Novel Adhesive Proteins of Pathogenic Staphylococci and Their Interaction with Host Proteins

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"Flying is learning how to throw yourself at the ground and miss."

Douglas Adams

Abstract

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In this thesis, interactions between bacterial and host proteins have been studied by a phage display approach. Affinity selection of a *Staphylococcus aureus* shotgun phage display library against human von Willebrand factor (vWf), led to the identification of a novel secreted vWf-binding protein (vWbp). Mature vWbp, which consists of 482 amino acids, could be recovered from an *S. aureus* culture supernatant. The specific interaction between vWbp and vWf is mediated by a region of 26 amino acids located in the C-terminal part of vWbp. Further characterisation revealed vWbp to be a bifunctional protein capable of inducing coagulation of plasma from several species, but with preference for human and porcine plasma. The coagulating activity was found to reside in the N-terminal part of vWbp, and to be depended on the interaction with prothrombin. Similarly, panning a *Staphylococcus lugdunensis* phage display library against human vWf resulted in the recognition of a novel vWf-binding protein (vWbl). This protein comprises 2060 amino acids, including a cell wall sorting signal with a surface-anchoring LPXTG-motif. The vWf-binding part of vWbl is located in repetitive domains. The incidence of the genes encoding vWbp and vWbl is very high in clinical isolates of the respective staphylococcal species.

In addition, the *S. aureus* library was sorted against *ex vivo* biomaterial, and six different staphylococcal protein were identified: coagulase, Efb, protein A, FnbpA, FnbpB, and Sbi. Fibrinogen-binding and β_2-glycoprotein I (β_2-GPI)-binding phagemid particles were dominating. Proteins adsorbed to different *ex vivo* central venous catheters were investigated using specific antibodies. Fibrinogen was found to be most abundant, but β_2-GPI was also detected on the investigated biomaterials. This is noteworthy in view of *S. aureus* adherence to biomaterials, but might also implicate a source for induction of disease-causing β_2-GPI-autoantibodies.

vWf is an essential molecule for platelet adherence and aggregation, especially during rapid blood flow. Thus, it is intriguing that two virulent staphylococcal species, both capable of causing life threatening endocarditis, have evolved proteins with affinity for vWf. Perhaps even more intriguing, both vWbp and vWbl presumably bind the same site in vWf, as indicated by cross-inhibition studies. However, the exact role for these staphylococcal proteins concerning staphylococcal pathogenesis remains to be elucidated.

Key words: *Staphylococcus aureus*, *Staphylococcus lugdunensis*, coagulase-negative staphylococci, phage display, vWf, von Willebrand factor-binding protein, vWbp, receptin, prothrombin activation, coagulase, vWbl, adhesin, LPXTG-motif, bacterial adhesion, intravascular device, biomaterial, beta2-glycoprotein I

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Abbreviations

Aap	Accumulation-associated protein
agr	Accessory gene regulator
APC	Antigen-presenting cell
β_2-GPI	β_2-glycoprotein I
Bap	Biofilm-associated protein
Bbp	Bone sialoprotein-binding protein
CHIPS	Chemotaxis inhibitory protein of *S. aureus*
ClfA/B	Clumping factor A/B
Cna	Collagen-binding protein
CoNS	Coagulase-negative staphylococci
CVC	Central venous catheter
ECM	Extracellular matrix
Ebh	ECM-binding protein homologue (*S. aureus*, ORF 31,5 kb)
Efb	Extracellular fibrinogen-binding protein
Emp	ECM protein-binding protein (*S. aureus*, ORF 1,0 kb)
Embp	ECM-binding protein (*S. epidermidis*, ORF 30,5 kb)
ET	Exfoliative toxin
E-tag	Expression screening tag
FAME	Fatty acid-modifying enzyme
Fbe	Fibrinogen-binding protein from *S. epidermidis*
FnbpA/B	Fibronectin-binding protein A/B
ICAM-1	Intercellular adhesion molecule-1
MHC	Major histocompability complex
MRSA	Methicillin-resistant *S. aureus*
ORF	Open reading frame
Pls	Plasmin-sensitive protein
SAG	Superantigen
SAK	Staphylokinase
SasG	*S. aureus* surface protein G
Sbi	*S. aureus* IgG-binding protein
SDR	SD-repeats
SE	Staphylococcal enterotoxin
SSSS	Staphylococcal scalded skin syndrome
TSS	Toxic shock syndrome
TSST-1	Toxic shock syndrome toxin-1
VISA	Vancomycin-intermediate-resistant *S. aureus*
VRE	Vancomycin-resistant enterococci
VRSA	Vancomycin-resistant *S. aureus*
vWbp	von Willebrand factor-binding protein
vWbl	von Willebrand factor-binding protein from *S. lugdunensis*
vWf	von Willebrand factor

Contents

Appendix

Papers I-IV

This thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Bjerketorp, J., Nilsson, M., Ljungh, Å., Flock, J.-I., Jacobsson, K. & Frykberg, L. 2002. A novel von Willebrand factor-binding protein expressed by *Staphylococcus aureus*. *Microbiology 148*, 2037-2044.

II. Bjerketorp, J., Jacobsson, K. & Frykberg, L. 2004. The von Willebrand factor-binding protein (vWbp) of *Staphylococcus aureus* is a coagulase. *FEMS Microbiol. Lett.* (In press).

III. Nilsson, M., Bjerketorp, J., Wiebensjö, Å., Ljungh, Å., Frykberg, L. & Guss, B. 2004. A von Willebrand factor-binding protein from *Staphylococcus lugdunensis*. *FEMS Microbiol. Lett.* (In press).

IV. Bjerketorp, J., Rosander, A., Nilsson, M., Jacobsson, K. & Frykberg, L. Sorting a *Staphylococcus aureus* phage display library against *ex vivo* biomaterial. (Submitted).

Introduction

"When you take stuff from one writer it's plagiarism;
but when you take it from many writers, it's research."

Wilson Mizner

General background

Of all living organisms, microbes are the most diverse products of evolution. Life itself stems from these microscopic beings, and together bacteria constitute the majority of all life. They have adapted to environments as diverse as boiling wells, ice and snow, the Dead Sea, bare rock, airborne particles, and upon or within living creatures. The norm is that you find bacteria wherever on earth you look. Consequently, bacteria exhibit an outstanding range of metabolic capabilities, but more than 99% of the existing bacterial species are still not possible to cultivate.

Even though the full range of bacterial species remains unknown, many are intimately involved in health and disease of humans and animals. The human body is colonised by bacteria at an approximate ratio of ten bacteria per human cell. Most are commensals, which benefit from living associated with us without causing harm. They might even protect us from disease by competing for nutrients with pathogenic bacteria. Disease-causing bacteria can be obligate pathogens, always causing disease, or opportunistic pathogens. The latter are normally commensals until some factor in the host-bacteria relationship changes, like malnutrition of the host, or if the bacteria enter an erroneous location in the host.

At the beginning of the last century, infectious diseases were the leading cause of death worldwide. However, this all changed due to better standards of living, the accidental discovery of penicillin by Fleming, and the many more antibiotics that followed. The development and use of antibiotics greatly reduced the death toll by bacterial diseases. A few decades ago, the battle against bacterial infections was generally believed to be won. However, the adaptive bacteria responded to the increased selective pressure introduced by the use and over-use of antibiotics. The microbes started to develop resistance against many antibiotics, and once more, bacterial infections emerged as a severe threat on the global scene. Some examples of important human bacterial pathogens are species of *Bacillus*, *Borrelia*, *Campylobacter*, *Clostridium*, *Enterococcus*, *Escherichia*, *Haemophilus*, *Helicobacter*, *Legionella*, *Mycobacterium*, *Neisseria*, *Salmonella*, *Staphylococcus*, *Streptococcus*, *Vibrio* and *Yersinia*, of which several show increasing resistance against many commonly used antibiotics.

The genus *Staphylococcus*

The focus of this thesis is on *Staphylococcus aureus*, the key member of the genus *Staphylococcus* with regard to human disease. Also, the more infrequently encountered opportunistic pathogen *S. lugdunensis*, and some other staphylococci

are discussed to some extent when considered appropriate. Staphylococci are Gram-positive, *i.e.* protected by a thick cell wall, composed of layers of peptidoglycan and additional teichoic, and lipoteichoic acid. They are coccoid, *i.e.* spherical in shape, and with a diameter of 0,5-1,5 µm, non-motile, non-sporulating, facultativly anaerobic, catalase-positive, highly salt- and lipid-tolerant, and their DNA contains 30 to 40% of guanine and cytosine residues. Staphylococci divide in more than one plane and are found as single cells, in pairs, tetrads, short chains, grape-like irregular clusters, or in a biofilm. Staphylococci were seen in human pus by Koch 1878, cultured by Pasteur 1880 and subsequently named by the Scottish surgeon Ogston. Staphyle denotes a bunch of grapes in Greek. They were formally described as *S. aureus* and *S. albus* by Rosenbach in 1884, based on the golden and white colour of the colonies, respectively. Since then, *S. albus* has been renamed to *S. epidermidis*, and today 36 species of staphylococci are recognised (Götz, Bannerman & Schleifer, 2004).

The genus is divided into coagulase-positive and coagulase-negative staphylococci (CoNS). This division is based on the occurrence of coagulase, or staphylocoagulase, an extracellular protein that has the ability to cause blood and plasma from various animals to clot. Only a few staphylococcal species are coagulase-positive and in clinical practise, coagulase-positive equals *S. aureus*, since this is the only coagulase-positive species colonising humans. Further, *S. aureus* is considered the most virulent of the staphylococci, and consequently the main pathogen. All members of the large and heterogeneous group of CoNS are in general significantly less virulent than *S. aureus*. Some CoNS, noted as important human pathogens, are still fairly virulent, *e.g. S. lugdunensis* and *S. epidermidis* (Lambe *et al.*, 1990). Staphylococci are normally found on skin and mucous membranes of mammals and birds. *S. aureus* is found in the nose of more than 30% of the healthy human population and *S. epidermidis* is found on the skin of all humans. The natural habitat of *S. lugdunensis* appears to be in the inguinal area of humans (van der Mee-Marquet *et al.*, 2003). At least 14 staphylococcal species are known to colonise man in different frequencies (Kloos & Bannerman, 1994). They are spread in hospitals and communities by direct contact, but also by indirect contact, due to the ability of staphylococci to survive for long periods in the relatively dry environment outside the host.

Staphylococci and disease

The infection process

Newly transmitted staphylococci have to adhere to the cells of the new host in order to colonise. If then an infection is to take place, the next task for the staphylococci is to pass the physical barriers of the host. The most frequent portals of entry are the sites where the comparatively dry, acidic and impermeable skin meets the moist and soft mucous membranes. Whenever the barriers are in any way injured, as during surgery, excellent entry sites are provided. Inside the body the staphylococci can once again attach to a suitable surface and establish a primary site of infection. As long as the staphylococci can evade the host immune system, they will multiply and spread, either directly through tissues, or via the

lymphatic system to the bloodstream causing septicemia. Staphylococci are now able to reach remote sites and cause a variety of clinical manifestations and diseases. The typical pathological finding of staphylococcal infections due to *S. aureus* is abscess formation. Patients with *S. aureus* septicemia are rarely cured unless the abscesses are drained. It is noteworthy that before the antibiotic era, the typical patient with *S. aureus* septicemia was young, previously healthy, had developed the infection from a superficial skin lesion, and died in more than 80% of the cases (Sjursen, 1999).

Staphylococcus aureus

S. aureus causes a broad range of diseases in humans, ranging from superficial skin infections, *e.g.* impetigo, to life threatening deep-seated infections, *e.g.* endocarditis (Lowy, 1998). Other examples, where *S. aureus* stands out as a serious pathogen, are soft-tissue, respiratory, bone, and joint infections, and in fact, *S. aureus* infections can involve any organ system. The wide variety of diseases caused by *S. aureus* is a result of its extensive spectrum of virulence factors, discussed in more detail later in this thesis. Several diseases caused by *S. aureus* involve toxin production, including impetigo and the much more severe form of disseminated skin disease named staphylococcal scalded skin syndrome (SSSS), as well as toxic shock syndrome (TSS) and food poisoning.

Staphylococcus lugdunensis and other CoNS

As highlighted in several case reports, *S. lugdunensis* infections are not very common, but can be aggressive in its nature and possibly, this is the staphylococcal species that comes closest to *S. aureus* with regard to virulence (Etienne *et al.*, 1989). *S. lugdunensis* has been reported to cause endocarditis, biomaterial-associated infections, septic arthritis, meningitis, and wound infections (Fleurette *et al.*, 1989; Kaabia *et al.*, 2002). Still, little is known why this species has the potential to cause more aggressive infections than other CoNS. *S. schleiferi* also seems to share some resemblance to *S. lugdunensis* and *S. aureus* concerning virulence. However, when it comes to the more numerous foreign body infections, *S. epidermidis* is the primary pathogen. Other important causative agents of infections in conjunction with indwelling medical devices are *S. aureus* and the CoNS as a group, quite often represented by *S. haemolyticus*, *S. capitis*, *S. hominis*, or *S. warneri* (Kloos & Bannerman, 1994).

Pathogens of medical progress

The number of staphylococcal infections has increased noticeably over the past decades. In part, this increase is due to an improved inclination to report cultures of CoNS as true pathogens, but in addition, a factual increase of the infection incidence is generally recognised. *S. aureus* and CoNS, have been reported as the most common pathogens of nosocomial, or hospital-acquired, infections, if urinary tracts infections are excluded (Emori & Gaynes, 1993). This is even more marked among the reported nosocomial bacteremias caused predominantly by CoNS, but also frequently by *S. aureus*, as noted in a recent review (von Eiff, Peters &

Heilmann, 2002). This trend parallels the increased use of intravascular devices, and there are reports that more than half of the *S. aureus* bacteremias are associated with such devices (Steinberg, Clark & Hackman, 1996).

