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The coagulase negative staphylococci

Molecular studies on *Staphylococcus haemolyticus* and novel treatment of staphylococcal biofilms *in vitro* and *in vivo*

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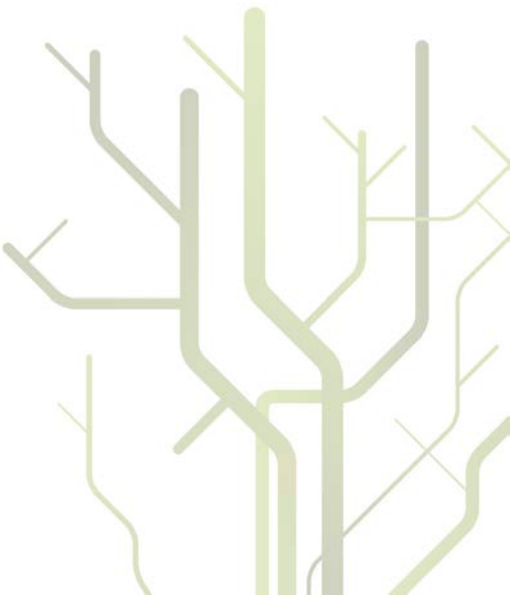

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List of papers

Paper I

Cavanagh JP, Klingenberg C, Hanssen AM, Fredheim EA, Francois P, Schrenzel J, Flægstad T, Sollid JE. Core genome conservation of *Staphylococcus haemolyticus* limits sequence based population structure analyses. J Microbiol Methods. 2012; 89: 159-66.

Paper II

Cavanagh JP, Hjerde E, Holden M, Klingenberg C, Flægstad T, Sollid JE. Whole genome sequencing reveals clonal expansion of multi-resistant *Staphylococcus haemolyticus* in European hospitals. In manuscript.

Paper III

Flemming K, Klingenberg C, **Cavanagh JP**, Sletteng M, Stensen W, Svendsen JS, Flaegstad T. High in vitro antimicrobial activity of synthetic antimicrobial peptidomimetics against staphylococcal biofilms. J Antimicrob Chemother. 2009; 63: 136-45.

Paper IV

Cavanagh JP, Granslo HN, Fredheim EA, Christophersen L, Jensen PØ, Thomsen K, Bjarnsholt T, van Gennip M, Klingenberg C, Høiby N, Svendsen JS, Stensen W, Flægstad T, Moser C. Efficacy of a synthetic antimicrobial peptide versus vancomycin in a *Staphylococcus epidermidis* device related murine peritonitis model. Submitted.

Abbreviations

Aap	Accumulation associated protein
AtlE	Autolysin/adhesin E
AMP	Antimicrobial Peptide
APHs	Nucleotidyltransferases
Bap	Biofilm associated protein
CC	Clonal complex
CFU	Colony Forming Unit
CoNS	Coagulase-Negative Staphylococci
CRISPR	Clustered regularly interspaced short palindromic repeats
CDS	DNA coding sequence
DNA	Deoxyribonucleic acid
eDNA	extracellular Deoxyribonucleic Acid
e.g.	exempli gratia
GI	Genetic island
IL	Interleukin
IS	Insertion Sequence
LTA	Lipoteichoic Acid
MGE	Mobile genetic elements
MSCRAMMs	Microbial Surface Components Recognizing Adhesive Matrix Molecules
MLST	Multi Locus SequenceTyping
MLVF	Multi locus number of Tandem repeat Fingerprinting
MRSA	Methicillin Resistant *Staphylococcus aureus*
NaCl	Sodium Chloride
Orf	Open reading frame
PAMP	Pathogen Associated Molecular Pattern

PFGE	Pulse Field Gel Electrophoresis
PGA	Poly-γ-Glutamic Acid
PIA	Polysaccharide Intercellular Adhesin
PRR	Pathogen Recognition Receptor
PSM	Phenol Soluble Modulin
QS	Quorum Sensing
RT	Repeat type
SAMPs	Synthetic Antimicrobial Peptidomimetics
SCC	Staphylococcal cassette chromosome
Ses	*Staphylococcus epidermidis* surface
ST	Sequence type
TLR	Toll like receptors
VNTR	Variable Number of Tandem Repeats
VRSA	Vancomycin resistant *S. aureus*
WHO	World health organisation

1 Introduction

Along with advances in medicine new problems arise. Previous innocuous bacteria become a source of distress, which is the case with the coagulase negative staphylococci (CoNS) including the species *Staphylococcus epidermidis* and *Staphylococcus haemolyticus*. Today CoNS are the most prevalent Gram positive bacterial species causing nosocomial infections (116). Baird-Parker reported in 1965 mucoid growth of *S. epidermidis,* in 1972 Bayston and Penny further observed an association between mucoid growth and cerebrospinal shunt infection (42). The concept biofilm was first introduced in the late seventies by Bill Costerton and his colleagues (43). It is now well accepted that biofilm formation is an important virulence factor of the staphylococci. Medical implants such as hip prostheses, contact lenses, artificial heart valves and catheters are considered as hot spots for biofilm formation and biofilm associated CoNS infections. These infections are mainly chronic, and removal of the colonised implant is often the only therapeutic option due to antibiotic treatment failure (93). The increasing use of implanted medical devices contributes to the fact that problems associated to biofilm infections are here to stay.

The research community is gradually beginning to unravel some of the many factors contributing to biofilm formation, the interplay with the host innate immune system, and why these infections tend to persist. The development of novel antimicrobial agents efficient towards bacteria encased in biofilm is important. In this study we investigated the action of synthetic antimicrobial peptides towards an *in vivo* implant infection, and the interplay between biofilm embedded *S. epidermidis* and the host immune system. We also investigated the genetic composition, epidemiology and genes involved in biofilm formation in *S. haemolyticus*. Understanding the possible role *S. haemolyticus* plays as a contributor to the common staphylococcal gene pool might result in increased focus on this previous modest pathogen.

1.1 Taxonomy of the genus *Staphylococcus*

The staphylococci are commensals mainly associated with the skin, mucous membranes and glands of warm blooded animals. The genus *Staphylococcus* belongs to the phylum *Firmicutes*, class of *Bacilli*, order *Bacillales* and the family *Staphylococcaceae.* The genus *Staphylococcus* was first defined by Rosenbach in 1884, in the same year, *Staphylococcus aureus* was classified into this genus. Today the genus *Staphylococcus* consists of 47 identified species and 24 subspecies. The most recent species *Staphylococcus agnetis* was included in 2012 (64, 231).The staphylococci are spherical, non-motile and appear singly, in small clusters or as short chains. They stain Gram positive, and their cell wall is composed of proteins, teichoic acid and peptidoglycan. *S. haemolyticus* and *S. aureus* have capsule formation ability. The staphylococci are usually catalase positive, oxidase negative and facultative anaerobe, they are salt tolerant, and able to sustain growth in the presence of 10 % NaCl (73, 88, 135, 214).

The staphylococci are separated in two main groups, the coagulase negative and the coagulase positive, depending on the presence of the enzyme coagulase which causes the fibrin of blood plasma to clot (16, 98). The coagulase positive bacteria comprise five different Staphylococci, amongst them *S. aureus*. The remaining group of the staphylococci are coagulase negative, among the most clinical important are *S.s epidermidis*, *S. haemolyticus*, *Staphylococcus saprophyticus* and *Staphylococcus lugdunensis* (52, 201).

1.1.1 Clinical significance

The Gram positive staphylococci, and primarily *S. aureus,* have for many decades been considered as an important human pathogen. *S. aureus* is responsible for a variety of infections, such as toxic shock syndrome, impetigo, toxic epidermal necrolysis, pneumonia, endocarditis and osteomyelitis (69, 77).

The CoNS were earlier regarded as innocuous inhabitants of the human normal skin flora, but are now emerging as opportunistic pathogens. In addition to their biofilm producing ability CoNS are

frequently multi-resistant, and a high prevalence of methicillin resistant CoNS are found (208). Among the CoNS, *S. lugdunensis* has several virulence factors in addition to biofilm formation and may cause severe disease. This CoNS species is not as frequent, but comparable in severity to infections caused by *S. aureus*. It accounts for 3% of the CoNS isolated, and has a propensity of causing native valve infections. Other clinically important species includes *S. saprophyticus*, which is a frequent cause of urinary tract infections in young adult females (84, 89, 247).

