been found in the deeper layers of the epidermis in samples from healthy *S. aureus* nasal carriers[118]. Bacteria such as *Clostridium botulinum* hemagglutin bind to E-cadherin and causes disruption of the epithelial barrier even without proteolytic cleavage of E-cadherin[321]. In addition, some adenovirus serotypes bind with Dsg2, causing a transient opening of the epithelial junction due to Dsg2 shedding[322,323]. Whether or not the SdrD-Dsg1 interaction results in Dsg1 cleavage is still elusive and our assay data from preliminary investigations were inconclusive (unpublished results).

Desquamation of the epithelium surface ensures the constant removal of bacteria from the nose. Dsg1 mediate keratinocytes differentiation via several signalling pathway such as suppression of mitogen-activated protein kinase (MAPK) pathway[324] (reviewed in[149]). Furthermore, Dsg1 is part of the corneodesmosomes and its proteolysis is required for efficient corneocytes desquamation[325,326]. Therefore, it is possible that SdrD-Dsg1 interaction aid in the extracellular persistence of *S. aureus* on the epithelial surface by interfering with the terminal differentiation of keratinocyte and desquamation of nasal epithelial surface. Since, *sdrD* expression is upregulated during nasal colonization[187], SdrD- Dsg1 interaction might be important for sustained nasal colonization. However, additional studies are required to confirm this.

Furthermore, bacteria can also target cell-cell junctions to facilitate the clinical manifestations of their infections[327,328]. Staphylococcal Exfoliative toxin (ET) cleaves Dsg1 resulting into loss of cell to cell adhesion between keratinocytes[165,329]. This is responsible for the clinical manifestations observed in staphylococcal scalded skin syndrome (SSSS)[165,329]. Whether the interaction between SdrD and Dsg1 contributes to *S. aureus* infections or diseases is unknown. However, in human blood *S. aureus sdrD* expression is upregulated[189] and also promotes survival of the bacteria in blood[190]. It is tempting to suggest that SdrD-Dsg1 binding and subsequent loss of cell-cell adhesion caused by *S. aureus* ET could facilitate movement of *S. aureus* into systemic circulation, where SdrD subsequently aids in the bacterial survival. Further studies are required to understanding the subsequent events following SdrD-Dsg1 interaction.

8.2 *S. aureus* CWA genes: genetic diversity and expression

Previous studies have evaluated the prevalence of *sdrC*, *sdrD* and *sdrE* genes among *S. aureus* isolates[179,180]. The *sdrC*, *sdrD* and *sdrE* genes are located in tandem arrangement within the *sdr* locus[186]. In **Paper II**, we confirmed the presence of *sdrC*, *sdrD* and *sdrE* genes in *S. aureus* isolated from the anterior nares of healthy adults. However, the prevalence of the genes within these *S. aureus* isolates differs. In agreement with another study[179], we found that *sdrC* gene is almost always present in within the strains. However, in **Paper II**, *sdrD* gene prevalence was lower than that observed by Sabat et al.[179]. Josefsson et al. reported at least two *sdr* genes were always present in all studied *S. aureus* isolates[186]. However, we found that *sdrC* and *sdrE* can occur singly in *S. aureus* isolates (**Paper II**). This might be due to the variations in the techniques adopted in our study and theirs. We have used a PCR approach as used in Sabat et al.[179] while Josefsson et al, have used a Southern blotting approach[186]. Primers used in PCRs are highly specific, therefore, the absence of some of these genes might be due to sequence variation in the genes.

Genetic variation in *fnb*, *hla, spa* etc. genes have previously been found[214,330,331]. Using *S. aureus* NCTC8325-4 as our model strain, we found that SdrD is involved in adherence to keratinocytes (**Paper I**). In **Paper II**, we revealed genetic variability within *sdrD* gene in *S. aureus* strains. These variations were predominantly in the SdrD A domain and R domain. The high sequence diversity observed within SdrD A

domain, is in agreement with previous studies[202,211]. In addition, we observed that variations within *sdrD* were lower within lineages compared between lineages (**Paper II**). This is in agreement with a study by McCarthy and Lindsay[202]. Variations within the sequence of CWA proteins could have functional implications on *S. aureus* colonization and/or infection. For example, a single nucleotide polymorphism (SNP) in FnBPA has been found to be associated with cardiovascular devices infection because of their increased binding to fibronectin[216,332]. In our study, there were significant differences in adhesion of two *S. aureus* expressing the different *sdrD* variants to human keratinocytes (**Paper II**). However, the functional effects of the *sdrD* gene variation are not fully understood yet and thus require additional studies.

Expression of *S. aureus* virulence genes can be altered by the environmental or growth conditions[201,203]. Analyses of virulence genes expression in conditions that represent the host milieu could suggest the importance of the virulence factor in *S. aureus* colonization and/or infection. For example, ClfB is expressed in nose and is major determinant during in nasal colonization of humans[101,175,201]. In **Paper III**, *sasG* expression is upregulated in human blood and SasG expression was induced by serum components. In addition, the protein expression occurred in the stationary phase of the bacterial growth. A previous study has shown that *sasG* is more prevalent in invasive isolates compared to carriage isolates[195]. In our study, SasG did not promote bacterial survival in human blood *ex vivo* (**Paper III**). This suggests that SasG might have some other roles in *S. aureus* invasiveness and/or infection. Further studies are required to delineate the molecular mechanism of SasG's contribution to *S. aureus* virulence.

A study showed that SasG expressing cells had fibrils structures on their surface[198]. These structures have been suggested to mask other *S. aureus* surface adhesins, causing reduced adherence of such cells to extracellular matrix components such as fibronectin and fibrinogen[198]. This could be the reason why SasG expression is delayed until stationary phase (**Paper III**). Perhaps ensuring that the other *S. aureus* surface proteins could carry out their virulence functions before SasG is finally expressed. However, this expression pattern might also be an indication of a yet to be characterized role of SasG in *S. aureus* virulence. Further studies are needed to properly understand this.