Modern medical practice continues to employ a rising number of artificial devices, both for permanent and more temporary use, *e.g.* artificial heart valves and central venous catheters, respectively. When a foreign material is implanted in the body, it interacts with the surrounding tissue and body fluids, and the surface of the implant is rapidly covered with different blood proteins. This adsorption depends in part on the physiochemical nature of the implanted material, and much effort has been spent on improving the inherent characteristics of biomaterials with regard to, *e.g.* tissue compatibility. The process of coating is dynamic, and the composition of adhered proteins will change over time, when the initially adsorbed proteins are replaced, or covered by other proteins (Courtney *et al.*, 1994; Vroman *et al.*, 1980). The adsorbed proteins can cause different problems such as activation of the coagulation system, and inflammation. Further, the insertion site is easily contaminated with staphylococci, and the coated biomaterial will promote their adherence. Once adhered, staphylococci readily colonise the biomaterial and establish an infection, which is difficult to cure, due to the formation of bacterial communities known as biofilm (von Eiff, Peters & Heilmann, 2002). This consists of multiple layers of bacteria that have embedded themselves in slime, *i.e.* a hydrated matrix made of extracellular polysaccharides and proteins. Biofilms attract a lot of interest since most bacteria probably live in biofilms on different suitable surfaces, which apparently include implanted medical devices (Schachter, 2003). The removal of the implant on which a biofilm has formed is usually required in order to cure this otherwise highly persistent form of infection. The ever-increasing use of biomaterials in modern medicine has given staphylococci in general, and CoNS in particular, the opportunity to step forward as the pathogens of medical progress (Rupp & Archer, 1994).

Antibiotic resistance

Another aspect of modern medicine is the use of antibiotics to combat bacterial infections. However, due to abuse of this miracle drug, the effectiveness of antibiotic treatment is waning. In clinical practice, most often the causative agent of an infection is not determined, instead broad-range antibiotics are used. Also, too often antibiotics are wasted on common colds caused by viral infections, which are not affected by antibiotics. Further, huge amounts of antibiotics are given as a growth enhancer in the feed of farm animals, enormous amounts of antibiotics are applied in agriculture as pesticides, and hundreds of everyday household products now contain antibacterial agents (Levy, 2001). Besides, many antimicrobial substances are intrinsically stable, and are found in the environment long after the actual antibiotic treatment. This massive onslaught of antibacterial compounds exerts a strong selection pressure on the resourceful microorganisms. The selection pressure is ingeniously met by the spread of existing resistance determinants, and the development of new ones.

The bacterial genomes are by no means fixed entities. A bacterium has the potential to quickly multiply, in an optimal environment in as short time as 20

minutes, and coupled to this short generation time is the possibility to mutate. Further, a bacterium has the ability to acquire foreign DNA elements with useful resistance genes in numerous ways, via conjugative plasmids, transposons, bacteriophages, or as naked DNA, directly from the surroundings. Many of these mobile elements, like the broad host range plasmids, have the capacity to move freely, not only between bacteria of the same species, but also between different species and genera. Besides, several resistance determinants are often occurring together on mobile elements, and this certainly facilitates the widespread distribution of multiple resistance genes among bacteria (Rice, 2000).

The current situation for nosocomial staphylococcal infections in some parts of the world, *e.g.* USA is that close to 60% of *S. aureus* isolates are resistant to methicillin (MRSA) (Anonymous, 2003). The corresponding figure for CoNS is close to 90%. Due to their nosocomial origin, the isolated staphylococci often carry resistance determinants against several important antibiotics. Vancomycin has for decades been the most important drug against these infections. However, vancomycin resistance appeared many years ago in some CoNS (Schwalbe, Stapleton & Gilligan, 1987). MRSA with heterogeneous or intermediate vancomycin resistance (VISA) also started to emerge some years ago (Hiramatsu *et al.*, 1997). Further, resistance to vancomycin is often found in clinical isolates of enterococci (VRE), and the transfer of the gene, *vanA*, encoding the vancomycin resistance determinant, to *S. aureus* has been observed in the laboratory (Noble, Virani & Cree, 1992). In 2002, the long anticipated reports appeared, describing the first cases of clinical isolates of *S. aureus* resistant to vancomycin (VRSA) (Anonymous, 2002a, b). For a relatively long time, no new types of antimicrobial compounds have been developed, with the exception of oxazolidinone, which is a novel type of protein synthesis inhibitor with activity against Gram-positive bacteria (Diekema & Jones, 2001). Some cases of resistance to linezolid has already been reported, both in VRE and in MRSA (Tsiodras *et al.*, 2001). If also these resistance determinants against vancomycin and linezolid become widespread among the hospital isolates on a global scale, the future situation for treatment of the nosocomial staphylococcal infections is grave.

Another clinically important aspect of bacterial resistance is the fact that bacteria in biofilms, *e.g.* on an indwelling medical device, are intrinsically less susceptible to antibiotics (Donlan, 2001). Microbes in a biofilm are 10 to 1000-fold less sensitive than planktonic, or free-floating, microbes, with regard to the same antimicrobial agent. As an example, in order to kill biofilm-living cells of *S. aureus* with chlorine, an oxidizing biocide considered as one of the most efficient antibacterial agents, a 600-fold higher dose is needed, compared to the dose that kills planktonic *S. aureus* (Luppens *et al.*, 2002). One explanation to biofilm resistance could be the adsorption of some antibiotics due to charge interactions with the biofilm-matrix, which might result in a slow or incomplete penetration of the antibiotics into the deeper layers of the biofilm (Stewart & Costerton, 2001). Another hypothesis focuses on the altered microenvironment within the biofilm, where the more actively growing and antibiotic-susceptible surface bacteria of the biofilm-community consume most of the nutrients available. This renders the deeper parts of the biofilm, where waste products might accumulate, more acidic and anaerobic. On the whole these deeper located biofilm dwellers are dormant

and non-growing, and as a result, less susceptible to antibiotics compared to the surface bacteria. An additional theory is the existence of a unique, and highly protected biofilm phenotype, where a sub-population of biofilm microbes express biofilm-specific genes, that induce changes in the morphology and biochemistry of these bacteria compared to planktonic bacteria (Costerton, Stewart & Greenberg, 1999; Davies, 2003). The observed phenomenon of biofilm resistance is likely to be of complex nature, and stems probably from a combination of some of the mechanisms proposed, and others, still to be found.

Antibiotic resistance that is caused by acquired resistance genes, is best escaped by removing the selective pressure promoting this carriage, an objective that will only be achieved through prudent use of antimicrobial substances. An alternative route to combat antibiotic resistance is the search for novel compounds that act upon the bacteria, preferably both in the planktonic and biofilm state, and without selecting for resistance, a task yet to be accomplished. Also a vaccine would be active against antibiotic-resistant bacteria, and a vaccine that targets staphylococcal adherence might also prevent the problem of biofilm formation, but the vaccine approach have so far proved difficult with regard to staphylococci.

Virulence factors

Capsule

The capsular polysaccharides are important surface components, forming a glycocalyx. It has been reported that more than 90% of *S. aureus* isolates produce one of 11 serologically distinct capsular polysaccharides (Sompolinsky *et al.*, 1985). The most common capsule types among clinical isolates of *S. aureus*, are the serotypes 8 and 5, found in 50% and 25% of the isolates, respectively. The presence of a capsule is thought to enhance staphylococcal virulence by impairing phagocytosis. Capsules have been shown to modulate adherence of *S. aureus* to endothelial cells *in vitro*, and to promote colonisation and persistence on mucosal surfaces *in vivo* (O'Riordan & Lee, 2004).

Peptidoglycan

The major cell wall component of *S. aureus*, peptidoglycan, is known to cause proinflammatory responses and platelet aggregation in the host, ultimately leading to disseminated intravascular coagulation in cases of severe septicemia (Kessler, Nussbaum & Tuazon, 1991). Peptidoglycan has also been reported to induce tissue factor expression in monocytes, and thereby activate the coagulation cascade, which will be discussed in more detail later in this thesis (Mattsson *et al.*, 2002).

Lysylphosphatidylglycerol

The most common bacterial membrane phospholipids, phosphatidylglycerol and diphosphatidylglycerol, are negatively charged. This makes bacteria susceptible to cationic antimicrobial peptides of the host defence, discussed later on in this thesis. However, some pathogens like *S. aureus* can modify their phospholipids by attaching positively charged lysine residue. This results in lysylphosphatidyl-

glycerol, which repels the cationic peptides and thus, can confer resistance to *S. aureus*. The gene product responsible for this modification of phospholipids is termed MprF and has been reported to be a virulence factor (Peschel *et al.*, 2001).

Receptins

Receptin denotes any microbial protein with affinity for a mammalian protein (Kronvall & Jönsson, 1999). The term receptin, parallels the term lectin, which is defined as a microbial protein with affinity for carbohydrates, a topic of great importance when studying adhesion of Gram-negative bacteria. Another term often used is adhesin, describing the determinant that provides adhesion, or attachment of cells, both prokaryotic and eukaryotic, to various structures and surfaces. An additional quite broad term is extracellular matrix-binding proteins, or ECMBPs, denoting soluble and surface-associated bacterial proteins binding to some component of the extracellular matrix (ECM) of the host (Flock, 1999). A related, but more narrow term is microbial surface components recognizing adhesive matrix molecules, or MSCRAMMs for short (Patti *et al.*, 1994).

Since this thesis deals both with secreted and cell wall-anchored staphylococcal proteins that bind to host proteins found in the blood, or associated with the extracellular matrix, the term receptins will be used throughout this thesis. *S. aureus* encodes a large number of receptin, many of which are well-studied. Much less is known about this class of molecules in other staphylococci, but the general view is that there are fewer to be found in other staphylococcal species compared to *S. aureus*. However, most *S. aureus* virulence factors, or equivalents probably also exist among the CoNS, but scattered between the different species. This thesis describes a novel surface-associated receptin of *S. lugdunensis*, and a novel secreted receptin of *S. aureus*, with many interesting features.

Secreted proteins

The secretory pathway, trafficked by most secreted proteins, recognises functional secretory signal peptides with certain characteristics. These comprise a few positively charged residues in the N-terminal of the protein to be secreted, followed by a central hydrophobic region, and a more polar region, which also includes the signal peptidase cleavage sequence, often involving alanines at the -3 and -1 positions (von Heijne, 1986). *S. aureus* produces a multitude of secreted proteins that fit into at least one of the categories toxin, enzyme, or receptin. Staphylococcal toxins are obvious virulence factors and they occur in many forms. One of the most striking features revealed about *S. aureus*, when the genome of two strains were sequenced a few years ago, was the existence of a large number of toxin genes (Kuroda *et al.*, 2001). About 20 toxin genes were known at that time, and another 21 putative toxin genes were found. A great diversity of superantigen (SAG) genes were noted, and it is assumed that gene duplication frequently occurs, resulting in a very large number of different SAGs at the global scale. By contrast, the only toxin genes found in the recently released genome of *S. epidermidis*, were those for β- and δ-hemolysin (Zhang *et al.*, 2003). Most strains of *S. aureus* secrete the commonly known cytotoxins, or hemolysins, designated α, β, γ, and δ, causing destruction of blood and tissue cells of the host

(Dinges, Orwin & Schlievert, 2000). α-toxin is hemolytic, cytotoxic, dermonecrotic, and one of the most potent toxins known (1 μg is often lethal to a rabbit). Erythrocytes, mononuclear immune cells, epithelial and endothelial cells, as well as platelets can be punctured by α-toxin. Monomers of this toxin insert either directly or via a high-affinity ligand into the lipid bilayer of a host cell, and polymerise to form cylindrical pores, usually with seven monomers each. The role of β-toxin is not clearly understood, but it acts as a type C phosphatase, hydrolysing sphingomyelin in the host cell membranes. It is often the most abundant protein in *S. aureus* culture supernatants, particularly of animal isolates, sometimes yielding 20 mg per litre. Leukocidin, or Panton-Valentine leukocidin, and γ-toxin belong to the family of two-component toxins. They are so closely related that the different γ-toxin and leukocidin units can be combined, thus forming different mixed bicomponent toxins, all capable of efficient lysis of leukocytes. Their role in staphylococcal disease remains unclear. This is also the case for δ-toxin, a small 26 amino acid peptide encoded by a part of the regulatory RNAIII of the accessory gene regulator (*agr*) system, which will be discussed briefly later in this thesis. Most staphylococci produce δ-hemolysin. It resembles the bee venom mellitin, and the mode of action of δ-toxin has been compared to the disruptive effect of detergents on cell membranes.

Common food poisoning occurs when staphylococcal enterotoxins (SEs), preformed in food contaminated with *S. aureus*, are ingested. If a localised *S. aureus* infection instead distributes the SEs systemically, they will act as SAGs, a group of powerful, pyrogenic and immunostimulatory exotoxins. Staphylococcal SAGs also include toxic shock syndrome toxin-1 (TSST-1), and they all induce a massive T-cell proliferation. SAGs may stimulate 5-30% of all T-cells, when present in concentrations measured in picogram ml^{-1}, while a normal antigen activates less than 0,01% of the T-cells under the same conditions. This highly unspecific T-cell stimulation is achieved by the simultaneous binding of a SAG to the T-cell antigen receptor and to the constant domains of a major histocompability complex (MHC) class II molecule of an antigen-presenting cell (APC), bypassing the need for any antigen to be processed and presented. This will lead to TSS manifested through an overwhelming release of host cytokines and inflammatory mediators, resulting in capillary leakage, hypotension, shock, multi-organ failure, and eventually death in approximately 5% of the cases. After the initial activation of the T-cells, most are eliminated through apoptosis and others become anergic, unable to respond to stimuli for a prolonged period of time. It is easy to envision that the role *in vivo* of the SAGs, must be to inhibit host immune responses to *S. aureus*. The main SAGs of staphylococci are TSST-1, responsible for menstrual TSS, and SE serotypes B and C, which together with TSST-1 are responsible for most nonmenstrual TSS (McCormick, Yarwood & Schlievert, 2001).