S. epidermidis is the leading cause of nosocomial infections followed by *S. haemolyticus* which is the CoNS second most frequently isolated from human blood cultures (65, 191). The increase in infections caused by these organisms is mainly foreign body related and/or associated with immunocompromised patients. e.g. patients with haematological disease and immature infants (185). Blood stream infections, prosthetic joint infections, vascular graft infections, endocarditis, and peritoneal dialysis catheter infections are complications caused by CoNS (57, 188, 204, 219). Complete removal of the infected device and prolonged antibiotic therapy is often necessary in order to remove the infection (140, 191, 194, 261) contributing to increased morbidity and prolonged hospitalisation associated with CoNs infections..

Due to the increasing number of patients requiring indwelling medical devices the number of biofilm related infections is rising (83, 220). It is now estimated that approximately 3% of all patients receiving medical devices develop biofilm infections, requiring revision therapy which represents a supplementary medical cost of € 800 mill. per year in Europe (63). Infections caused by *S. haemolyticus* are often difficult to treat. The ability to produce biofilm and the notoriously multi-resistance to antimicrobial agents, favours *S. haemolyticus* as an emerging cause of nosocomial infections (49, 86, 111, 116, 142). The biofilm mode of growth confers many additional advantages for the bacterial persistence such as immune evasion, tolerance towards desiccation and antimicrobial agents (4, 57, 128). Staphylococcal biofilm, resistance and immune evasion will be discussed in the following chapters.

1.2 Molecular epidemiology

According to the World Health Organisation (WHO) epidemiology is defined as "*the study of the distribution and determinants of health-related states or events (including disease), and the application of this study to the control of diseases and other health problems*"(255). Molecular epidemiology could also be described as characterising bacterial isolates causing disease based on their genetic composition, the study of distribution, transmission and occurrence in a population (78).

Molecular identification of bacterial lineages, more prone to causing disease is important in understanding the establishment and transfer of bacterial infectious epidemiology (193). The tracking of bacterial isolates belonging to a defined clone allows implementation of preventive measures, such as patient isolation and bacterial sanification. Close examination and comparative analyses of different lineages allows an understanding of bacterial evolution which is caused by mutations, recombination and/or the acquisition of foreign DNA by horizontal gene exchange (11, 100, 113, 223).

By applying different methods, genomic divergence is used to discriminate between closely related bacteria. The degree of resolution, time consumption and cost, varies depending on the different methods applied. Techniques currently most commonly used in typing of staphylococci will be described in the following sections.

1.2.1 Pulsed field gel electrophoresis (PFGE)

Pulsed field gel electrophoresis has been considered the gold standard in molecular typing of bacteria (92). By enzymatic restriction cutting the total genome is fragmented and separated on an agarose gel by applying alternating electrical fields (71, 173). Acquisition or loss of genes is reflected in alterations of the banding pattern. Analyses of banding patterns can be performed both visually and by a computerised system. Computerised comparison of the banding patterns by using a system of band – based similarity coefficients is often applied to determine isolate

relatedness. In order to allow accurate band matching between two lanes in the gel, the band position tolerance can be adjusted. Assignment of isolates to different clusters, are often based on a threshold where isolates with >80% similarity are grouped together (28).

PFGE is suitable for the investigation of isolates temporally and spatially associated such as outbreak situations. Due to the sensitivity towards rapid genomic variation, the method is considered highly discriminatory as a global view of the bacterial genome is obtained (23). Albeit reproducible, PFGE is time consuming and the results are not directly comparable between different labs, due to variations in the electrophoresis conditions, and subjective interpretation of the banding patterns (253).

1.2.2 Multi locus variable number of tandem repeats analysis (MLVA)

Analysis of whole genome sequences has revealed the presence of short tandem repeated DNA sequences. The repeat copy number at each repeat sequence locus can vary, defining the so-called variable number of tandem repeats (VNTR). Variations in the repeat loci are used to study strain relatedness (159, 237). Typing schemes using variable number of tandem repeats usually uses several tandem containing genes. MLVA is a PCR based method. PCRs with specific primers based on flanking repeats, detects fragments of different lengths. The different fragments are either fluorescently labelled for automatic size detection, or products are separated on an agarose gel and visually interpreted (81, 115). When using an automated DNA analyser, the number of repeats is automatically calculated based on the size of the PCR product and the individual repeat size. When the PCR products are separated on a gel, variation is based on the size difference, and the method has also been named multi locus variable tandem repeat fingerprinting (MLVF). The possibility of separating PCR products on an agarose gel, reduces costs and promotes the application, but introduces the problem of inter laboratory variation due to the subjective interpretation and electrophoresis conditions. MLVA schemes have been developed for *S. aureus*, *S. epidermidis* and *S. haemolyticus*, and have shown a good resolution capacity comparable to PFGE, apart from in *S. haemolyticus* (80, 81, 132, 213, 238). MLVA is suitable for outbreak situations, but it is questionable how suitable it is for long term or broad phylogenetic analysis,

due to the variable nature of the tandem repeats. A careful selection of repeat genes is important in order to find appropriate stability over time (3, 158).

1.2.3 Multi locus sequence typing (MLST)

MLST is a sequenced based method, and has been widely used in the study of bacterial evolution and pathogenicity (169). MLST creates an allelic profile based on combinations of single nucleotide polymorphisms found in sections of usually seven housekeeping genes present in all isolates. The analysed isolates are assigned a sequence type (ST) after comparing their sequences with known alleles. By selecting slowly evolving housekeeping genes, the evolutionary relationship can be studied, as well as the establishment and spread of specific bacterial clones (67). The eBURST algorithm groups isolates sharing high genetic similarity into clonal complexes. Isolates which have six out of seven identical loci are grouped together. A clonal complex is defined by the ST believed to be the primary founder, from which the other STs have evolved. The primary founder is predicted as the ST with the highest number of single nucleotide variants (68).

MLST sequencing data is portable, easily comparable between labs, and accessible through the MLST.net database where 477 *S. epidermidis* and 2297 *S. aureus* STs are presently listed (accessed 25.09.2012) (181). The major disadvantages with MLST are sequencing costs, and a lack of discriminatory ability between closely related isolates (193). A recent study demonstrated the use of next generation sequencing technology to obtain large numbers of high quality MLST sequences at reduced sequence costs (24).

1.2.4 Whole genome sequencing

Whole genome sequence data are being produced in tremendous quantities due to the application of next generation sequencing techniques, which offers whole genome sequencing at low cost

and at high through put. The first large scale comparative analysis of the clonal lineage ST239 of methicillin resistant *S. aureus* (MRSA) was performed in 2010. This study clearly demonstrated the discriminatory power of full genome analysis, discriminating between isolates of the same ST. Phylogenetic analysis based on single nucleotide polymorphisms (SNPs) in the core genome separated 63 isolates of sequence type (ST239) according to geographic origin. The level of resolution was so high, that it assigned isolates to separate wards in the same hospital (105). Genome sequencing of several *S. aureus* genomes have shown a well conserved core genome, with introduction of variation due to mobile genetic elements (113, 207). A study sequencing the highly resistant and transmissible strain MRSA TW20 identified as ST239, gave evidence of which mobile genetic elements contributing to the success of this strain in the hospital environment (114). Full genome sequencing is comparable to PFGE in such that the whole genome is studied, but the resolution power is higher as it allows insight into differences at nucleotide level. As technology advances, full genome sequencing performed by bench top equipment will be commonly used in microbial epidemiological typing. The challenge to date is to analyse and interpret the huge amount of data obtained.

1.3 Genomic composition

Thirty-eight staphylococcal complete genomes have been fully sequenced to date, of which five are coagulase negative strains (60, 91, 209, 259). In addition several genomes have been sequenced using the Solexa/Illumina Sequencing Technology.

Studies comparing the staphylococcal genomes revealed a larger genome size in *S. aureus*, and several protein families encoding virulence genes only found in the *S. aureus* genomes. There was also evidence of lineage specific gene loss and horizontal gene transfer (91, 228). There is a large degree of conservation of genes among the staphylococci, but an area designated the *oriC* environ shows little homology between the species. Genes encoded on mobile genetic elements carrying virulence factors and antibiotic resistance genes, confers large variation between different isolates and staphylococcal strains. Whole genome sequencing of *S. haemolyticus* JCSC

1435 revealed a large number of IS elements, probably causing the frequent rearrangements observed in this isolate (230).