Understanding genetic and expression variation in *S. aureus* virulence genes is complex and challenging. Allelic variation in bacterial virulence genes could be some form of adaptation geared towards different host species specificity. This mechanism has been observed in FimH, the type 1 fimbrial adhesin in *Salmonella typhimurium* where certain SNPs drive specificity of the adhesin for either humans or animals host[333]. Though, McCarthy and Lindsay[202] reported no difference in sequences of *S. aureus* surface proteins of animal or human lineages. It is likely that the genetic variations within *S. aureus* virulence genes or lineages represent adaptation to selective pressure such as host immune responses or antibiotic encountered at the within different individuals. Successful *S. aureus* colonization of different hosts has been suggested to be a result of a series of co-evolutionary processes involving the host, pathogen and the environment[57]. Therefore, genetic and expression variations in *S. aureus* CWA proteins as observed in **Paper II** and **III**, might be a means of achieving this.

8.3 *S. aureus* CWA proteins: evasion of host immune response

S. aureus can evade the host immune response in several ways[49]. In **Paper I**, we showed that *S. aureus* SdrD interacts with the host cell adhesion molecule Dsg1. Internalization and cytoskeleton rearrangement have been indicated in Pemphigus, an autoimmune skin disorder caused by antibodies directed against Dsg1 and Dsg3 ectodomains[334,335]. Moreover, interaction between the bacterial surface protein and host adhesion molecules can facilitate their internalization by the host cells (reviewed in[328]). Bacteria such as *Listeria monocytogenes* binds to E-cadherins via its surface protein InlA and the interaction mediate its internalization by the epithelial cell[336]. Intracellular localization protects the bacteria from the host immune sentinels and also reduces the effect of antibiotics on them[337]. This can further aid in persistence of *S. aureus* colonization or recurrent infection[116,119]. Thus, one can speculate that interaction between SdrD and Dsg1 could lead to the endocytosis of the bacteria. *S. aureus* internalization by keratinocytes via FnBP dependent or independent pathway have been reported[338]. Our preliminary data indicated that compared to the isogenic *sdrD* mutant, the presence of SdrD contributed to the internalization by HaCaT cells (unpublished results). Further studies are required to if this is a result of SdrD-Dsg1 interaction.

We found that SasG promotes bacterial aggregation when grown in presence of serum (**Paper III**). *S. aureus* ClfA mediated bacterial aggregation and protected the *S. aureus* from clearance by phagocytes such as neutrophils and macrophages[272,339]. Previous studies indicated that SasG does not contribute to sepsis[340] or abscess formation[192]. However, in mice, SasG contributed to bacterial deposition at the kidney following intravenous inoculation with *S. aureus*[192]. SasG expression caused formation of fibrils and reduced adherence to extracellular matrix[198]. Bacterial aggregation could be a way of moving from the site of infection to other organs in the host and also evading immune evasion mechanisms. Additional studies are ongoing to properly understand this.

In **Paper II**, we reported variations within the *sdrD* gene from different *S. aureus* isolates. In addition, we showed that variations within the SdrD A domain are surface associated (**Paper II**). Microbes can also alter their surface protein via antigenic variation to evade the host immune system[341,342]. For example, variations within *S. aureus* FnBPA and FnBPB A domains have been observed to reduce host immuno-cross reactivity within the different FnBP isotypes[214,215]. In addition, glycosylation of serine aspartate (SD) repeats of CWA proteins protect the protein from neutrophil and macrophages mediated proteolysis[343]. It contributed to ClfA virulence in a bloodstream infection model[344]. It has been suggested that glycosylation increased with the number of SD repeats present[343,344]. We found that variations were also high with SdrD SD repeats (**Paper II**). Although the virulence capabilities of the different *sdrD* variants is not fully understood, it is tempting to speculate that certain variations within the SdrD functional domains of *S. aureus* isolates might contributes to evasion of the host immune defence mechanisms. However, this requires further studies.

9 CONCLUSION

Though our understanding of *S. aureus* interaction with humans has improved greatly since the discovery of the bacteria, there are still a lot of questions regarding the molecular determinants involved. Despite this, *S. aureu*s remains an efficient colonizer and an important human pathogen. Host colonization and/or infection by *S. aureus* is both complex and multifactorial. This study has characterized the role of *S. aureus* cell wall anchored proteins in ensuring its adhesion and/or immune evasion.

We showed that CWA proteins SdrD and SasG mediate bacterial adhesion to host cells (**Paper I**, **II** and **III)**. Furthermore, we identified Dsg1 as the host ligand of SdrD and show that this interaction promotes bacterial adhesion to host cells (**Paper I).** In addition, we found that genetic variations within the *sdrD* gene is concentrated within its SdrD A domain and R domain (**Paper II).** Furthermore, we revealed that expression of *S. aureus* SasG is upregulated in human blood and in the presence of serum components (**Paper III).** However, we found that SasG does not promote bacterial survival in human blood *ex vivo* (**Paper III).**

Our findings are consistent with the multifactorial nature of *S. aureus*-host interaction. We have provided increased knowledge about the molecular mechanism *S. aureus* uses in its interaction with the host. Additional studies into the subsequent events following this interaction is needed in order to further clarify the mechanism used by *S. aureus* to colonize and invade host cells.

In the future, our findings (**Paper I**, **II** and **III)** should help in providing molecular mechanistic knowledge required in developing alternative therapeutics to combat *S. aureus* colonization and/or infection.

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