Both in bullous impetigo and SSSS the typical flaking blisters of the superficial epidermis are caused by the action of staphylococcal exfoliative toxin (ET) (Farrell, 1999; Melish, Glasgow & Turner, 1972). The occurrence of similar symptoms, in an autoimmune disorder called pemphigus foliaceus, was explained some years ago (Mahoney *et al.*, 1999). This work, in conjunction with an observed similarity between ETs and the serine protease superfamily, solved the

enigma of skin peeling in SSSS, and led to the identification of desmoglein 1 as the target of ET (Amagai *et al.*, 2000; Rago *et al.*, 2000). Desmosomes are the major type of intercellular adhesive junctions, and desmoglein 1 is a member of the cadherin family of calcium dependent cell-to-cell adhesion molecules, and the only desmosomal glycoprotein expressed in the upper epidermis. Consequently, when desmoglein 1 is specifically cleaved by the protease activity of ETs (either A, B, or D), the superficial layer of the skin detaches, leaving deeper granular layers exposed and highly susceptible to secondary infections (Amagai, 2003).

Even though some toxins obviously are enzymes, these are generally not grouped with the staphylococcal exoenzymes, *e.g.* lipases, proteases, and nucleases. Exoenzymes are considered to be secondary virulence factors, whose main function is to convert local host tissues into nutrients required for staphylococcal growth. These proteins are found both in *S. aureus* and CoNS, *e.g. S. epidermidis* and *S. lugdunensis*. Certain lipases, fatty acid-modifying enzymes (FAME), from different staphylococcal species, have the potential to inactivate the bacteriocidal lipids found in staphylococcal abscesses (Arvidson, 2000). Various proteases can, besides provide nutrients, inactivate important host proteins, as exemplified by the V8 serine protease that can cleave all human immunoglobulin classes, thus putatively impairing the humoral host defence against the bacteria. Hyaluronidase, or more specifically hyaluronate lyase, is produced by *S. aureus*, but not by *S. epidermidis*. This exoenzyme is considered to contribute more to virulence than other, as it disrupts hyaluronic acid, one of the central components of the extracellular matrix, thus facilitating for *S. aureus* to penetrate into tissues.

Secreted receptins are generally regarded as virulence factors. One important example is the extracellular fibrinogen-binding protein (Efb), which has been shown to be a virulence factor that delays wound healing in a rat model of *S. aureus* infection (Palma *et al.*, 1996). Other important secreted receptins are coagulase and staphylokinase (SAK), which are sometimes considered as exoenzymes. However, since they are not true enzymes, but instead receptins, that bind and activate host enzymes through complex-formation with these, they will be discussed in the following paragraphs.

Coagulase is a bifunctional receptin, with the capacity to bind fibrinogen via several C-terminal repeats, besides its well-documented ability to cause blood and plasma from several mammals to clot (Bodén & Flock, 1989). Coagulase is secreted by most strains of *S. aureus*, and is known to form an equimolar reactive complex with prothrombin (Hemker, Bas & Muller, 1975; Kawabata *et al.*, 1985). The normal physiological activation mechanism of the zymogen prothrombin is by enzymatic cleavage into thrombin, the key effector enzyme in the blood coagulation cascade, which is described in more detail later in this thesis. Thrombin subsequently cleaves fibrinogen into fibrinopeptides and fibrin, which results in fibrin clotting, the ultimate product of coagulation. However, coagulase does not cleave prothrombin into thrombin, instead it acts as a cofactor, which induces a conformational change in prothrombin, resulting in an active complex, which also converts fibrinogen into fibrin. Virulence studies have been performed in different animal models with an isogenic *S. aureus* mutant, in which the gene encoding coagulase has been deleted. In two murine models of subcutaneous and

intramammary infection, no diminished virulence was noticed (Phonimdaeng *et al.*, 1990). When the same mutant was used in a different mouse model of blood-borne staphylococcal pneumonia, a possible role for coagulase was suggested in the later stages of infection (Sawai *et al.*, 1997). Although the role of coagulase in pathogenesis is not resolved, the mechanism of prothrombin activation was recently explained in detail, when the crystal structure of coagulase in complex with thrombin was determined (Friedrich *et al.*, 2003). This is discussed in more detail later on, as the novel secreted von Willebrand factor binding-protein (vWbp) of *S. aureus*, described in this thesis, also is a coagulase.

As with coagulase and prothrombin, a reactive equimolar complex is also formed between SAK and plasmin(ogen), the host target of SAK (Collen, 1998). Plasminogen, the precursor form of plasmin, is very effectively activated by the SAK-plasmin complex. Activated plasmin is a broad spectrum serine protease that degrades fibrin clots and ECM proteins, except collagens. Instead, plasmin can activate latent procollagenases and other proteolytic enzymes, which in turn cause the degradation of collagens and ECM proteins in concert with plasmin. An assumed role for SAK during an infection is to release staphylococci trapped in fibrin clots, and to support bacterial metastasis, thereby promoting virulence and spread of bacteria to new sites in the host (Lähteenmäki, Kuusela & Korhonen, 2000). Apart from *S. aureus*, some CoNS, *e.g. S. lugdunensis*, are reported to produce SAK (Sawicka-Grzelak *et al.*, 1993). Evidence of SAK as a virulence factor of staphylococci is still lacking, but a large number of invasive bacterial pathogens express different receptins that use the host plasmin(ogen) system, thus enabling them to degrade host tissues, a feature assumed to contribute substantially to bacterial virulence (Lähteenmäki, Kuusela & Korhonen, 2001).

Recently, a secreted chemotaxis inhibitory protein of *S. aureus* (CHIPS) was reported (De Haas *et al.*, 2004). This putative receptin specifically impairs neutrophil chemotaxis towards excreted bacterial formylated peptides (FP), and C5a derived from the host complement system, both potent chemoattractants present during staphylococcal infections. The ligands of CHIPS are not yet identified, but possible candidates are the receptors for the above mentioned chemoattractants, FPR and C5aR. This suggestion was made since CHIPS was observed to bind specifically to neutrophils and monocytes expressing these receptors, and in amounts corresponding to the known levels of receptor-expression by these cell types. The gene coding for CHIPS was isolated from *S. aureus* strain Newman, and was found to be located on the same bacteriophage as the genes encoding SAK, and also SEA, inserted into the chromosomal gene for β-toxin. As both SAK and SEA, which show pronounced human-specific action, CHIPS inhibits chemotaxis of human cells 30-fold more efficiently, compared to mouse cells (Dohlsten *et al.*, 1993; Gladysheva *et al.*, 2003; De Haas *et al.*, 2004).

Another very interesting secreted receptin, almost exclusively built up of repeated 110 amino acids domains, has been independently isolated and studied by different groups. Therefore the same protein is known as p70, and as the MHC class II analogous protein (Map), as well as the extracellular adhesion protein (Eap) (Jönsson *et al.*, 1995; Palma, Haggar & Flock, 1999; Yousif *et al.*, 1994). In a recent review, the numerous possible functions of this multifunctional protein

were outlined (Harraghy *et al.*, 2003). Most of the produced Eap is secreted from the bacteria, and has been reported to bind several different host plasma proteins, *e.g.* fibrinogen, fibronectin, and prothrombin. Eap can also form oligomers and bind back to several structures on the surface of *S. aureus*. Thus, Eap could aid *S. aureus* adhesion to *i.e.* an indwelling medical device coated with host proteins, as well as promote intercellular aggregation of *S. aureus*. Further, Eap can facilitate both adherence and internalisation of *S. aureus* to, and into, epithelial cells and fibroblasts. Another target for Eap is ECM proteins and Eap also binds and blocks an endothelial cell receptor, intercellular adhesion molecule-1 (ICAM-1), which thus prevents the extravasation of leukocytes, making Eap like CHIPS, an anti-inflammatory agent (Chavakis *et al.*, 2002). The affinity of Eap for ICAM-1 might also disturb APCs in their interaction with T-cells, and moreover, Eap induces apoptosis, when interacting with T-cells (Lee *et al.*, 2002). Especially the immuno-suppressing properties may be of significance for the outcome of an *S. aureus* infection. In one study, none of the mice infected with an *S. aureus* strain lacking Eap developed heart abscesses eight weeks post-infection, whereas almost 60% of the mice that received wild-type bacteria did (Lee *et al.*, 2002). Taken together, this implies that Eap is an important virulence factor of *S. aureus*, at least in chronic infections. Eap exemplifies very well the inherent complexity and biological importance of this heterogeneous family of secreted receptins, that appears to have several independent, but to some extent overlapping, or even opposed functions, which in the end are elaborately interwoven.

Cell surface associated proteins

The partial redundancy and complexity described above is definitely also found among the rich repertoire of receptins, which are secreted and subsequently seized at the staphylococcal cell surface. Many surface proteins are anchored to the cell wall by a mechanism, that requires a C-terminal cell wall sorting signal, usually consisting of a conserved LPXTG-motif, followed by a hydrophobic domain and a tail of mostly positively charged residues (Mazmanian, Ton-That & Schneewind, 2001). The LPXTG-residues of an exported protein are recognised by sortase, an enzyme on the outside of the bacterial membrane, which cleaves the protein between the T- and G-residues, and subsequently anchors the protein covalently to the pentapeptide cross-bridge of the cell wall peptidoglycan (Mazmanian *et al.*, 1999). From analysis of staphylococcal genomes sequenced so far, at least 22 *S. aureus* , and 11 *S. epidermidis* surface proteins, have this motif (Mazmanian, Ton-That & Schneewind, 2001; Ponnuraj *et al.*, 2003; Roche *et al.*, 2003). The method of anchoring surface proteins via LPXTG, or an equivalent motif, is found to be very wide-spread among Gram-positive bacteria, and more than one sortase gene is usually found within each bacterial species (Pallen *et al.*, 2001). For example, *S. aureus* has a second sortase, that recognises a different and to *S. aureus* unique NPQTN-motif situated on a protein of so far unknown function but of importance for staphylococcal persistence in infected tissue (Mazmanian *et al.*, 2002). *S. aureus* sortase-deletion mutants failed to process and display this type of surface proteins, and in mouse models, the virulence of the mutants was found to be attenuated (Jonsson *et al.*, 2002, 2003; Mazmanian *et al.*, 2000). In staphylococci, many of these surface proteins anchored by sortase have been

studied, and much is known with regard to their binding capacities, while others of still unknown function are regarded as putative receptins.

Protein A, the now classical prototype of a cell wall-associated receptin, was studied when the sorting mechanism to the cell wall was elucidated in *S. aureus* (Schneewind, Model & Fischetti, 1992). Protein A is primarily recognised because of its specific binding of the Fc-domain of IgG via five homologous domains, named E, D, A, B, and C, which are composed of ~60 amino acids each (Sjödahl, 1977; Uhlén *et al.*, 1984). The binding of IgG in a non-immunogenic manner, appears to disguise the bacteria, and thereby protect them from phagocytosis by host immune cells. Further, protein A was recently demonstrated to bind von Willebrand factor (vWf), and also the protein gC1qR, *e.g.* found on the surface of adhered platelets (Hartleib *et al.*, 2000; Nguyen, Ghebrehiwet & Peerschke, 2000). Consequently protein A may also serve as an adhesin. Animal model experiments with isogenic *S. aureus* mutants, without the gene encoding protein A, have confirmed this receptin to be a virulence factor (Gemmell *et al.*, 1997; Patel *et al.*, 1987). Since this thesis describes two novel staphylococcal receptins with affinity for vWf, this will be discussed in more detail later on.

Recent data, derived from crystal structure determinations, implicate that the structural organisation of staphylococcal cell wall-associated receptins, may be better represented by the fibrinogen-binding "clumping factors" (ClfA and B), from *S. aureus*, and by the fibrinogen-binding protein Fbe, also known as SdrG, from *S. epidermidis* (Hartford *et al.*, 2001; McDevitt *et al.*, 1994; Ni Eidhin *et al.*, 1998; Nilsson *et al.*, 1998; Ponnuraj *et al.*, 2003). The N-terminal surface-directing signal peptide is followed by a fairly large and non-repetitive A-domain, where the fibrinogen-binding is located. After the A-domain follow the characteristic SD-dipeptide-repeats (SDR), which force the receptin to stretch away from the surface of the bacteria, due to the alternating hydrophilic and negatively charged amino acids. Finally, next to the SDR, the C-terminal cell wall sorting signal is found. In the SdrG/Fbe protein, two B-repeats of so far unknown function are located between the A-domain and the SDR. Another four surface-associated proteins from *S. aureus* are also included in the SDR-family of putative receptins. These are SdrC, SdrD, SdrE, and the plasmin-sensitive protein (Pls) (Josefsson *et al.*, 1998; Savolainen *et al.*, 2001). In *S. epidermidis*, SdrF, and the untypical and LPXTG-less SdrH have been recognised (McCrea *et al.*, 2000; Mazmanian, Ton-That & Schneewind, 2001). For all these, perhaps excepting SdrE, which is ~85% identical to the bone sialoprotein-binding protein (Bbp), the function is unknown (Tung *et al.*, 2000). Recent work on the mechanism of ligand-binding by SdrG/Fbe has revealed a structural model that is applicable on many surface-associated receptins of staphylococci and other Gram-positive bacterial species (Ponnuraj *et al.*, 2003). The crystal structure of the binding region of SdrG/Fbe, with and without ligand has been solved, and a dynamic "dock, lock, and latch" mechanism was suggested. The A-domains of many surface-anchored receptins are composed of two or three IgG-like folds, and in a cleft between two of the domains, short linear sequences of their respective ligands are recognised, and bound. Following this docking of the ligand, a β-strand of one of the IgG-like domains passes over the bound polypeptide, thereby locking it in place. Finally, the β-strand inserts itself as a latch into a gap between

two of the β-strands of the other domain, thus stabilising the overall structure. The previously observed conserved sequence motif, TYTFTDYVD, constitutes the back of the gap where the latching strand inserts itself (Josefsson *et al.*, 1998; McCrea *et al.*, 2000; Ponnuraj *et al.*, 2003). Also, a conserved structural feature, with alternating small residues, which face the larger side chains of the TYTFDYVD-motif, is observed in the latching β-strand. Preliminary crystal structures and computer modelling place nine *S. aureus*, and five *S. epidermidis* surface receptins in this novel family, sharing the dock, lock, and latch mechanism of ligand binding. In *S. aureus* the members of this group of receptins are ClfA and B, the fibronectin- and fibrinogen-binding proteins (FnbpA and B), SdrC, SdrD, SdrE, and Bbp, and the collagen-binding protein, Cna (Jönsson *et al.*, 1991; Patti, Boles & Höök, 1993; Signäs *et al.*, 1989). Like SdrG/Fbe, with the exception of ClfA and B, these receptins have a few repeated B-domains after the A-domain, where additional ligand binding might occur. A major feature of FnbpA and B is their fibronectin-binding D-repeats, next to the cell wall sorting signal. However, based on data from NMR spectroscopy an extended tandem β-zipper model was recently proposed for the binding between several sequential domains of fibronectin, and β-sheets of FnbpA, both from within and outside the D-repeats (Schwarz-Linek *et al.*, 2003).