Genome based analyses gives valuable insight into the genetic composition of bacteria. Of important note is the selection of isolates. Strains are mostly selected due to their pathogenic potential, and might not be representative of the species as a whole. With the advances in sequencing technology and reduction in sequencing costs, a more diverse range of isolates could be included in order to create a more correct picture of bacterial diversity (157, 193).

1.3.1 Population structure

Analyses of MLST sequence data have revealed that hospital associated MRSA has a clonal population structure, and that certain successful clones are associated with disease (66, 176). The population structure of *S. epidermidis* is less clonal, and a higher degree of diversity has been observed as compared to *S. aureus* (178). However, there still seems to be a limited number of clones associated with disease (17, 140). A recent multicentre study observed dissemination of multidrug resistant *S. epidermidis* clones across the whole US. The most prevalent strains from this study had also been identified in other countries, suggesting an international dissemination of a successful clone (95).

1.4 Biofilm

Biofilms are defined as organised communities of bacteria, encased by a self-produced polymeric matrix (43). The ability to colonise and make biofilms on implanted medical devices in combination with multi-resistance has promoted CoNS as opportunistic pathogens. In this section advantages of the biofilm mode of growth and molecular mechanisms involved in biofilm formation by CoNS will be discussed.

1.4.1 Biofilm properties

Growth of bacteria in structured biofilms are most likely the preferred mode of growth for most bacterial species (57). The structured organisation of bacteria results in different physio chemical properties, such as low oxygen levels and altered pH inducing a differentiated mode of growth, resulting in a population of dormant persisters and dividing bacteria (149, 225, 256).

The life in a biofilm represents several advantages for the bacteria. It protects against several external factors such as desiccation, shear stress, the host immune system, antimicrobial agents and disinfectants (183, 190, 252). It has also been indicated that the transfer of resistance genes are more efficient in biofilms due to the close proximity of bacterial cells, contributing to increased adaption towards antimicrobial stress and structural stability of the biofilm (107, 182).

1.4.2 Biofilm formation

Biofilm formation is a multi-step process, regulated by several mechanisms. *S. epidermidis* biofilm formation, mediated by the polysaccharide intercellular adhesin (PIA), is extensively studied, and will be used as a model in the description of biofilm formation. The different stages; i) attachment, ii) accumulation and growth and iii) detachment are further described in the text and summarised in Figure 1. A mature *S. epidermidis* biofilm grown on silicone implants is depicted in Figure 2.

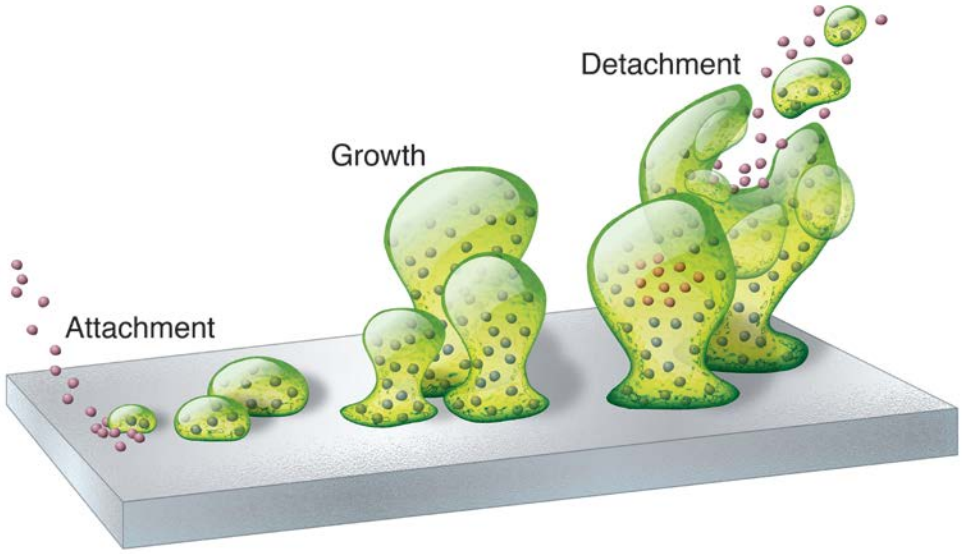

Figure 1. The schematic presentation of biofilm formation in a stepwise manner (figure by Roy-Andre Lysså).

The initial stage, **attachment,** involves binding to a surface, which in CoNS infections typically are plastic surfaces used in medical implants (168, 192). Surfaces can be either unmodified, or modified due to deposition of host matrix proteins. The passive binding to unmodified surfaces is mediated by nonspecific interactions, such as Van der Waals, electrostatic and hydrophobic forces (8, 70). The hydrophobic nature of implanted medical devises also attracts the hydrophobic staphylococcal cell surface (206).

Two staphylococcal surface proteins (SSP-1 and SSP-2) were early identified as important in bacterial adhesion to polystyrene surfaces (240). Proteins such as the autolysins AtlE and Aae are involved in binding both to unmodified and modified surfaces (108, 109). In addition to the adhesive properties they also have autolytic properties involved in cell lysis, resulting in the release of extracellular DNA (eDNA) which plays an important role in biofilm formation (48, 196).

Upon entrance to the human body, artificial devices become coated with host matrix and plasma proteins. Host surface matrix molecules, such as fibrinogen, collagen and fibronectin act as

bacterial binding sites. Microbial surface components recognising adhesive matrix molecules, MSCRAMMs, are involved in the first step of attachment to a modified surface. Common features of the MCRAMMs are an exposed binding domain, a cell wall spanning domain and a bacterial cell wall attachment domain. Well-studied MSCRAMMs in *S. epidermidis* is the fibrinogen binding proteins SdrG and SdrF (9, 70, 119, 190, 191).

Accumulation and growth. Following the initial attachment the bacterial cells accumulate by attachment to each other, and the biofilm matures. Important in the accumulation phase of *S. epidermidis*, is the production of the polysaccharide intercellular adhesin (PIA) synthesized by enzymes encoded by the *icaADBC* operon (165). PIA is a beta-1 6 linked N-acetylglucosamine. The partly de-acetylated polymers, results in a net positive charge which enables binding to the negatively charged bacterial cell surface by electrostatic interactions (206). Genes encoding PIA are present in many CoNS and are also found in *S. aureus* (166). However, the discovery of clinical isolates of *S. epidermidis* and *S. haemolyticus* producing a PIA-independent biofilm lead to the identification of other proteins involved in biofilm accumulation and maturation. The accumulation associated protein (Aap) was identified in *icaADBC*-negative *S. epidermidis* isolates still capable of biofilm formation. Enzymatic processing of Aap by endogenous proteases renders the active form responsible for intercellular adhesion. It has also been speculated that activation of Aap by host proteases, results in a host elicited immune evading strategy (162, 205).

Over the last 5-10 years other components and mechanisms that promote biofilm formation have also been identified. The extracellular matrix binding protein (Embp) was identified as being responsible for biofilm formation in *aap*- and *ica-ADBC* negative isolates. This large protein has both intercellular adhesive properties and extracellular matrix binding abilities. It was also demonstrated to protect *S. epidermidis* against phagocytosis (38). The biofilm associated protein (Bap) is also associated with the formation of proteinacious biofilms in *S. epidermidis*. The switch towards a Bap dependent biofilm was found in PIA positive isolates where IS*256* inserted into the *ica*- operon (110, 235). Recent studies have shown that the biofilm matrix is also composed of eDNA (50), found in both in PIA dependent and in dependent isolates. AtlE is involved in bacterial lysis as well as playing an important role in initial attachment (196). The

finding of several biofilm encoding genes highlights the importance of biofilm as a bacterial survival strategy.

The tight cell aggregation in biofilms creates micro environments differing in oxygen and nutrient levels, inducing different metabolic states. Two-component regulator systems such as the LytS/R, responds to environmental factors by leading to up regulation of cell death and transcriptional alterations, which is believed to play an important role in the biofilm formation (199, 260). The biofilm is also associated with a structured organisation of cells, where fluid filled channels are believed to function in the distribution of nutrients (226). The regulation of this structuring and biofilm formation is not well described in staphylococci yet, but is believed to depend upon a wide range of factors, such as environmental factors and bacterial density, in part coordinated by quorum sensing systems (e.g. *agr*), transcriptional regulators of the *sarA* family and stress sigma factor σ^B regulation (8).

Biofilm formation by clinical *S. haemolyticus* isolates seems to be common (85). However, a PIA-independent biofilm is prevalent. Studies have shown involvement of proteins and extracellular DNA in the composition of the *S. haemolyticus* biofilm (85).

Detachment. Little is known regarding factors promoting structuring and detachment in staphylococcal biofilms. Detachment of bacterial cells from the biofilm is believed to be regulated by the quorum sensing system *agr*, and may in addition be regulated by mechanical forces and reduction in biofilm building material. Recent studies have shown that phenol soluble modulins (PSM) play an important role. Detachment of bacteria from the biofilm mediated by PSM leads to the spread of bacteria from the biofilm, which was demonstrated in an *in vivo* catheter model (190, 249).

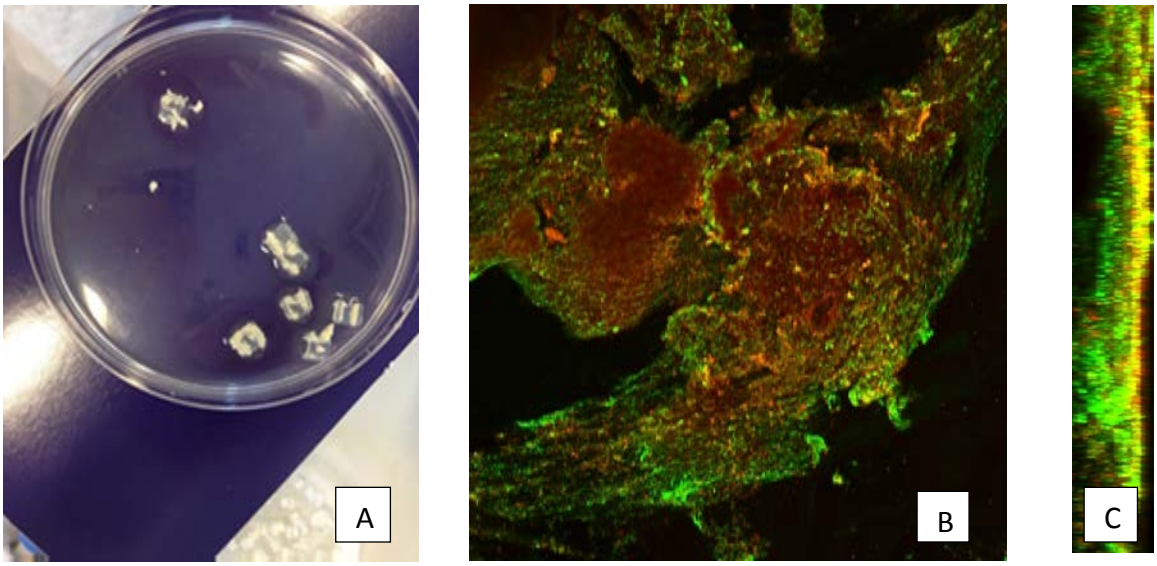

Figure 2. Images of mature (120 hours) *S. epidermidis* biofilm, grown on silicone implants. Image A; biofilm attached to silicone implants after incubation. Image B; confocal laser scanning microscopy image of biofilm stained with Live/Dead stain, (Invitrogen). Red colour reflects dead bacteria and green colour reflects intact bacteria. Image C is a cross section of biofilm image B. (Image, Pauline Cavanagh)

1.5 Host immune response

The skin and the gastrointestinal tract of the human body are colonised with commensal microorganisms. These microorganisms are mainly harmless, but may cause disease upon entrance in the blood stream or other usually sterile body compartments. Even though CoNS are part of the human normal flora they are able to persist and cause infections, not usually lethal but conferring substantial morbidity. Persistence is mainly attributed to their biofilm mode of growth, and the mechanisms responsible are now being unravelled. Protection and elimination of pathogens from our body is the key role of our immune system. It can be divided into the innate and the adaptive immune response. The innate immune response is the first line of defence,

which acts immediately upon encounter with infectious agents, such as bacteria, viruses, fungi, and parasites. In contrast, the adaptive immune response responds later but develops an immunological memory enabling a rapid response upon subsequent reinfection with the same pathogen. The innate and the adaptive immunity is interdependently connected through dendritic cells and toll like receptor stimulation, secretion of interleukins (ILs) and several other factors (160).

1.5.1 Innate immunity

The innate immune system is composed of several defence mechanisms, but can roughly be divided into two main groups. The first group is the epithelium which act at all times as the first line of defence against invading pathogens, functioning as barriers secreting mucus, antimicrobial peptides and enzymes. The second group is activated upon encounter with pathogens, and comprises the granulocytes, the monocytes, and the complement system. Monocytes circulating in the blood differentiate into macrophages when entering the surrounding tissue. The main role of the macrophages and the granulocytes is to phagocytise invading pathogens. These innate immune cells can recognize bacterial surface molecules (e.g. lipoteichoic acid); so called pathogen associated molecular patterns (PAMPS). Recognition of PAMPS occurs with specific pattern recognition receptors (e.g. toll like receptors). This may induce phagocytosis and subsequent killing of the pathogens by antimicrobial proteins and reactive oxygen (25, 101). In addition, a number of cytokines and chemokines are released upon recognition and binding of PAMPS to these immune cells, further stimulating the maturation and recruitment of more immune cells and initiating the inflammatory response (171).

1.5.2 Toll like receptors

Toll like receptors (TLRs) are present on the surface of several immune and non-immune cell types. Upon binding to PAMPs an intracellular cascade of reactions leads to the activation of transcription factors resulting in the production of several cytokines and chemokines, up-regulation of phagocytosis and maturation of leucocytes. TLR2 recognises several molecules associated with the Gram positive bacterial surface such as lipoproteins, lipoteichoic acid and

peptidoglycan (137). Typical cytokines produced by stimulation of the TLR and subsequent activation of transcription are TNF-α, IL-1β and IL-6 and chemokines such as CCL-2 (IL-8). Chemokines migrates from the foci of infection, becomes immobilised on endothelial cell surfaces and attracts neutrophils circulating in the blood, by creating a concentration gradient. The recruited neutrophils are guided to the site of infection, where they can aid in the destruction of the pathogen (47). In Table 1 different cytokines and chemokines assessed and analysed in this thesis are presented.

Table 1. Cytokines assessed and analyses in studies included in this thesis.

Cytokines	Producer cells	Cytokine function
GM-CSF (Granulocyte macrophage stimulating factor)	Fibroblasts and monocytes	Stimulates neutrophil development and differentiation.
IL-1β (Interleukin -1β)	Monocytes-Macrophages and epithelial cells	Fever, T-cell and monocyte-macrophage activation
TNF-α (Tumor necrosis factor)	Macrophages, NK cells and T cells	Promotes inflammation and endothelial activation
CXCL, IL-8	Macrophages and epithelial cells. T cells	Neutrophils, basophils, T cell subset and endothelial cells. Stimulates neutrophil recruitment

Based on reference (121) and references therein.

1.5.3 The complement system

The complement system is composed of approximately 30 different proteins in plasma and on cell surfaces (248). The main function of the complement system is to mark pathogens for

recognition and destruction by macrophages, to induce a series of inflammatory responses and to bridge the innate and the adaptive immune response. The complement system can be activated by three pathways, dependent on different molecules for their activation; the classical pathway, the alternative pathway and the lectin pathway, summarised in Figure 3 (2, 77). The **classical pathway** is activated by binding of protein C1q to bacterial surface molecules, C-reactive protein or antibody-antigen complexes, herby bridge the innate immune system to the adaptive immune system. The **alternative** pathway is activated by binding of C3b, which is spontaneously produced. Further activation in order to produce C3 convertase is only obtained through binding to a bacterial surface. **The lectin** pathway is activated by binding of carbohydrates such as *N*-acetylglucosamine, and mannose containing carbohydrates found on the bacterial surface, to the ficolin and mannose binding lectin (MBL) receptors.

All three pathways converge in the activation of the protease C3 convertase, from where they follow a common pathway. The products of the common pathway are C3a, C5a, C3b and terminal complement complex (TCC-C5b-C9). The three main effects of the products from complement activation are; i) opsonisation of pathogens by binding of C3b, inducing phagocytosis ii) disruption of the bacterial cell wall (predominantly in Gram negative bacteria) by insertion of TCC (also called membrane attack complex-MAC), produced by C5b-C9 and iii) peptide mediators of inflammation, C5a and C3a (98, 121).