Despite their proposed shared dock, lock, and latch mechanism of ligand-binding, these receptins obviously have distinct properties, *e.g.* with regard to their preferred ligands and different effects exerted upon the host. For example, ClfA binds the γ-chain of fibrinogen in a manner that inhibits adhesion of platelets to immobilised fibrinogen, indicating that the binding sites for an important platelet integrin and this staphylococcal receptin overlap (McDevitt *et al.*, 1997). SdrG/Fbe binds and blocks the β-chain of fibrinogen (Davis *et al.*, 2001). This interferes with fibrin clot formation and release of fibrinopeptide B, which otherwise act as chemoattractants on leukocytes. ClfB binds both the α- and β-chain of fibrinogen (O'Brien *et al.*, 2002b). In addition, ClfB binds to cytokeratin 10, which is found in the exposed nasal epithelium, and thus ClfB is believed to be a major determinant in nasal colonisation and carriage of *S. aureus*. Animal model studies on some types of *S. aureus* infections have established ClfA and Cna as virulence factors (Patti *et al.*, 1994; Hienz *et al.*, 1996; Moreillon *et al.*, 1995; Rhem *et al.*, 2000). FnbpA and B may play an important role in the fibronectin-dependent internalisation of *S. aureus*, even though *S. aureus* is mainly regarded as an extracellular parasite (Fowler *et al.*, 2000; Joh *et al.*, 1999). The internalisation is probably achieved by the formation of a fibronectin bridge between Fnbps and integrins on non-phagocytic host cells, in which the staphylococci may find a protected niche. Further, FnbpA was recently demonstrated to inhibit fibrin assembly during thrombin-induced clot formation (Matsuka *et al.*, 2003). Also, FnbpA was shown to be a functional substrate for coagulation factor XIII, which stabilise fibrin clots by introducing covalent bonds between fibrin molecules. This finding indicates that *S. aureus* might be able to attach covalently to fibrinogen and fibronectin under certain conditions.

The above described surface proteins are known to mediate adhesion of *S. aureus* to various host structures. The picture is a little more complex for the intriguing protein Pls, which instead prevents adhesion of intact *S. aureus* to

ligands like fibronectin, even when the fibronectin-binding adhesins are present (Hildén *et al.*, 1996; Savolinen *et al.*, 2001). The gene coding for Pls is not found in all *S. aureus* isolates, but is closely associated with the *mecA* gene coding for a penicillin-binding protein in MRSA (Ito *et al.*, 2001). The expression of Pls thus explains the puzzling report on *S. aureus* strains defective in the adhesion to fibronectin and fibrinogen when a *mecA* element was present, even though the cell wall-associated receptins that bind these ligands were found in normal amounts (Vaudaux *et al.*, 1998). Structurally, Pls does not have the TYTFDYVD-motif discussed above, but has a few repeated B-domains between the A-domain and SDR, and also several short repeats between the signal peptide and the A-domain. Pls is homologous to *S. aureus* surface protein G (SasG), and the accumulation-associated protein (Aap) from *S. epidermidis*, which is of importance for the biofilm formation following the initial adhesion event (Hussain *et al.*, 1997; Roche, Meehan & Foster, 2003). Pls has also been reported to promote bacterial aggregation and moreover, to bind lipids (Huesca *et al.*, 2002). The SasG gene was found to positively correlate with isolates of *S. aureus* causing disease. However, no protein ligand has yet been found, neither for Pls, nor for Aap, but both Pls and SasG were found to promote bacterial adherence to nasal epithelial cells (Roche, Meehan & Foster, 2003). Preincubation with Pls, SasG or Aap inhibited this binding, indicating a common ligand for these adhesins, found to share a conserved domain in the A-domain. This conserved domain was also found in yet another LPXTG protein, SasA, exhibiting an untypical SX dipeptide repeat region. As mentioned earlier, Pls is sensitive to the proteolytic action of plasmin, and the cleavage site is immediately N-terminal of the conserved domain reported to bind nasal epithelium. It appears that bacteria expressing Pls can under certain conditions prevent adhesion and under other conditions promote adhesion. Possibly cleavage of Pls are involved in the regulation of these different qualities. Another surface anchored protein, the biofilm-associated protein (Bap), is composed of a relatively short non-repeated A-domain, which is preceded by a couple of short repeats, and followed by numerous longer repeats (Cucarella *et al.*, 2001). Bap is found in some strong biofilm producing bovine isolates of *S. aureus*, and is involved in adherence to inert surfaces, and intercellular adhesion of staphylococci. In an foreign body mouse infection model, Bap was found to be involved in the pathogenesis of persistent infections. Further, expression of Bap prevented initial attachment of *S. aureus* (Cucarella *et al.*, 2001, 2002).

Iron is an essential component for bacterial growth, and some genes are expressed when free iron is limited. This occurs during infection, since the iron inside the host is bound to different host proteins, like transferrin, haptoglobin, or hemoglobin. In this context four genes encoding additional putative receptins of *S. aureus* have recently been described, three thereof in close proximity to each other. These are the iron-responsive surface determinants, IsdA and IsdB, both containing the LPXTG motif, and IsdC, which contains the alternative sorting motif NPQTN mentioned previously (Mazmanian *et al.*, 2002). In the same operon as the gene encoding IsdC are the gene for the second sortase and genes encoding a ferrichrome transporter. An alternative name for IsdA is StbA, staphylococcal transferrin-binding protein, which more clearly describes the function of this receptin, and yet another name is FrpA, for fur-regulated protein A (Morrissey

et al., 2002; Taylor & Heinrichs, 2002). Recently, IsdA was reported to possess a distinct domain with broad-spectrum ligand-binding activity recognising fibrinogen and fibronectin (Clarke, Wiltshire & Foster, 2004). IsdB is reported to bind hemoglobin, and even though IsdC binds heme-iron, the function of IsdC is not yet clear (Mazmanian *et al.*, 2003). Separated from this gene cluster, is the fourth iron-regulated gene encoding a LPXTG-receptin, IsdH or HarA, which recently was demonstrated to bind haptoglobin (Dryla *et al.*, 2003).

Several receptins present on the surface of *S. aureus* lack the LPXTG motif but may still serve as adhesins. Among these is the second IgG-binding protein in *S. aureus* (Sbi), which also binds to β_2-glycoprotein I (β_2-GPI) (Zhang *et al.*, 1998, 1999). Sbi does not have the typical cell wall sorting signal and membrane spanning domain, but in the C-terminal end Sbi has a region with proline residues repeated every fifth amino acid, which is similar to other cell wall spanning domains (Uhlén *et al.*, 1984; Guss *et al.*, 1986). Further, an *S. aureus* receptin binding elastin, EbpS, has been reported, which does not even possess an N-terminal signal peptide, but is instead an integral membrane protein (Downer *et al.*, 2002; Park *et al.*, 1996). Yet another class of staphylococcal surface receptins has been proposed, the autolysins/adhesins, which show both autobacteriolytic enzymatic activity and affinity for host ligands. The autolysins/adhesins from *S. epidermidis* are Aae, demonstrated to bind vitronectin, fibrinogen and fibronectin, and AtlE, which binds to polymers, and vitronectin (Heilmann *et al.*, 1997, 2003). Proteins homologous to AtlE from other species of staphylococci are reported to bind fibronectin (Allignet *et al.*, 1999; Hell, Meyer & Gatermann, 1998). Another receptin, closely associated with the cell surface, is a recently described protein with a broad affinity for fibrinogen, fibronectin, collagen and vitronectin, named ECM protein-binding protein (Emp) (Hussain *et al.*, 2001). Also, a major portion of Eap, previously mentioned under secreted proteins, binds back to the surface of *S. aureus*, and the surface-located neutral phosphatase has been identified as one docking ligand for Eap (Yousif *et al.*, 1994; Flock & Flock, 2001). Surface located receptins are sometimes found to be otherwise well-known proteins, which do unknown things at a surprising location. Plasmin(ogen) binding receptins, in both streptococci and *S. aureus*, were recently revealed to be the α-enolase, known as an intracellular key enzyme in the glycolytic pathway (Mölkänen *et al.*, 2002; Pancholi & Fischetti, 1998). This is at first perplexing, but since glycolytic enzymes developed very long ago, it is not that odd that some have evolved extra functions. In fact, an increasing number of these moonlighting proteins are found to perform more than one function (Jeffery, 1999).

The most remarkable proteins recently reported are the giant fibronectin-binding proteins of *S. aureus*, and *S. epidermidis*, encoded by ORFs ~31,5 and ~30,5 kb in size, respectively (Clarke *et al.*, 2002; Williams *et al.*, 2002). The one in *S. aureus* is called Ebh, for ECM-binding protein homologue, and the other in *S. epidermidis* is called Embp, for ECM-binding protein, and at least one part, corresponding to 30% of both proteins predicted sequences, is identical (Zhang *et al.*, 2003). Ebh is predicted to be a surface protein of ~1100 kDa, and has been extracted from the cell wall fraction of lysed *S. aureus* bacteria. However, no size marker is available to estimate the actual size of the mature protein. As possible means to attach to the

bacterial surface, Ebh has in its C-terminal part a putative membrane-spanning domain and a putative peptidoglycan-binding repeat region, similar to those found in other Gram-positive species (Foster, 1993). Ebh has three repeat regions following the signal peptide and the non-repeated A-domain. The first, and third region only have a couple of repeats each, while the second consists of over 40 repeats, each 126 amino acids long, thus constituting the major part of this huge protein. These repeats confer the observed fibronectin-binding capacity, and show homology to a major adhesin, an ECM-binding protein of a *Streptococcus* species, hence the name Ebh (Manganelli & van de Rijn, 1999). The coding sequence is present in the reported complete genome sequences of *S. aureus*, but it appears that a frameshift mutation has separated the coding sequence into two ORFs in some *S. aureus* strains (Kuroda *et al.*, 2001). Analysis of mRNA from *S. epidermidis* also revealed that the gene coding for Embp was expressed (Williams *et al.*, 2002). Binding studies on Embp indicated that the fibronectin-binding is separated from the site in fibronectin where FnbpA and B of *S. aureus* bind. Why some staphylococci express such large surface-associated receptins is currently an open question, but since they exist, some advantage must be gained.

Gene regulation

Since staphylococci can exist as commensals, causing disease is merely one of several possible lifestyles of these facultative pathogens. Thus, the virulence genes and the factors they express simply represent an adaptation to one specific environment, the one inside the hostile host. Also the differentiation into a protected biofilm community and back to planktonic bacteria again whenever suitable, might be regarded as another state of existence, which promotes staphylococcal survival in the host. When affronted with the numerous aspects of staphylococcal virulence, where the many potentiating factors comprise *e.g.* toxins, exoenzymes, both secreted and surface-associated receptins, it is obvious that the precise regulation of the genes encoding the virulence determinants is crucial. The accessory genes involved in pathogenesis are collectively known as the virulon. The correct sub-set of the virulon is expressed due to the action of an extensive regulatory network, which orchestrates the amount of expression of any given gene, at the appropriate time, location and nutritional status of the bacteria. Most of the work on virulence gene regulation in staphylococci has been done on *S. aureus*. Some important players identified include four two-component systems, *agr*, *Sae*, *arlRS* and *SrrAB*, an alternative stress-induced sigma factor, σ^B and numerous transcription factors, *e.g. SarA* and its homologues (Novick, 2003).