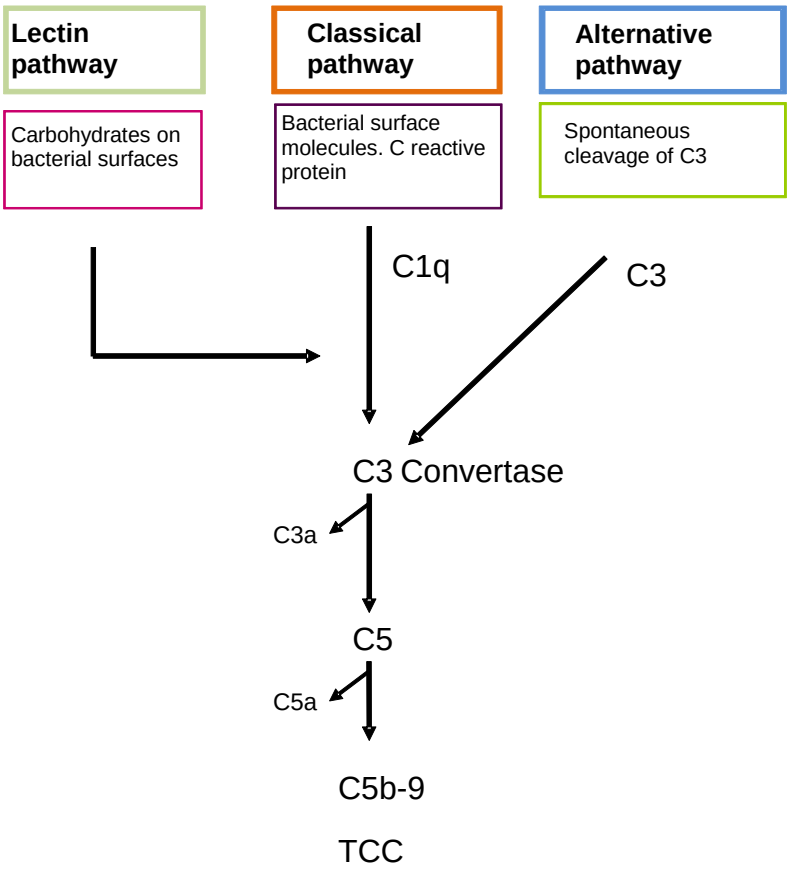

Figure 3. The three initial pathways of the complement cascade, the lectin pathway, the classical pathway and the alternative pathway. Adopted from Fredheim *et al*. (2).

1.6 Bacterial immune evasion

Albeit efficient in the protection from invading pathogens, bacteria are able to circumvent recognition by the immune defence by several mechanisms. Biofilm infections are often persistent, and remains unresolved despite activation of both the innate and the adaptive immune system (129).

Several studies have investigated the mechanisms enabling establishment of chronic infections caused by CoNS in association with medically implanted devices**.** Reduced macrophage killing by reduced phagocytic uptake in both PIA-dependent and PIA-independent (Aap, Embp) biofilms was demonstrated by Schommer *et al*. (216). Biofilm structure offers differential physiological conditions, inducing bacterial persisters and dormant cells (148). A recent study by Cerca *et al.* (29) showed reduced macrophage activation and cytokine production in biofilms with high proportions of dormant cells. Another recent report revealed induction of macrophage apoptosis by S. *haemolyticus* (144).

Evasion of killing by human neutrophils was demonstrated by Cheung *et al.* (34). The *S. epidermidis* protease SepA, and the antimicrobial peptide sensor (Aps) were involved in degradation of human antimicrobial peptides employed by neutrophils when killing phagocytised bacteria (147). SepA is under control of the quorum sensing systems *agr* and *sarA* which are also global regulator systems, coordinating adaptations to a stationary growth phase, in response to stress and high cell density (186).

The PSMs are a new class of antimicrobial peptide toxins, with cytolytic activity. PSMs are important mediators of neutrophil destruction in *S. aureus* infections, and have been identified in several CoNS, including *S. haemolyticus* and *S. epidermidis*. Functional studies have been performed with *S. epidermidis* where they did not play the same important role as in *S. aureus* infections, reflecting the less virulent nature of *S. epidermidis* (33, 94, 201).

Several animal studies have demonstrated the importance of PIA in the establishment and persistence of *S. epidermidis* biofilm infections (151, 210, 211). On the other hand, other in vivo studies have shown that lack of PIA did not abolish the ability of *S. epidermidis* to colonize and persist, which indicate the involvement of additional mechanisms (35, 74, 82).

However, in a biofilm, PIA plays a central role in host immune evasion. Studies have reported a decrease in antibody and complement deposition on PIA producing strains resulting in decrease phagocytosis (244, 245). Fredheim *et al*. (2) demonstrated that a PIA biofilm induced a

significantly lower activation of leukocytes, as well as a lower secretion of cytokines, compared to a PIA-negative biofilm.

1.7 Antimicrobial agents and resistance mechanisms

1.7.1 Biofilm and antimicrobial resistance

Bacteria surrounded by the extra cellular polymeric biofilm matrix are more resistant to killing by several antimicrobial agents compared to their planktonic counterparts (56). Several theories have been launched in order to explain the protective effects of the biofilm towards antimicrobial agents; reduced efficiency of the antimicrobial agents due to reduced penetration, the creation of a drug concentration gradient, charge repulsion, an unfavourable biofilm environment reducing the effects of the antimicrobial agent and differential growth creating a combination of dormant, dividing and persisting cells (76, 149, 150, 197). The biofilm is also a favourable environment for horizontal gene transfer due to the close proximity of bacterial cells which may facilitate the spread of resistance genes (164). CoNS and *S. haemolyticus* in particular are also resistant to several antimicrobial agents when analyses in planktonic growth. A combination of several factors is most likely contributing to the observed resistance found in bacteria inhabiting biofilms. An overview of the different target sites, resistance genes and mechanisms found in staphylococci, including means of transfer of resistance genes will be presented in the following paragraphs.

1.7.2 Antimicrobial agents and their mode of action

Since penicillin was discovered by Flemming in 1928, and introduced as the “miracle drug” during the Second World War, a wide array of different antimicrobial agents has been introduced in order to combat bacterial infections. Based upon the target site the different antimicrobial agents can be classified into five major groups; (i) inhibitors of cell wall synthesis, (ii) inhibitors

of cytoplasmic membrane function, (iii) inhibitors of protein synthesis, (iv) inhibitors of nucleic acid synthesis and (v) inhibitors of folic acid synthesis [132]. The different modes of action of antimicrobial agents are depicted in Figure 4. In the treatment of staphylococcal infections, agents affecting the cell wall, protein synthesis, folic acid pathway and nucleic acid synthesis are commonly used.

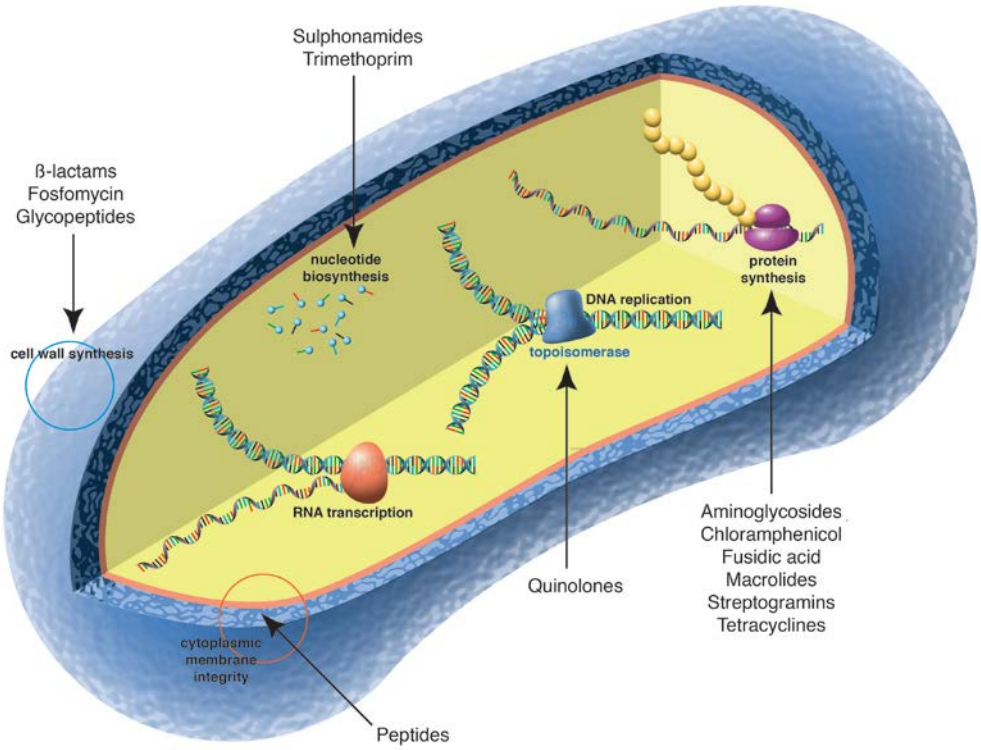

Figure 4. Targets of different antimicrobial agents used in the treatment of staphylococcal infections (figure from Roy-Andre Lysså).

In the following section resistance mechanisms towards the most commonly used antimicrobial agents used in the treatment of staphylococcal infections will be briefly presented. Genes conferring resistance, central in our study are listed in Table 2.

1.7.3 B-lactam resistance

The most Useful anti staphylococcal agents have been the β-lactam antibiotics (penicillins, cephalosporins, carbapenems and monobactams). The β-lactams are cell wall inhibitors, and block peptidoglycan biosynthesis by binding to cell wall synthesising enzymes also designated as penicillin binding protein (PBPs). Since the introduction in 1944, resistance evolved rapidly and today more than 90% of clinical isolates of *S. aureus* are resistant to penicillin (161). Penicillin resistance is mediated by β-lactamases encoded by *blaZ*, which hydrolyse the β-lactam ring. In 1959 the first penicillinase resistant antibiotic, methicillin was introduced (61). Already by 1961 there were reports of MRSA. Today as many as 70 % of the CoNS and 40-60 % of hospital adapted *S. aureus* in certain parts of Europe, USA and Japan are resistant to methicillin (54, 75, 96). Methicillin resistance in conferred by the gene *mecA* encoding an alternative PBP2a with a lower affinity for β-lactams (212). The *mecA* gene is usually carried on the staphylococcal cassette chromosomal SCC*mec*.

1.7.4 Aminoglycoside resistance

The aminoglycosides play an important role in the treatment of staphylococcal infections. They act by inhibiting protein synthesis through irreversible binding to the 30S subunit of the ribosome.

In staphylococci resistance to aminoglycosides are caused by enzymatic drug modification, conferred by four classes of enzymes depending of the modification they induce; Acyltransferases (AAC), Phosphotransferases (APHs), Nucleotidyltransferases (ANTs) and Adenyltransferases (AADs). The three most common aminoglycoside modifying enzymes in staphylococci are:

i) 6-N-acyltransferase aac2”-O- phosphotransferase encoded by *aac (6’)-Ie-aph (2”)*, conferring resistance to gentamicin, kanamycin, tobramycin and amikacin.

ii) 4'-O- adenyltransferase I encoded by *ant(4')-Ia* conferring resistance towards neomycin, kanamycin, tobramycin and amikacin.

iii) 3'-O-phosphotransferase III encoded by *aph (3')-IIIa* conferring resistance to kanamycin and amikacin (134, 156, 215).

The genes encoding the most commonly found resistance mechanism encoded by *aac (6`)-I2-aph (2")*, are carried on transposon Tn*4001*, which might be located chromosomally or on a plasmid. Prevalence of gentamicin resistance is generally higher in methicillin resistant CoNS, 73% compared to 9% resistance in *S. aureus* (116), this concurs well with other studies where 80 % of the *S. haemolyticus* isolates were found to be resistant towards gentamicin (141).

1.7.5 Macrolide–lincosamide-streptogramin B (MLS_B) resistance

The macrolides, lincosamides and streptogramins are functionally and structurally related and act by inhibiting protein synthesis by binding to the 50S subunit. MLS_B resistance is caused by three mechanisms in staphylococci (163, 203, 251).

i) Target site alteration by methylation encoded by the *erm* genes (*ermA, ermB, ermC*). Expression of *ermC* is nominally inducible, but mutations upstream of the *ermC* coding sequence cause a constitutive expression.

ii) Transportation of the antimicrobial agent out of the bacteria by efflux pumps encoded by the *msrA* gene.

iii) Enzymatic modification of the drug, encoded by the *mph*C gene.

1.7.6 Glycopeptide resistance

The glycopeptide antibiotics vancomycin and teicoplanin inhibit cell wall biosynthesis. In treatment of MRSA, vancomycin is often the drug of choice. However intermediate resistance to vancomycin was first reported in CoNS and is associated with a thickened cell wall. Genes responsible for the up regulation of cell wall precursors and alteration of the cell wall composition have been detected. *S. aureus* with intermediate vancomycin resistance (VISA) or heterogeneous vancomycin resistance (hVISA) have been isolated worldwide (27, 145). In 2002 the first report of a *vanA* containing *S. aureus* came from Michigan. *vanA* is transferable and encodes high levels (vancomycin MIC $\geq$ 128 mg/l) of inducible resistance towards vancomycin and teicoplanin (44). At the moment it appears that the incidence of acquired vancomycin resistant CoNS are low, and the number of VRSA caused by *vanA* acquisition is still low (224). However the future development needs close monitoring.

Table 2. Antimicrobial agents, target sites and resistance genes found in staphylococci.

Antimicrobial agent	**Mode of action**	**Resistance gene**	**Resistance mechanism**
Penicillin	**Cell wall synthesis**	*blaZ*	Enzymatic inactivation of β-lactam ring
β-lactams	Cell wall synthesis	*mecA*	Target site modification by using alternative PBP2a or PBP2`
Fosfomycin	Cell wall synthesis	*fosB*	Enzymatic inactivation
Glycopeptides Vancomycin	**Cell wall synthesis**	*vanA*	Target site modification
Aminoglycosides	**Protein synthesis**	*aacA-aphD*	Enzymatic drug modification
Gentamycin/Kanamycin/Tobramycin/Amikacin	**30S ribosomal subunit**		
Neomycin		*Ant(4`)-Ia*	Enzymatic drug modification
Kanamycin/Tobramycin/Amikacin			
Kanamycin		*aph(3`)-IIIa*	Enzymatic drug modification
Amikacin			
Macrolides/lincosamides Streptogramin B	**Protein synthesis 50S ribosomal subunit**	*ermA*	Target site modification
Macrolides/lincosamides Streptogramin B		*ermC*	Target site modification
Macrolides/lincosamides Streptogramin B		*ermB*	Target site modification
Phenicols/ lincosamides		*cfr*	Target site modification
Streptogramin A		*vga*	Enzymatic inactivation

Macrolides		*mphC*	Enzymatic inactivation
Macrolides		*msrA*	Active efflux
Glycopeptides Vancomycin	**Cell wall synthesis**	*vanA*	Target site modification
Tetracyclines	**Protein synthesis**	*tetK/tetL*	Membrane associated efflux pumps
	30S ribosomal subunit		
Tetracyclines		*tetM/tetO*	Ribosomal protection
Tetracyclines		*mepA*	Multidrug efflux pump
Quinolones	**Inhibition of type V topoisomerase**	*parC/grlA*	Target site modification
Quinolones		*gyrA*	Target site modification
Quinolones		*norA*	Multidrug efflux pump
Mupirocin	**Isoleucyl t-RNA synthetase**	*mupA*	Target replacement
Quaternary ammonium compounds		*qacA*	Multidrug efflux pump
Quaternary ammonium compounds	**Cytoplasmic membrane**	*qacG*	Multidrug efflux pump
Fusidic acid	**Elongation factor**	*fusB*	Binding to ribosomal elongation factor
		fusC	Binding to ribosomal elongation factor

Based on references (45, 122, 134, 156, 203, 217) and references therein.