As an example of an important virulence gene regulation mechanism the *agr* system will be discussed to some extent. The identification of a global regulator, *agr*, which affects the expression of most of the accessory genes, was a central advance in the field of staphylococcal virulence gene regulation research (Morfeldt *et al.*, 1988; Peng *et al.*, 1988; Recsei *et al.*, 1986). The *agr* locus contains two divergent transcription units, RNAII and RNAIII. Under laboratory conditions, these are expressed during exponential phase in a manner dependent on bacterial density through quorum sensing, *i.e.* when the extracellular concentration of an autocrine octapeptide has accumulated to a certain level. The

RNAII transcript contains *agrB*, *D*, *C*, and *A* genes, in that order. The *agrD* gene encodes the propeptide AgrD, which is converted into the octapeptide, and subsequently secreted. In this process, the membrane protein AgrB, encoded by *agrB*, is likely to participate. The *agrC* and *agrA* genes encode a classical two-component signal transduction system, where AgrC is the membrane-bound receptor for the autoinducing octapeptide, and AgrA is the cytoplasmic response regulator, which is required for the induced expression of both transcripts in the *agr* operon. The RNAIII transcript encodes the δ-hemolysin, but it is the RNAIII molecule, which is the effector of *agr* regulation. The RNAIII has a complex secondary structure with 14 hairpin loops, but the mechanism by which RNAIII regulates transcription is unknown. The net effect of RNAIII expression is that the genes encoding cell surface receptins, *e.g.* protein A, which are produced during early exponential phase, are down-regulated. Instead, genes encoding secreted toxins, *e.g.* α-toxin, and exoenzymes are switched on, and consequently these proteins are mainly produced during post-exponential growth. In the light of *agr* regulation, a model situation of a localised infection can be envisioned where newly adhered staphylococci, thus present at a low density, need their surface adhesins to hold on to the tissue. As the bacterial density and the octapeptide concentration increase extracellularly, the available nutrients are likely to decrease. At some point, the autoinducing octapeptide initiates the switch, resulting in a staphylococcal phenotype expressing, among other things, proteases capable of degrading both the surrounding tissues to release nutrients, and the anchoring adhesins of the staphylococci. This makes it possible for staphylococci to escape from the present location, to again up-regulate the expression of adhesins, and to seed into new sites. As indicated earlier, this and other effects are not by any means achieved solely by the regulatory action of the *agr* locus, but by the combined inputs from the extensive regulatory network as a whole.

The *agr* locus is widely distributed among the staphylococcal species, and several subtypes of the locus are even found within a species (Dufour *et al.*, 2002). However, considerable sequence variation is found in the components AgrB, C and D from different species, subspecies, and subtypes. The consequence of this variety is that the octapeptide, which is autoinducing for one species or subtype, is instead inhibiting for other staphylococci (Ji, Beavis & Novick, 1997; Otto *et al.*, 2001). Competition and staphylococcal cross communication appear to occur on our skin between, *e.g. S. aureus* and *S. epidermidis*, and this peptide argumentation seems to generally favour the existence of *S. epidermidis*. The importance of *agr* in staphylococcal virulence is confirmed in animal models, where *agr* mutants are less virulent (Abdelnour *et al.*, 1993; Gillaspy *et al.*, 1995). However, conflicting data are reported on the importance of *agr* expression during infections (Goerke *et al.*, 2000, 2001). The *in vivo* expression of *agr*, and some genes known to be *agr*-regulated *in vitro*, were examined by reverse transcription-PCR. From the observed expression patterns the *agr* activity appeared nonessential for the expression of α-toxin. Further, low levels of protein A expression was observed in spite of low levels of RNAIII. Instead, the mutation of *sae* resulted in severe downregulation of α-toxin expression, and thus, also other regulatory circuits than those characterised *in vitro* might be used by *S. aureus* during infections.

Host factors

Immune defence

The host protection scheme against microbial infections includes an innate part, and an acquired, or adapted response (Figure 1). The innate part includes both the physical, chemical and microbiological barriers, and the innate branch of the immune system. The very first line of defence consists of the intact skin and mucous membranes, which generally are impermeable to infectious agents. A range of anti-microbial factors contributes to the barrier function, *e.g.* low pH secretions containing lactic acid and fatty acids. Also, several antimicrobial peptides are found in sweat, or on epithelial surfaces, like dermcidin, β-defensins and cathelicidin (Nizet *et al.*, 2001; Schittek *et al.*, 2001). Further, lysozyme is secreted into the tear fluids, and so is secretory IgA that is encountered very early in the defence of the mucous membranes, although IgA is a component of the acquired immune system, discussed in the next paragraph. In the lower respiratory tract, ciliated cells efficiently remove airborne bacteria entrapped in the mucus in a process called the mucocilliary escalator. Also the abundant resident microbial flora should be considered as part of the innate defence barrier against pathogens, both through competition for available nutrients, and by the production of antimicrobial compounds directed against other species.

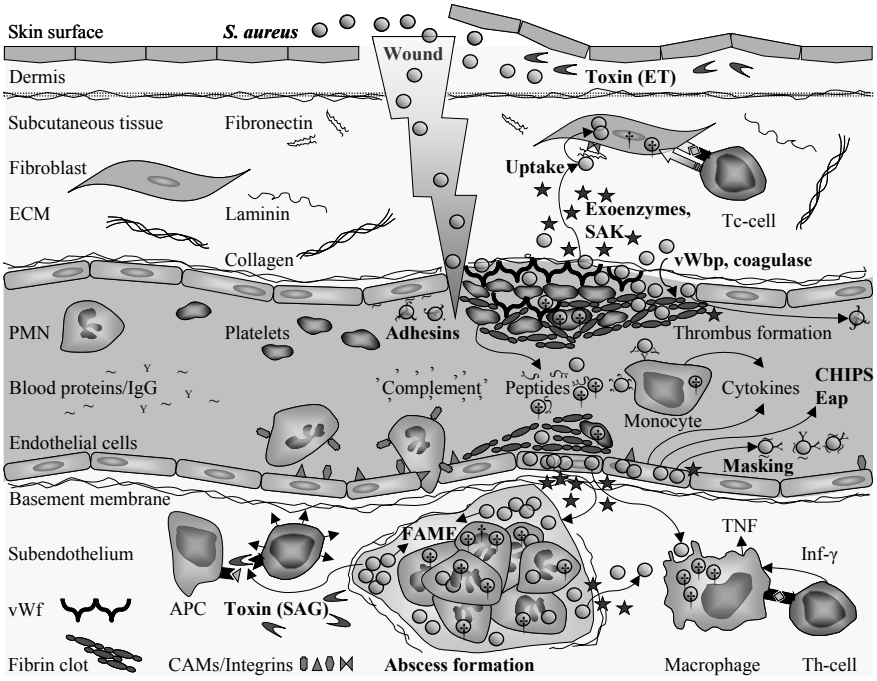


Figure 1. The host protection scheme against bacterial infections includes both innate and adapted immunity for which ***S. aureus*** has several countermeasures (indicated in bold). When the skin barrier is breached, bacteria enter the wound. If the wound is shallow, staphylococci might not be able to penetrate further into the subcutaneous tissue, but local production of **ET** can cause skin blistering. If the wound extends deeper to also include blood vessels, this will initiate hemostasis and the innate immune system through thrombus formation. Via vWf, platelets adhere to the collagen in the basement membrane of the subendothelium, become activated and aggregate. The coagulation cascade is also activated, leading to fibrin clot formation. Activated platelets release chemokines, which attracts leukocytes, as well as antimicrobial peptides that kill bacteria (indicated by †). Bacteria can also be phagocytosed and killed by platelets. Thrombin, the key effector enzyme of the coagulation cascade, activates platelets efficiently and converts fibrinogen into fibrinopeptides and fibrin, which attract leukocytes and polymerise into a clot, respectively. *S. aureus* uses **adhesins** to attach to host molecules like vWf and fibrin(ogen). Other receptins like **vWbp** and **coagulase,** initiate coagulation without converting prothrombin into thrombin, thereby possibly avoiding release of platelet peptide. The expression of **SAK**, instead lead to the degradation of the fibrin clot and release of *S. aureus*. **Exoenzymes** which degrade ECM can also be expressed by *S. aureus*, thus facilitating the spread through host tissue. **Uptake** of *S. aureus* by non-professional phagocytic host cells, like fibroblasts and endothelial cells, occurs through indirect interaction via a fibronectin bridge, formed between *S. aureus* adhesins and host cell integrins. The presence of *S. aureus* in blood activates circulating complement, which opsonises the bacteria and attracts leukocytes. Also antibodies in the blood can opsonise *S. aureus*, leading to ingestion of bacteria by professional phagocytes like monocytes, resulting in the release of different cytokines. This will cause upregulation of cell adhesion molecules (CAMs), both locally on endothelial cells and on recruited circulating leukocytes like polymorphonuclear neutrophils (PMNs). *S. aureus* can express proteins like **CHIPS** and **Eap** which inhibit the recruitment and extravasation of leukocytes, respectively. To avoid being opsonised and engulfed, *S. aureus* can bind different blood proteins *e.g.* antibodies, in a non-immune manner, thus **masking** themselves from phagocytes. *S. aureus* that have spread into the tissue often give rise to **abscess formation** in which the PMNs are less effective in killing staphylococci. The bacteria can produce **FAME** in order to protect themselves from bacteriocidal lipids. Bacteria that escaped from the abscess, can be ingested and killed by phagocytes like macrophages, which present bacterial antigens for Th-cells from the adaptive immune system. These will make macrophages more efficient in their killing, and cause them to release proinflammatory molecules. Th-cells also activate B-cells to produce specific antibodies. Even intracellular bacteria can be killed by Tc-cells. *S. aureus* sometimes produces **SAGs**, that stimulate T-cells in an unspecific manner, leading to massive cytokine release, severe inflammation, and possibly **TSS**. See text for further details and references. Adapted from Lowy (1998).

The second line of defence is formed by the innate immune system, which comprises phagocytes, complement, cytokines and acute phase proteins (Parkin & Cohen, 2001). Two main types of phagocytes are encountered circulating in the blood, macrophages and polymorphonuclear neutrophils, which will act when physical barriers are breached. Neutrophils are the dominant type of white blood cells, although quite short-lived, while macrophages are more long-lived cells of monocyte lineage. During the very early stages of infection, activated macrophages release cytokines, which will stimulate division of precursor cells from the bone marrow, resulting in elevated levels of neutrophils. Chemoattractants and proinflammatory molecules cause neutrophils, and also other leukocytes, to home to the infected site, where they attach through upregulated adhesion molecules, present both on the leukocytes and on the cells of the infected tissue. Most of the tasks executed by the phagocytes, can also be performed by platelets. This is discussed later on in connection with the

coagulation process. Phagocytes have receptors recognising structures specific to microbes termed pathogen-associated molecular patterns, *e.g.* lipoteichoic acid. Phagocytes engulf microorganisms, forming intracellular vesicles that fuse with cytoplasmic granules with toxic content, which kills the microbes by a respiratory burst of oxygen radicals. This process is 100-fold more efficient if the microbe first is tagged, or opsonised, with specific antibodies or complement, for which the phagocytes have receptors. Some encapsulated organisms are protected from phagocytosis, unless opsonised. The complement system is activated in a cascade-like manner and has multiple roles, *e.g.* acting as chemotactic and bacteriolytic compounds. Proinflammatory cytokines and bacterial products cause cells in general to produce particular cytokines, called inflammatory chemokines, which act as chemoattractants with longer duration than *e.g.* complement. Inflammation causes swelling of the affected tissue, allowing easier access for the recruited phagocytes. Collectively, these defence mechanisms are unspecific.

The third line of defence, the antigen-specific immunity needs some time, measured in days, or even weeks, in order to gain momentum, but in the later phases of infections it is of major importance as it is adapted to the present infectious agent. The adaptive part of the immune defence consists of a cell mediated immune response through T-lymphocytes divided into T-cytotoxic (Tc) and T-helper (Th) cells, and an antibody-mediated immune response through B-lymphocytes (Parkin & Cohen, 2001). Those T- and B-cells that specifically recognise a foreign antigen are activated to undergo proliferation. T-cells destined to become Tc-cells must have an antigen presented bound to a MHC I molecule on a nucleated cell harbouring an intracellular infectious agent, and if the T-cell receptor recognises the antigen it may become a Tc-cell capable of killing infected cells, or intracellular pathogens (Kaufmann, 1999; Stenger *et al.*, 1999). T-cells meant to be Th-cells must instead recognise an antigen presented bound to a MHC II molecule on an APC in order to become a Th-cell. A subpopulation of the Th-cells can activate macrophages, and in this way help killing the pathogens. Another subpopulation of the Th-cells supports the activation of B-cells, that recognise the antigen specifically. Activated B-cells are transformed into plasma cells, which secrete huge amounts of specific antibodies, that most often can neutralise their antigens and stimulate phagocytosis. Memory cells formed after an infection will cause a stronger and faster acquired immune response, if the infectious agent is encountered another time.

However, sometimes a encountered pathogen is not so easily defeated. The hallmark of an *S. aureus* infection is abscess formation. An abscess can form *e.g.* when *S. aureus* enters into the host tissue, but without being able to penetrate into the circulation. This gives rise to a localised infection, screened off by the formation of a capsule of ECM components produced by the host in order to contain the infection, and thus prevent it from reaching the circulation. Immune cells, predominantly neutrophils, will be recruited to a localised infection through chemoattractants, and these cells can enter into the abscess and ingest the bacteria. However, once abscesses have formed, they tend to be chronic in nature, and neutrophil localisation and chemotaxis have been reported to be impaired at this stage (Bamberger, Bettin & Gerding, 1987; Bamberger *et al.*, 1989). Moreover, neutrophils have a diminished capacity of neutralising staphylococci in the abscess

milieu, which probably contributes to the fact that the bacteria often persist inside an abscess until it is drained (Bamberger & Herndon, 1990).

Extracellular matrix

The ECM comprises any molecule secreted and immobilised outside cells. Accordingly, ECM is an extremely complex and varying mixture of proteins, glycoproteins, proteoglycans, and hyaluronic acid, which provides both structure and function. ECM contributes most significantly to the different and unique structure of each type of tissue in our body (Reichardt, 1999). The components in ECM can broadly be divided into fibrous elements, *e.g.* collagen and elastin, into link proteins, *e.g.* fibronectin and laminin, and into space filling molecules, like glycosaminoglycans. These molecules, in company with many other, provide tissues with three-dimensional structure, tensile strength, and attachment sites for cells. Further, ECM holds a reservoir of signalling factors, *e.g.* cytokines and growth factors, that modulate as diverse functions as inflammation, cell migration, orientation, differentiation, proliferation, angiogenesis, wound healing and immune responses. ECM is a dynamic scaffold for tissue morhpogenesis, maintenance, and reconstruction of tissue following an injury (Badylak, 2002). The most abundant proteins in ECM are different types of collagens, which together constitute ~1/3 of the total protein content in mammals. In the ECM underneath the barriers posed by the skin and mucosal layers in the body, mainly type I collagen is found. The ECM forming the basement membrane, directly beneath the endothelial cells of blood vessels, is instead mainly composed of collagen type IV and laminin. As long as the tissues are intact, these are major obstacles that an invading pathogen has to penetrate to reach the blood circulation.