1.8 Synthetic antimicrobial peptidomimetics

Antimicrobial peptides (AMPs) are widespread in nature, and play an important role in the host immunity against pathogenic bacteria (184). AMPs have a broad spectrum of activity, against bacteria, fungi, parasites and enveloped viruses. There are several modes of action, which reduces the risk of resistance development. Due to resistance towards many conventional antibiotics AMPs are promising as new drugs against pathogenic bacteria. Cationic antimicrobial peptides (CAP) are generally large molecules with a net positive charge, and contain approximately 50% hydrophobic residues. There are four main secondary structures; α – helical, β – sheet, loop and extended peptides, the two first are most widespread in nature. There are many structural scaffolds of the antimicrobial peptides, but their amphipathic nature seems to be the common hallmark. Due to their amphipathic nature they bind to bacterial membranes. This induces pore formation, lipid flip-flop and loss of membrane potential. At higher concentrations cell lysis is observed. AMPs have also been demonstrated to act on intracellular targets inhibiting translation, DNA, RNA and protein synthesis. The effect on the different targets seems to be concentration dependent (102, 222). In the following sections bacterial responses towards CAPs are discussed.

Naturally occurring AMP are usually large, susceptible for protease digestion and expensive to produce (15). By identifying the active site of CAPs, short cationic peptides have been produced. The new compounds have been named synthetic antimicrobial peptidomimetics (SAMPs), and harbour the attractive properties of the AMPs. For anti-staphylococcal activity two cationic units and two lipophilic bulky units is the minimum required. Studies have shown rapid bacterial killing, presumably by membrane disruption (106). Compared to conventional used antimicrobial agents the SAMPs and AMPs have shown high anti-biofilm activity *in vitro* (14).

1.8.1 Bacterial defence towards antimicrobial peptides

As bacteria have co evolved with their hosts, several bacterial defence mechanisms against antimicrobial peptides have developed. *S. aureus* has a range of mechanisms in the battle against antimicrobial peptides, such as alterations of bacterial surface charge, trapping of the peptides and proteolytic cleavage. The cationic PIA and the anionic poly glutamic acid (PGA) creates both a mechanical and an electrochemical barrier in *S. epidermidis* which may explain the observed resistance (143). In *S. epidermidis* up regulation of the three component system antimicrobial peptide sensing system (*aps*) upon release of CAPs contributes to alteration of amino acids in the cell wall components. This alters the charge of the peptidoglycan and the cell membrane, which results to increased resistance (153). Due to the small size and synthetic modification, SAMPs are poor substrates for proteolytic cleavage, and the general opinion is that there are few resistance mechanisms against antimicrobial peptides, as they act on several targets. However as resistance mechanisms towards host cationic peptides exist, one does not know if resistance mechanism will evolve if these drugs become more commonly used. As history has repeatedly shown; when bacteria join forces, we are outmanoeuvred.

1.9 The accessory genome

When challenged with antimicrobial agents and a constantly changing environment, bacteria have a remarkable ability to adapt. Plasmids, transposons, insertion sequence (IS) and phages, denoted the accessory genome, comprises non-essential genes, but plays and important role in bacterial evolution an adaption (154). Staphylococci have accumulated genes conferring resistance towards almost all commonly used antimicrobial agents used in treatment of staphylococcal infections. It has been suggested that the staphylococci share a common flexible gene pool indicated by sequence similarity between resistance genes (86, 90, 96). In *S. aureus* as much as 15% of the genome is denoted the accessory genome, including genes encoding virulence and resistance to antimicrobial agents and substrate utilisation (157). Many genes found in the

accessory genome are carried on mobile genetic elements which enables transfer and rapid adaption. In the following sections conveyors responsible of gene translocation are described.

1.9.1 Plasmids

Plasmids are usually extra chromosomal circular DNA molecules. They usually do not encode essential functions, but provide genes which might benefit the bacterial host (31). Staphylococci commonly carry one or more resistance plasmids. Large staphylococcal plasmids usually carry resistance to several antibiotics, metals, antiseptics or disinfectants as well as virulence genes (40, 55, 131, 218). Based on their conjugation and replication ability the plasmids are taxonomically grouped in three families (22, 72):

i) the rolling circle replicating (RCR) plasmids.

ii) the theta replicating plasmids which are subdivided into the

pSK41-like conjugative and non-conjugative metal and antimicrobial resistance plasmids.

The small RCR plasmids usually carry one resistance gene, whereas the larger theta replicating plasmids carry multiple resistance genes which are often located on one or more transposon like elements. The modes of transmission are by conjugation and transduction (6, 79, 239). Plasmids can also recombine, form co integrates and integrate into other plasmids, and in this manner form new multi resistance plasmids (217). The largest plasmids encode their own mobilisation, and often mediate the co-transfer of smaller plasmids. A study performed by Shearer *et al*. showed that 90 % of the investigated staphylococci carried one or more plasmids (218).

1.9.2 IS elements and transposons

Transposons are genetic elements able to move from one DNA molecule to another. The smallest transposons are the IS which only encode the enzymes necessary for their own movement. Composite transposons are formed when two IS elements brackets genes. The composite

transposons enable the movement of genes conferring antimicrobial agents or heavy metals (31, 189, 202). In the fully sequenced *S. haemolyticus* JSCS 435, 82 IS elements were detected, of which 60 were intact. This is a large number compared to *S. epidermidis* ATCC 12228 and *S. aureus*, where only 18 and 13 IS elements were intact respectively. Two IS groups, IS*1272* and ISSha*1* comprised 68% of the IS elements found. The large amount of IS elements is believed to contribute to genome plasticity, by means of rearrangements or deletions. Transposons can integrate in the chromosome at various sites or in plasmids. The latter enables transfer to a new host where it can excise and re-integrate in to the recipient chromosome (7, 157). There are several examples of transposons mediating resistance.Tn*1546* transferred from enterococci to staphylococci have introduced vancomycin resistance in *S. aureus* (32). Two transposons carrying antibiotic resistance were found in the genome of JCSC 1435, Tn*552* encoding ß-lactamase and Tn*4001* known to encode aminoglycoside resistance (230).

1.9.3 Staphylococcal cassette chromosome

The staphylococcal cassette chromosome (SCC) is a family of mobile elements first described in the staphylococci (125). SCCs act as vehicles transporting antibiotic resistance genes as well as other genes, in addition to integrated transposons and plasmids. SCC carrying *mec*A encoding resistance to methicillin has caused the emergence of MRSA. The origin of SCC*mec* is unknown, but it has been hypothesised that it were originally transferred from *S. haemolyticus*, as IS *1272* found in the class B *mec* complex is prevalent in *S. haemolyticus* (112, 113, 124). The SCC element integrates at a sequence specific site (*attB*scc) in the chromosome, located near the origin of replication at the 3' end of an open reading frame (ORF) of unknown function, designated *orfX* (125, 221). Movement by excision and integration is carried out by specific cassette chromosome recombinases. Classification of the SCC*mec* elements is based on the combination of one of the eight allotypes of recombinases (*ccr*AB 1-6, *ccr*C 1-2) and the one of the five types of the *mec*-complex (A-E). The *mecA*-complex is composed of the intact copy of the *mecA*- gene, and complete or truncated copies of the regulatory genes *mecI* and *mecR1* and one copy of IS *431or* IS *1272* (36, 126, 221). In *S. haemolyticus mec*- complex C is most prevalent, but frequent rearrangements of the *ccr*- genes leads to new SCC*mec* types, indicating extensive diversity of

SCC in CoNS (26, 103). In *S. aureus* 11 SCC*mec* types are currently identified (http://www.sccmec.org, accessed 26.09.2012).

1.9.4 Bacteriophages

Bacteriophages are viruses infecting bacteria. In the process of infection bacteriophages are capable of transferring DNA at high frequencies, either by generalised or specialised transduction. Generalised transduction is assumed to be the most common mode of horizontal gene transfer in staphylococci (236). In the genomes of *S. aureus*, one to three prophages often carrying virulence genes, are usually found (157). Prophages have also been found in the genomes of both *S. haemolyticus* JCSC 1435 and *S. epidermidis*. The two prophages found in *S. haemolyticus* were associated with β-lactam resistance and a truncated mercury reductase homolog (230).

1.10 Barriers to horizontal gene transfer

Although the staphylococci have several means of acquiring new genes, defence mechanisms conferring immunity against integration of foreign DNA such as phages and plasmids have been identified. The acquisition of mobile DNA is not necessarily beneficial, and it can have deleterious effects on host fitness (200). Two systems maintaining genomic integrity in staphylococci will be described. The recently discovered clustered regularly interspace short palindromic repeats (CRISPR) systems is based on sequence directed immunity. Clusters of short DNA repeats separated by spacers are found in the genomes of several bacteria. They are usually flanked by a set of CRISPR associated protein coding genes. The CRISPR operon consists of multiple direct repeats, separated by stretches of variable sequences called spacers. The spacers are short pieces of DNA mostly corresponding to DNA sequences of interfering DNA. Adjacent CRISPR associated genes encodes proteins such as helicases, nucleases, polymerases and RNA binding proteins, involved in maintenance of the CRISPR function (97, 117, 127, 172). DNA

uptake is regulated in a three step process, where foreign DNA is integrated, expressed and removed (133, 170). CRISPR loci have been found in the genomes of approximately 40% of all bacteria (172).