Platelets and coagulation

In order for the host to maintain the vital hemostasis and to defend itself against pathogens, a self-sealing defence mechanism has evolved that responds to any vascular damage, and also to bacteria entering the vasculature. This system of blood coagulation follows a complex scheme comprising an extensive interplay between vascular endothelium, platelets, plasma proteins promoting coagulation, other proteins inhibiting coagulation, and fibrinolytic proteins (Pasi, 1999). These components are ready to be rapidly activated, but normally the system is balanced in favour of anticoagulation to assure the free flow of blood in the vascular system. Following a blood vessel injury, the first response is an immediate vasoconstriction, both of the damaged vessel and of vessels in adjacent tissues, thus reducing the blood flow into the damaged area. At the point of damage, the endothelial cells no longer cover the subendothelial ECM, which instead is exposed to the components of the blood. A couple of crucial proteins in the blood will bind to the uncovered ECM, predominantly to collagen, and also to specific integrin receptors on the circulating platelets, thus serving as interconnecting adhesion molecules. The bridging proteins, *i.e.* vWf and fibrinogen, will in concert contribute to the initial adherence and the subsequent aggregation of platelets, but to a varying degree depending on the velocity of the blood within the damaged vessel (Ikeda *et al.*, 1991; Savage, Saldivar & Ruggeri, 1996). Since these proteins

are of central importance for this thesis, they will be discussed in some detail in the following paragraphs in the context of hemostasis.

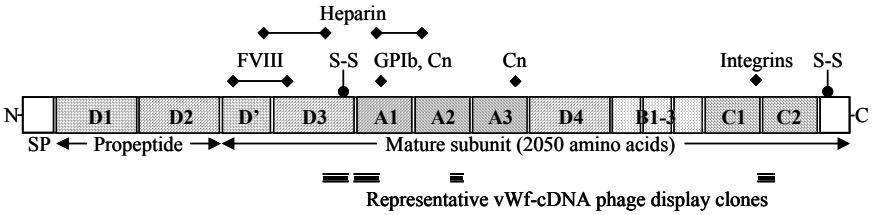


Figure 2. Schematic representation of vWf with the different repeated domains indicated (A-D), and some of their binding capacities specified above: FVIII, factor VIII; GPIb, glycoprotein Ibα; Cn, collagen; Integrins, *e.g.* $\alpha_{IIb}\beta_3$. Abbreviations: N, N-terminal; SP, signal peptide C, C-terminal; S-S, disulphide bridges responsible for multimerization and dimerization. Indicated below are clones representative for the four major groups of clones isolated when a vWf-cDNA phage display library was panned against polyclonal anti-vWf antibodies, as discussed later in this thesis. Adapted from Vischer & de Moerloose (1999).

Endothelial cells produce vWf, which is stored in intracellular granules called Weibel-Palade bodies, and secreted both into the ECM, and into the blood, which holds a vWf-concentration of ~10 μg ml^{-1}. Megakaryocytes, the cell type responsible for platelet production, synthesise the vWf stored within the α-granules of platelets (Vischer & de Moerloose, 1999). The mature form of vWf is a large multifunctional glycoprotein, consisting of 2050 amino acids arranged in four different types of repeats, which are denoted A-D (Figure 2). Homodimers of vWf, ~540 kDa in size, are most often combined to multimers of different sizes, the largest up to several thousand kDa (Ruggeri, Dent & Saldivar, 1999). One important function of vWf is to bind and protect factor VIII of the blood coagulation cascade from degradation. Further, vWf is absolutely essential for the initial attachment of platelets during rapid arterial blood flow, even though fibrinogen also participates in the platelet aggregation that follows, which results in the formation of the primary hemostatic plug (Ruggeri, Dent & Saldivar, 1999; Matsui *et al.*, 2002). vWf mediates platelet adhesion through two distinct platelet receptors, the glycoprotein Ibα in the glycoprotein Ib-V-IX complex and the glycoprotein IIb-IIIa, also called integrin $\alpha_{IIb}\beta_3$. The former interaction causes the platelets to slow down and roll across the subendothelial surface. This transient type of interaction activates the integrin $\alpha_{IIb}\beta_3$, causing platelets to bind irreversibly to the RGD-sequence of vWf. During the process, platelets become activated, which results in degranulation and a dramatic change in shape, features that promote further platelet aggregation and activation.

Parallel to the process of platelet plug formation, the circulating components of the blood coagulation cascade are activated from their resting pro-forms into activated serine proteases, assembled in complexes at the lipid surfaces derived from the inner leaflet of activated platelets (Lentz, 2003). This is mainly initiated through an integral transmembrane protein called tissue factor, found in abundance on the surface of vascular cells, but also to some extent on nonvascular cells. The tissue factor protein on damaged cells binds to the circulating factor VII, some of which already exists in an active form. This particular complex formation triggers coagulation by cleaving the factors IX and X into their active states, IXa

and Xa (Butenas & Mann, 2002; Dahlbäck, 2000). The factors IXa and Xa bind the activated factors VIIIa and Va, respectively (see below), and thus form the complexes which activate factor X and prothrombin into Xa and thrombin, respectively. Thrombin is the principal effector enzyme of the coagulation cascade (Figure 3). Most importantly, thrombin causes the conversion of fibrinogen into fibrin by removal of the highly anionic fibrinopeptides A and B. This modification exposes otherwise hidden positively charged binding structures with affinity for negatively charged sites on neighbouring molecules (Doolittle, 2003). This causes fibrin monomers to rapidly polymerise into a fibrin clot, the gel-like network that reinforces the primary platelet plug and hopefully stops the bleeding. Other vital functions of thrombin concern activation of platelets, and the feedback amplification through activation of several factors involved in the coagulation cascade, *e.g.* factors V, VIII, and also XI that activate factor IX (Dahlbäck, 2000; Butenas & Mann, 2002). Further, thrombin activates factor XIII, which catalyses the reaction that covalently cross-links fibrin molecules, thus hardening the initially soft fibrin clot. The coagulation system is under tight regulatory control through the serine protease inhibitors termed tissue factor pathway inhibitor and antithrombin, as well as by the activation of protein C on intact endothelial cells. Activated protein C inactivates the active factors Va and VIIIa by proteolytic degradation. Recently β_2-GPI, was demonstrated to inhibit the activation of factor XI (Shi *et al.*, 2004). In order to ensure unimpeded blood flow, a fibrinolytic system exists, that will degrade and remove any surplus deposited fibrin (Collen & Lijnen, 1991). This system consists of inactive plasminogen, the plasminogen activators, *e.g.* tissue plasminogen activator, plasmin, the active fibrinolytic serine protease, and plasmin inhibitors. This system will ultimately act to remove the temporary repair patch, the fibrin clot, as the wound heals beneath it.

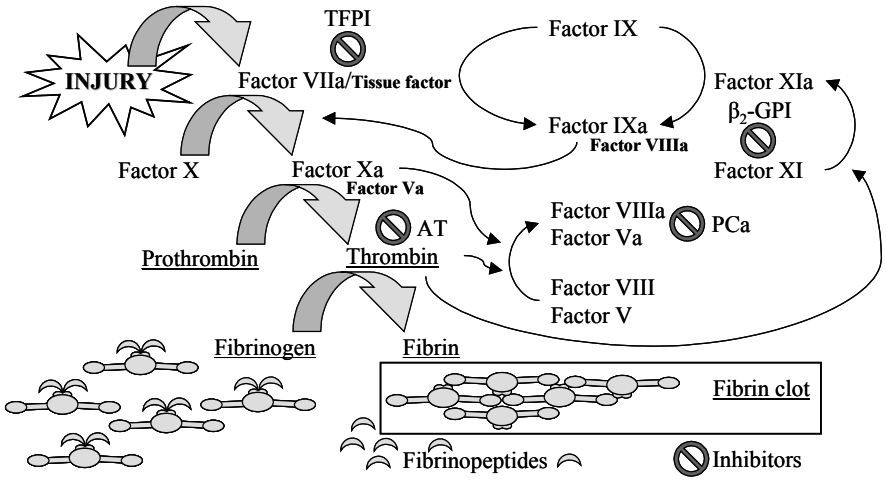


Figure 3. A simplified outline of the revised coagulation cascade. The most central components with regard to this thesis are underlined. Tissue factor and factors Va and VIIIa act as cofactors (shown in bold), which greatly enhance the enzymatic rate. The inhibitors indicated are: TFPI, tissue factor pathway inhibitor; β_2-GPI, β_2-glycoprotein I; AT, antithrombin; PCa, activated protein C. See text for details. Adapted from Pasi (1999).

Platelets have multiple roles, one of which is to participate in the antimicrobial host defence. They circulate in abundance and respond to chemotactic stimuli. Consequently they are an important part of the innate immune defence at sites of microbial colonisation of vascular endothelium (Yeaman & Bayer, 1999). Platelets have receptors and granules comparable to leukocytes, and are capable of internalising microbial pathogens. Further, they generate antimicrobial oxygen metabolites, and upon activation by thrombin, they release various antimicrobial peptides (Tang, Yeaman & Selsted, 2002). Platelets aid the recruitment of leukocytes to a vascular injury, and in conjunction, they form a defence against pathogenic bacteria, *e.g.* staphylococci, that might enter the bloodstream through a wound. However, despite these host defence efforts, staphylococci are sometimes able to evade the immune system, colonise and cause septicemia.

Other factors

How an opportunistic pathogen like *S. aureus* is met by the host depends on many factors. The course and outcome of an infection will be affected by the general condition of the host, including factors as nutritional status, age, underlying disease, general immune status, and more specifically, if the host has been infected by this organism before, and if that infection gave rise to a protective acquired immunity. Generally, humans have antibodies directed against antigens from staphylococci as a result of colonisation. However, it is not certain that the immune response was of such quality that it is able to protect the host during a subsequent infection, since in spite of antibodies elicited in response to an invading pathogen can be of protective, non-protective, or even disease-enhancing character (Casadevall & Scharff, 1995). In the case of *S. aureus*, antibodies raised often seem to be non-protective, since the presence of specific antibodies, people get reinfected. Further, *S. aureus* can affect immune cells of the host in several ways, some of which are discussed earlier in this thesis.

One thing that seems to greatly influence the risk of acquiring an *S. aureus* infection, *e.g.* during surgery, is the nasal carrier state (Herwaldt, 2003). Some people are persistent carriers of *S. aureus*, some are intermittent carriers, whereas others appear to be permanent non-carriers (VandenBergh *et al.*, 1999). One explanation to these differences might be variations in certain host factors, *e.g.* cytokeratin 10, supposed to be of importance for nasal adherence of *S. aureus* (O'Brien *et al.*, 2002b). If host factors, important for *S. aureus* carriage or non-carriage, could be established, new and exiting possibilities for anti-staphylococcal therapies would open.

Present investigation

"If we knew what it was we were doing, it would not be called research, would it?"

Albert Einstein

Aims of the study

The specific objectives of this thesis were to identify novel receptins from some important pathogenic staphylococci, and to study their interactions with host proteins. These goals were set up against the background of the increased antibiotic resistance observed among staphylococci at a global scale, which has prompted new efforts to elucidate the virulence mechanisms and to develop vaccines against these persistent opportunistic pathogens. In this regard, all receptins are of interest, since these are the contact points between bacteria and host. By targeting such key events, the infection process might be prevented, either by passive, or active immunisation. Although the genome of *S. aureus* now is sequenced, there is still a need to uncover the actual function of putative gene products. In our search for novel receptins, the powerful method of phage display was applied. Libraries derived from *S. aureus* and *S. lugdunensis* were constructed and affinity selected against ligands of interest with regard to severe infections, like catheter-related septicemia and endocarditis.

Phage Display

Basic phage display

Phage display has evolved as a dynamic and resourceful technique for the identification and characterisation of interactions between proteins, since the proof of concept was first established (Smith, 1985). This was achieved by insertion of a foreign gene fragment in fusion with a gene encoding a coat protein of a filamentous phage, resulting in display of the corresponding fusion protein on the surface of the phage particles produced. By affinity selection, against an antibody specific for the foreign peptide, these phage particles could be isolated and enriched more than 1000-fold from a background of wildtype phage particles. One of the fundamental aspects of phage display is the direct physical link between displayed phenotype, and genotype of a phage particle.

Filamentous phage of the Ff group infect *Escherichia coli* via the F pili. However, the infected cells are not killed, instead they continue to grow and divide, while secreting progeny phage particles (Russel, Linderoth & Sali, 1997). The phage genome encodes 11 proteins, five are coat proteins. Protein III (pIII) is a minor coat protein, important for infectivity, and located in a few copies at one end. A few thousands copies of the major coat protein, pVIII, cover the surface of the phage particle. In different phage display applications, these two coat proteins are generally employed (Azzazy & Highsmith, 2002; Smith & Petrenko, 1997).

Insertion of foreign DNA directly into one of the coat protein genes of the phage genome makes every copy of the corresponding coat protein a fusion protein. This practice has severe limitations concerning the range of tolerated foreign peptides. Instead, phagemid vectors with features from both phage and plasmid enables display of just one, or a few fusion proteins on the surface of phagemid particles, when the other wild type phage proteins are supplied *in trans* through a helper phage (Bass, Greene & Wells, 1990). Alternatively, a second copy of the gene encoding the coat protein can be inserted into the phage genome and used for the cloning of foreign DNA. The most common application of phage display is affinity selection, or panning of large libraries displaying short random peptides, or the variable domain of antibodies against some ligand of interest. The ligands employed range from defined ligands to exceedingly complex ligands, *e.g.* targeting organs *in vivo* (Pasqualini & Ruoslahti, 1996).

Shotgun phage display

A special variant of the general phage display method is shotgun phage display, which we have employed as a forceful instrument for studying interactions between bacterial and host proteins (Jacobsson & Frykberg, 2001; Jacobsson *et al.*, 2003). Such libraries are constructed from bacterial chromosomal DNA, which is randomly fragmented and inserted into a phagemid vector. If large enough, the resulting library should theoretically consist of phagemid particles expressing polypeptides corresponding to all genes encoded by the organism of choice. By panning such a library against a ligand of interest, binding polypeptides can be isolated and putative receptins identified. Also panning against a complex mixture of ligands, *e.g.* serum, is feasible (Jacobsson & Frykberg, 1998). From an bacterial gene already cloned, or eukaryotic cDNA, it is possible to further map a binding domain by isolation of overlapping peptides.

The phagemid vector pG8SAET, which gives rise to multivalent display of pVIII fusion proteins, has been used in this thesis. Libraries made with this vector often result in a high frequency of correct clones, when panned against an appropriate ligand. This vector also contains an expression screening tag (E-tag) useful for identification of correct clones.

Results and discussion

A novel vWf-binding protein and coagulase of S. aureus (I, II)

Intravascular infection is a grave complication of invasive *S. aureus* disease, which requires bacterial attachment to thrombi, or subendothelium, for colonisation to occur. A few years ago, *S. aureus* was reported to bind to vWf-coated surfaces, suggesting a role for vWf-binding in the pathogenesis of intravascular infections (Herrmann *et al.*, 1997). Thus, we used shotgun phage display to identify vWf-binding receptins in *S. aureus*. Our work with the characterisation of a vWf-binding receptin was well underway, when an article appeared, describing protein A as the vWf-binding adhesin of *S. aureus* (Hartleib *et al.*, 2000). However, we had instead found a secreted vWf-binding protein (**I**).

The library derived from *S. aureus* strain Newman was panned against human recombinant vWf. This resulted in a specific enrichment of binding phagemid clones. Of 32 clones isolated and sequenced, 26 clones had overlapping inserts, suggesting that a gene encoding a hitherto unknown vWf-binding receptin had been identified. The gene was named *vWb*, and the corresponding protein vWbp, for von Willebrand factor-binding protein. With the aid of available genome sequence from *S. aureus* strain COL, the complete *vWb* gene was PCR-cloned from strain Newman. This gene is present in all *S. aureus* genome sequences publicly available today, as well as in the seven other strains we have examined. There seems to exist at least three different variants, with the deduced gene products being approximately 80% identical to each other. In the genome of *S. aureus* strain COL, and other strains, the *vWb* gene is located between the genes encoding ClfA and Emp, already discussed in the introduction of this thesis. The two genes that follow next encode a nuclease, and a small putative receptin of so far unknown function. This putative receptin is of special interest to this study since the sequence is homologous with the C-terminus of vWbp, as discussed later on. Virulence genes are quite often encountered in groups (Hacker *et al.*, 1997), and since ClfA is a recognised virulence factor, the mentioned neighbouring genes can be considered to encode putative virulence factors, guilty by association.

vWbp comprises 508 amino acids, with an N-terminal signal peptide of 26 amino acids. From the panning experiment the vWf-binding region was mapped to 26 amino acids, corresponding to amino acids 333 to 358 of the mature receptin. The lack of any apparent surface retaining features suggested that vWbp is a secreted protein, which was confirmed by purification and subsequent N-terminal sequencing of vWbp from the culture supernatant.

Several independent lines of evidence imply that the interaction between vWbp and vWf is specific. Phagemid particles, displaying a major part of vWbp, were panned separately against different immobilised ligands, and generally, 1000 to 10000-fold more phagemid particles bound to vWf than to the other tested ligands. This binding was inhibited by vWbp-specific antibodies in a dose-dependent manner. Also, a recombinant peptide, comprising the minimal binding domain of 26 amino acids from vWbp, inhibited the interaction between vWbp and vWf in a dose-dependent manner (Bjerketorp, unpublished observation). Further, vWbp from an *S. aureus* supernatant also bound to vWf in a Western blot assay. Moreover, a part of vWbp, corresponding to amino acids 124-392 of the mature vWbp, could capture vWf from human serum when immobilised on a column. Accordingly, vWbp can bind both immobilised and soluble vWf.

As vWf is an essential component in blood clotting and interacts with many different molecules, we wanted to locate the domain in vWf to which vWbp binds, as this might help to elucidate the biological function of this interaction during infection. Three different strategies were used to identify the binding motifs in vWf. First, a random peptide library was panned against vWbp, to find binding peptides that perhaps resembled amino acid motifs in vWf. However, no vWbp-binding peptides were obtained, instead some peptides with very good plastic-binding ability were enriched. Secondly, cDNA encoding vWf was used to make a phage display library. Unfortunately, no enrichment was obtained when this

library was panned against vWbp. Thus, most likely, vWbp binds to a domain in vWf, which is not correctly folded at the phage surface, or that requires posttranslanational modifications for the interaction. Thirdly, we also had access to polyclonal antibodies against vWf, that efficiently inhibited the binding between vWbp and vWf. Thus, a significant fraction of the antibodies must be directed to the domain bound by vWbp. The cDNA library was panned against the antibodies, and a specific enrichment of binding phagemid particles was obtained. The insert of a large number of clones was determined, and almost all inserts were derived from four distinct regions in vWf (Figure 2). Two groups mapped in close proximity to crucial regions located in the domains A1 and C1 in vWf. These regions are *e.g.* involved in initial platelet adherence and aggregation, respectively. However, we have not yet been able to demonstrate that one of these important regions of vWf is the target for vWbp.

If vWbp does bind to one of these key domains of vWf, this might hamper platelet plug formation, or platelet activation. As vWbp can initiate coagulation without converting prothrombin into thrombin (as discussed later), which is a powerful platelet activator, it might be possible for *S. aureus* to divert the release of bacteriocidal platelet peptides in a wound. It has been shown that the fibrinogen-binding receptins coagulase and Efb can bind platelets (Heilmann *et al.*, 2002). Coagulase also avoids thrombin-formation when initiating coagulation, and Efb stimulates fibrinogen-binding to activated platelets, but simultaneously delays wound healing by inhibiting fibrinogen-dependent platelet aggregation (Palma *et al.*, 2001). Acting together, these secreted receptins might make thrombi less bacteriocidal and more suitable for *S. aureus* colonisation. However, the scheme is very complex and far from understood, since several surface-anchored *S. aureus* adhesins can promote platelet aggregation by binding to platelets, which is reported to be important in the pathogenesis of endocarditis (Nguyen, Ghebrehiwet & Peerschke, 2000; O'Brien *et al.*, 2002a; Sullam *et al.*, 1996).

Interestingly, a moderate sequence similarity was observed between vWbp and coagulase. When vWbp was mixed with rabbit plasma from a commercially available coagulation test, it was found that the homology reflected a conserved function. Thus, like coagulase, vWbp is a bifunctional protein with ability to both bind a protein relevant for host hemostasis, and to induce coagulation (**II**).

To further investigate the coagulating ability of vWbp, plasma from different animal species were mixed with vWbp. Plasma from several of the species tested were sensitive to the action of vWbp, but to a varying degree. Most strikingly, human plasma and also porcine plasma coagulated within one minute. Rabbit plasma was found to coagulate quite poorly and plasma from *e.g.* mouse not at all. In order to investigate this species variation, human plasma was added to plasma from *e.g.* mouse and rabbit, to a final concentration of 1%. The vWbp-induced coagulation time was now reduced to 10 minutes. Thus, a species-specific determinant was responsible for the observed differences in coagulation time. Since it is known that coagulase interacts with prothrombin (Hemker, Bas & Muller, 1975; Kawabata *et al.*, 1985), we tested if prothrombin was the component with which vWbp interacted in a species-specific manner. Plasma from *e.g.* mouse and rabbit was mixed with vWbp and human prothrombin. Now coagulation

occurred within one minute, whereas the same experiment with bovine prothrombin did not reduce the time for coagulation. From these experiments it appears to be the effectiveness of prothrombin activation, that determines the species-specific coagulation by vWbp. Interactions between pathogens and their hosts eventually lead to evolutionary effects on both parties. Thus, the emergence of bacterial virulence factors, tuned to their hosts, is to be expected.

The coagulation was easily monitored by mixing human plasma and vWbp in a spectrophotometer cell. Coagulation was inhibited in a dose-dependent manner by addition of antibodies against vWbp, but not by addition of antibodies against coagulase. When vWbp and coagulase were mixed in equine plasma, specific antibodies against both these proteins were needed in order to inhibit the reaction. Although vWbp and coagulase have similarities, antibodies against the respective protein show almost no cross-reactivity in a Western blot assay. As a consequence, antibodies against both vWbp and coagulase, might be required in order to fully inactivate the coagulating ability of *S. aureus*.

When truncated variants of vWbp were expressed and examined, only the first 250 amino acids were needed for coagulation, while an N-terminally truncated vWbp was inactive. These findings are in accordance with the recent study published during the course of our work (Friedrich *et al.*, 2003). This reported on the crystal structure of coagulase bound to human prothrombin and thrombin, the mechanism of prothrombin activation by coagulase, and also proposed a domain organisation of vWbp based on homology to coagulase (Figure 4). The first 150 amino acids of coagulase, the D1 domain, activate prothrombin by inserting the N-terminal amino acids into an activation pocket of prothrombin. The following 111 amino acids, the D2 domain, are required for the proper binding of prothrombin. The corresponding D1 and D2 domains in vWbp were proposed to comprise the first 131, and the following 130 amino acids, respectively. A novel fold with a three-helix bundle in each domain was described, and the fold was also observed in other putative receptins from *Streptococcus agalactiae* and *Streptococcus pyogenes*. Kinetic experiments stressed the importance of an intact N-terminal of coagulase, since addition or removal of one amino acid, significantly reduced the prothrombin activating ability. The N-terminal amino acids of the recombinant vWbp used in our study were determined, and found to be identical to the native vWbp. However, our recombinant coagulase unfortunately contained an additional alanines residue (A-coagulase), introduced due to cloning constraints in the expression system. Most likely, the coagulating activity of A-coagulase does not reflect the full activity of native coagulase. The A-coagulase variant with an extra alanine, a residue with a small side chain, could still be more active than the coagulase reported by Friedrich *et al.* (2003), which had an extra methionine, a residue with a larger side chain. Also, the known species-specificity of coagulase are retained, since A-coagulase coagulates rabbit plasma better than plasma derived from any other species.

The similarities between vWbp and coagulase were found to extend even further. When mixing vWbp or coagulase with human prothrombin, the respective receptin is trimmed in the C-terminus, releasing 77 or 136 amino acids, respectively. The part released from vWbp corresponds to roughly half the

putative receptin encoded by the last gene in the *clfA-vWb* gene cluster earlier mentioned (Figure 4). The similarities between the two proteins are high in their C-terminals (~70%), but drops to below 20%, immediately upstream of the cleavage site. Conceivably, this small homologous protein originated through gene duplication of vWbp, followed by deletion of the domains responsible for coagulation and vWf-binding. In coagulase, the C-terminal cleavage corresponds to the release of all fibrinogen-binding repeats. It remains to be elucidated whether these fragments are released also *in vivo*, and what functions they possibly serve.

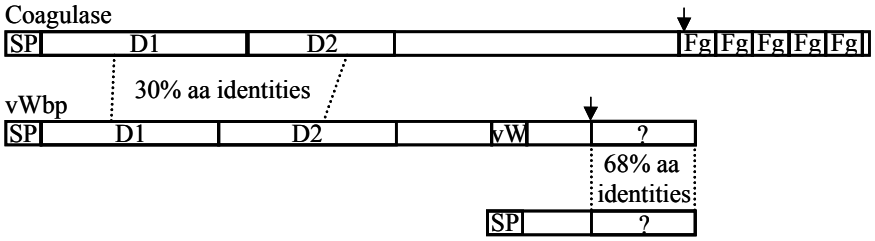


Figure 4. Alignment of vWbp from *S. aureus* strain Newman and coagulase from *S. aureus* strain 8325-4. Abbreviations: SP, signal peptide; aa, amino acids; vW, von Willebrand factor-binding region; Fg, fibrinogen-binding repeats. The human prothrombin cleavage sites are indicated (arrows), as are the domains (D1 and D2), responsible for activation and binding of prothrombin, respectively. Adapted from Friedrich *et al*. (2003). vWbp is also aligned against the small putative receptin located in the same gene cluster as vWbp. The question mark indicates an unknown function.

SAK-activation of human plasminogen dissolves fibrin clots, thus counteracting the clotting reaction induced by vWbp and coagulase. This is particularly interesting in view of a recent study, showing that the expression of SAK is inversely correlated to virulence of *S. aureus* in human infections (Jin *et al.*, 2003). Thus, isolates of *S. aureus* from nasal carriers, or non-lethal bacteraemia, had a higher SAK-expression than *S. aureus* isolated from patients with lethal bacteraemia. It is possible that the SAK-expression counteracts an otherwise deleterious coagulating effect of vWbp and coagulase in these patients. Apparently, *S. aureus* benefits from short-circuiting the host systems related to coagulation, otherwise receptins like vWbp, coagulase and SAK would not exist. Notably, both vWbp and coagulase have additional binding capacities, not required for coagulation, but perhaps still linked to coagulation, since both vWf and fibrinogen are of exceptional importance for hemostasis.

A vWf-binding protein from S. lugdunensis (III)

In some CoNS, *e.g. S. epidermidis* and *S. haemolyticus*, vWf-binding proteins have been reported (Li, Lundberg & Ljungh, 2000). A phage display library derived from another CoNS, *S. lugdunensis*, was panned against vWf. This resulted in the identification of a gene, present in the clinical strains tested, which encoded a vWf-binding protein of 2060 amino acids, termed vWbl (**III**).

The hallmarks of a cell-surface receptin, an N-terminal signal peptide and a cell wall sorting signal, are present in the deduced sequence of vWbl. After the signal peptide follows an A-region of unknown function, encompassing approximately

two thirds of the protein. Next to the A-region follows a repeat (R)-region, comprising 10 imperfect repeats, R1-R10, each consisting of 67 amino acids. The signal peptide contains the motif YSIRK-G/S, demonstrated to aid efficient secretion (Bae & Schneewind, 2003). In the C-terminal part of the A-region, an RGD-motif is located. This motif is found in numerous host ECM-molecules and mediates integrin-binding (Ruoslahti & Pierschbacher, 1987). The R-region shows homology to repeats in a cell wall anchored protein associated with the virulence of *Streptococcus suis* (Smith *et al.*, 1992; Vecht *et al.*, 1991). The R-region of vWbl is also the vWf-binding region. From the panning experiments, a minimal binding region was mapped to 24 amino acids in the C-terminus of R2. Three separate panning experiments resulted in clones mapping to this repeat only, although nine of the ten repeats are 65% identical. In a fourth panning experiment, the elution condition was changed from lowering of the pH, to allowing infection of the bacteria directly. This resulted in inserts recovered also from R5. Some phagemid particles were evidently not released by lowering the pH, and possibly other repeats are recovered by other elution conditions.

A phage stock prepared from a representative clone, was panned separately against several different immobilised ligands., Approximately 1000 to 10000 more phagemid particles bound to vWf than to any other ligands tested. A recombinant protein, corresponding to R1-R3 of vWbl, could inhibit this interaction, as could specific antibodies against vWbl, but not pre-immune antibodies. Also various ELISAs were performed, and the binding between recombinant vWbl and immobilised vWf could be specifically inhibited by soluble vWf. Thus, both soluble and immobilised vWf are recognised by vWbl. Interestingly, and a bit surprising, a recombinant peptide comprising the minimal binding domain of vWbp (**I**), inhibited interaction between vWbl and vWf in a dose-dependent manner.

The minimal binding domains of both vWbp and vWbl are small and they show no apparent sequence similarities. It is intriguing that these pathogenic staphylococci both have evolved proteins that seem to bind to the same site in such a large protein. Since vWbp is secreted, the primary function is not to confer adhesion of *S. aureus*, but instead to exercise some influence on hemostasis, or maybe innate immunity, via interaction with vWf. However, vWbl is cell wall anchored, and probably acts as an adhesin. Theoretically, vWbl might also affect the function of vWf. Since vWf is the critical bridging molecule required for platelet adherence to exposed subendothelium, as well as platelet aggregation during arterial blood flow, vWbl and its interaction with vWf is interesting because *S. lugdunensis* is reported to cause aggressive cases of endocarditis. The RGD-motif found in vWbl implicates the possible adherence of *S. lugdunensis* directly to eukaryotic cells and platelets. Direct staphylococcal binding to integrins through an RGD-dependent mechanism has not been reported, but other microorganisms use this way of adherence to host cells (Isberg & Tran Van Nhieu, 1994). However, indirect binding of *S. aureus* to integrins via fibronectin has been reported to result in the internalisation of staphylococci in non-professional phagocytic cells (Joh *et al.*, 1999; Fowler *et al.*, 2000). If an equal mechanism exists for *S. lugdunensis* remains to be investigated.

Sorting an S. aureus phage library against ex vivo biomaterial (IV)

As discussed previously in this thesis, the use of biomaterials increases in modern medicine. *S. aureus* is recognised as one of the most important pathogens of nosocomial bloodstream infections (Lowy, 1998). A major risk factor for being affected by this type of infection is having an intravascular devise, *e.g.* a central venous catheter (CVC) inserted. Various plasma proteins adsorb to a CVC present in the bloodstream, enabling *S. aureus* to adhere by means of cell surface associated receptins, thus initiating infection. To further investigate the receptins possibly involved in this adherence, an *S. aureus* phage display library was panned against a CVC removed from a patient two days after insertion (**IV**).

The numbers of eluted phagemid particles, as well as the frequency of E-tag positive clones, increased in three consecutive cycles of panning, thus indicating an enrichment of correct clones. From each panning, the insert of ~50 E-tag positive clones were sequenced. Six different receptins were identified: coagulase, Efb, protein A, FnbpA and B, and Sbi. The majority of the isolated phagemid particles encoded the fibrinogen-binding part of coagulase, or the β_2-GPI-binding part of protein Sbi. The library was also panned against plasma proteins coated for one hour *in vitro*. Of 24 E-tag positive clones isolated, no β_2-GPI-binding phagemid particles were found, while fibrinogen-binding phagemid particles were dominating. Thus, fibrinogen is the main host protein recognised by *S. aureus* receptins after a short *in vitro* coating, whereas other host proteins like β_2-GPI, possibly deposited later than fibrinogen, might be important *in vivo*.

To find out whether the high number of β_2-GPI-binding phagemid particles isolated from the panning against the *ex vivo* CVC was the result of β_2-GPI being a major host component on biomaterial, or perhaps just more accessible. CVCs removed from patients after various implantation times, between 6 and 18 days, were investigated. Western blot analysis were performed on proteins released from the *ex vivo* biomaterials, using antibodies against fibrinogen, fibronectin, IgG, and β_2-GPI. Fibrinogen was found to be the major component on all CVCs, but fibronectin and β_2-GPI were also detected on all tested biomaterials, whereas the presence of IgG varied among the CVCs. To our knowledge, the presence of β_2-GPI on *ex vivo* biomaterials has not been demonstrated prior to this study. For bacterial adherence to biomaterial, the total amount of adsorbed host proteins is not as important as the surface-available components.

To study the accessibility of fibrinogen, IgG, and β_2-GPI on the different CVCs, three different phage stocks with these binding specificities were used. Several factors might affect the number of phage particles obtained after a panning experiment. However, it is conceivable that the accessibility of the ligand is the most important factor. Subsequently, the number of released phage particles in panning against *ex vivo* biomaterial should give an indication of the accessibility of the different proteins. The different phage stocks bound all CVCs, but not to an uncoated CVC. Thus these three host proteins were present on the surface of all *ex vivo* biomaterials tested. The number of phagemid particles binding to β_2-GPI on the biomaterial was similar to those binding to fibrinogen. The number of phagemid particles binding to these proteins in pure form, results in ~50 times more phagemid particles binding to fibrinogen, than to β_2-GPI. This means that

although, β_2-GPI is present in much lower amounts than fibrinogen on the CVCs, the surface accessibility of β_2-GPI might be higher. Taken together, our result suggests that fibrinogen is the protein most accessible at the surface after a short *in vitro* coating with plasma, but after a long *in vivo* coating the surface accessibility of other proteins like β_2-GPI increases compared to fibrinogen. These data are in accordance with a previous study, which reported on the lower contribution of fibrinogen in *S. aureus* adherence, when fibrinogen was deposited on biomaterial during hemodialysis, compared to when pure fibrinogen was coated onto a hemodialysis tubing *in vitro* (Francois *et al.*, 2000). In that study, the presence of an additional unidentified host component was suggested to be of importance for the adherence of *S. aureus* to biomaterial. Thus, in view of our results, this component could be β_2-GPI, which *S. aureus* can bind via protein Sbi.

Affinity selection of an *S. aureus* phage display library against an *ex vivo* CVC revealed several interactions of supposed biological significance for *S. aureus* adherence to biomaterials. However, during panning, it is possible to identify protein interactions of high affinity, regardless of the localisation and function of the binding protein, *i.e.* to encounter a interaction without biological meaning. Also, it is not realistic that all hypothetically possible interactions are identified. Although the main selection factor of phagemid particles should be affinity and accessibility of the ligand, other factors might also influence which phagemid particles that are found after a panning. For example, various phagemid clones might replicate to different numbers, and also, non-identical polypeptides sharing the same binding specificity could be displayed with different efficiency. Consequently, some phagemid particles displaying certain domains will lose in the competition with other binding phagemid particles.

Sorting an S. epidermidis phage library against ex vivo biomaterials

CoNS are the bacteria most frequently isolated from biomaterial infections (von Eiff, *et al.*, 2002). Of these, *S. epidermidis* is the most common pathogen isolated. Therefore, a library constructed from a clinical isolate of *S. epidermidis* was panned against different *ex vivo* biomaterials, *e.g.* CVCs, and screws removed from bone (Bjerketorp *et al.*, unpublished). The most frequently isolated phagemid particles contained overlapping inserts of the gene encoding dihydrolipoamide acetyltransferase, an intracellular glycolytic protein of the pyruvate dehydrogenase complex. Further, phagemid particles encoding Fbe were found (Nilsson *et al.*, 1998). To investigate if the acetyltransferase might be a moonlighting protein, also located on the cell surface of *S. epidermidis*, antibodies against the protein were raised. Non-covalently bound surface proteins from the clinical strain were extracted, and analysed by Western blot. A protein of expected size was detected by the specific antibodies. The surface expression of the presumed acetyltransferase peaked during early exponential growth phase. The identity of the acetyltransferase interacting factor on the *ex vivo* biomaterials is unknown, as the biological importance of this interaction during infection.

Concluding remarks

"Facts are the air of scientists.
Without them you can never fly."

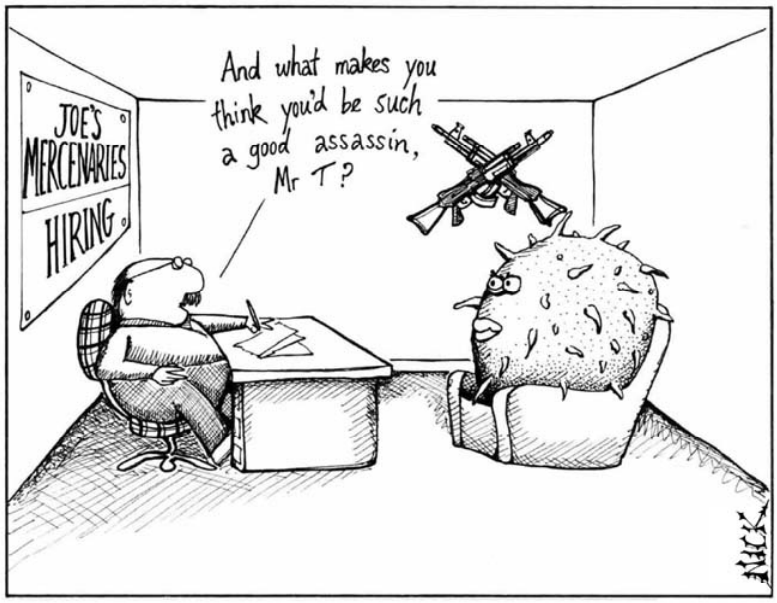
Linus Pauling

In this thesis, interactions between bacterial proteins and host proteins have been studied, mainly using phage display. Panning an *S. aureus* phage display library against *ex vivo* biomaterial led to the identification of β_2-GPI on the surface of CVCs. This is interesting in view of *S. aureus* adherence, but might also have significance in the induction of β_2-GPI-autoantibodies found in the autoimmune disease called antiphospholipid syndrome. Two novel vWf-binding receptins have also been identified, one secreted from *S. aureus*, and one surface-anchored from *S. lugdunensis*. Further characterisation revealed vWbp to be a bifunctional protein capable of inducing coagulation of plasma from several species, with specific species tropism for human and porcine plasma.

The coagulation system and innate immune response are merely different aspects of the overall host integrity scheme. Obviously, *S. aureus* must benefit from affecting some of the key events of this scheme to its own purpose, since *S. aureus* has evolved an large number of receptins recognising many different components in the host ECM, and of the coagulation system/innate immune response. A common feature of many *S. aureus* receptins is that they often possess multiple functions, some of which are unique, and some that instead overlap with the function of other receptins. Most likely, no single receptin is indispensable for the overall virulence of *S. aureus*, since other receptins can compensate the loss. Of special interest would be to construct an *S. aureus* deletion mutant lacking the genes encoding vWbp, coagulase and SAK. By complementing these receptins, in different combinations, and in an appropriate animal model, it should be possible to clarify this potentially important aspect of *S. aureus* virulence. However, the contribution of the interactions identified in this thesis to the pathogenesis of *S. aureus* and *S. lugdunensis* remains to be elucidated.

As staphylococcal resistance against available antibiotics continues to increase, research efforts have to proceed to find novel functional treatments against these unrelenting opportunistic pathogens. A lot of future promise is seen in the exploration of antimicrobial peptides, used by organisms from insects to man as part of their innate immune system (Hancock, 2001). Apparently, microbes do not easily develop resistance against these peptides, but so far systemic use has been hampered, *e.g.* by unwanted side effects. All efforts to develop staphylococcal vaccines have so far met with limited success, and today it is hard to say which components that in the end will be included in a vaccine, that gives prolonged protection. Passive immunisation using different antibody preparations are currently being evaluated, to be used for certain groups at high risk of staphylococcal infection, *e.g.* premature infants (Hall *et al.*, 2003; Vernachio *et al.*, 2003). The strategy of passive immunisation has historically been successful in some part of the world, even for the treatment of severe cases of staphylococcal

infection caused by resistant *S. aureus* (Kelly, 2000). A total eradication of the commensal staphylococcal flora is not desirable, since this might result in the colonisation by even worse opportunistic pathogens. Possibly it might be feasible to replace virulent and resistant staphylococcal strains with avirulent strains in a probiotic manner. Regardless of the approach chosen to overcome the problem of bacterial resistance, man will benefit from gaining more knowledge about the individual virulence factors, the regulation of virulence genes, and about the ecological interplay between bacteria and host.



As each disease is finally eradicated, redundant lymphocytes increasingly find themselves looking for other work.

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