The second system representing a barrier for horizontal gene transfer is a “restriction modification system”. This system acts by methylating DNA at specific sequences. If foreign un-methylated DNA sequences are detected, it will be digested. Type I and II restriction systems are found in *S. aureus* (41, 227, 246). It is believed that efficient transfer of genetic information is greatly reduced by the presence of these mechanisms, something that was clearly demonstrated when comparing DNA transfer frequencies in isolates of *S. aureus* with and without mutations in the *Sau1* restriction modification system (227).

Systems restricting uptake of foreign DNA are themselves found on mobile elements. In addition to restricting uptake of foreign DNA such systems are also believed to be involved in elimination of unstable genomes and to maintain intact genome (123, 257).

1.11 The use of animal models

Biofilm associated infections are complex. There are several factors contributing to the persistence of these infections, such as reduced effect of antimicrobial agents, host immune evasion and the differential mode of growth of bacteria inhabiting the biofilm. The concerted actions of all these factors are impossible to mimic in an *in vitro* laboratory experiment. The *in vitro* conditions work well enough in the study of an isolated effect, such as comparing effects of antimicrobial therapy, or the effect of knocking out a gene in order to study gene function. However, in a living organism, several regulatory systems function in parallel, and cross talk between the systems induces effects which are difficult to perceive. Laboratory animals such as mice are widely used to study staphylococcal infections owing to (i) the presence of many well characterised inbreed and outbreed strains, (ii) the presence of strains lacking or over expressing genes of interest, (iii) the existing knowledge of the murine immune system and (iv) the development of staphylococcal infections in the mice (39). Commonly used mouse strains for the purpose of animal experiments are the BALB/c and the NMRI mice. The BALB/c mice are

inbred mice resulting in a homogenous population, where the responses towards treatment are similar. The NMRI mice are outbred resulting in a more heterogeneous population. The animal to animal variation might be larger, and a more diverse response might be observed. Ideally one should include both inbreed and out breed mice, in order to fully examine the response, however by using inbred mice the statistical power might be higher, and the number of mice needed in each experiment might be reduced.

Animal experiments should only be conducted after thorough *in vitro* studies. In order to ensure the maximal outcome of experiments including the use of animals, detailed planning is important. The 3Rs principle was first introduced in the book "The principle of human experimental technique" from 1959 by Russel and Burch (198). The three Rs stand for Replacement, Reduction and Refinement. Replacement refers to alternatives replacing the use of research animal. Reduction refers to methods reducing the number of animals used, by refining methods and statistical calculations. Refinement involves methods reducing animal suffering and distress. The guidelines in; Animals in research; reporting *in vivo* experiments (ARRIVE) consists of a checklist of 20 items which describes the minimum information that all scientific publications using laboratory animals should include. Information such as animal numbers, strain, sex, age and weight should be included, in order to ensure inform and guide future research. Good reporting is essential as detailed reports maximises the output of the animal experiment, and serves as important guidelines for further studies (138).

2 Aims of study

The main objectives of this study were to;

1. Identify genetic markers of *S. haemolyticus* useful for molecular typing schemes and through deep sequencing data, gain insight in the molecular background for antimicrobial resistance in this species.

2. Determine the effect of synthetic antimicrobial peptidomimetics (SAMPs) on staphylococcal biofilms *in vitro* and the clinical effect *in vivo* in an animal model.

Paper I: This work aimed to find a molecular typing method suitable for molecular epidemiology of *S. haemolyticus*. The discriminatory power of Pulsed Field Gel Electrophoresis (PFGE), Multi Locus Sequence Typing (MLST) and Multi Locus Variable number of tandem repeat Fingerprinting (MLVF) were investigated and compared.

Paper II: This work aimed to answer questions concerning the core genome conservation observed for *S. haemolyticus* in paper I. It also aimed to reveal the genetic basis of multi-resistance found in *S. haemolyticus*. High throughput sequencing was applied to determine the complete genome of 134 *S. haemolyticus* isolates.

Paper III: This work aimed to determine the antimicrobial effect of different antibiotics and synthetic antimicrobial peptidomimetics (SAMPs) on established staphylococcal biofilms *in vitro*. A simple screening method to quantify the metabolic activity of biofilms was evaluated.

Paper IV: This work aimed to apply a device related peritonitis model *in vivo* to investigate the efficacy of a SAMP (Ltx 21) compared to vancomycin with a particular focus on biofilm clearance and host innate immune response.

3 Material and methods

3.1 Materials

Paper I:

172 *S. haemolyticus* isolates were obtained from national and international collaborators. The isolates were collected during the period 1989 to 2010. The collection comprised 164 human clinical isolates (blood, urine, catheters, nose and wound), four human community acquired isolates and four isolates of animal origin (bovine, equine and porcine). The isolates were defined as community acquired if they were recovered within 48 hours of hospitalisation or isolated from healthy individuals without prior hospitalisation the past year (136). The age of the patients varied from zero to 45 years. Geographically the isolates originated from Belgium (n=2), Germany (n=13), Greece (n=1), Japan (n=17), Norway (n=74), Spain (n=2), Switzerland (n=50) and United Kingdom (n=12), USA (n=1). MLST and MLVF were performed on a selected collection of diverse isolates (n=45).

Paper II:

From the collection of 172 *S. haemolyticus* isolates described in paper I, a selection of 134 isolates of *S. haemolyticus* from geographically diverse origins (Belgium, n = 2; Germany, n = 10; Japan, n = 13; Norway, n = 54; Spain, n = 2; Switzerland, n = 43; UK, n = 9; USA, n = 1) was included.

Paper III:

Six staphylococcal isolates, two *S. haemolyticus*, two *S. epidermidis* and two *S. aureus* isolates were chosen based on their biofilm producing ability. One strong and one weak biofilm producer were included for each of the staphylococcal species. All isolates were blood culture isolates apart from the *S. aureus* isolates which were isolated from joint fluid.

Paper IV:

Bacterial isolates: The PIA producing *S. epidermidis* 1457 was used. This isolate was originally isolated from an infected central venous catheter at the university of Hamburg-Eppendorf, Germany (167) and is commonly used as a *S. epidermidis* biofilm producing reference strain.

Mice: Inbred female BALB/c mice of 8-9 weeks were used (Taconic M&B A/S Ry, Denmark). On average the mice weighed 20 Grams. In total 132 mice were used. Animals were randomly separated into treatment groups after insertion of the pre-colonised implants (vancomycin, SAMP or placebo). Twelve animals were sacrificed from each treatment group at day 3, 6 and 8 after insertion of the implants. Three control groups were included i) animals without surgery (n=6), ii) animals with clean implants treated with NaCl (n=9) and iii) animals with clean implants treated with peptide (n=9). Three mice from control groups ii) and iii) were sacrificed at day 3, 6 and 8 after insertion of the implants. No selection was made on the days of sampling.

3.2 Methods

3.2.1 Species identification

Paper I and IV:

Species identification was performed by Gram staining, catalase test and coagulation assay by Staphaureux plus® (BioMerieux, Marcy l'Etoile, France) followed by partial 16S rRNA gene or *rpoB* sequencing (58, 195). Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) was used in order to determine species of small colony variants observed (19, 59).

3.2.2 Antimicrobial susceptibility testing

Paper I-IV:

Antimicrobial susceptibility testing was performed using Etest according to the manufacturer's description (AB BIODISK, Solna, Sweden). The antimicrobial breakpoints were interpreted according to the EUCAST guidelines (62). Minimal biofilm inhibitory (MBIC) concentration was determined by using Alamar blue (Invitrogen, Carlsbad, CA, USA) as an indicator of metabolic activity. Complete suppression of metabolic activity was obtained if a certain agent caused a reduction of Alamar blue ≤ negative control +2 standard deviations. A strong suppression was obtained when the reduction of Alamar blue were ≤75% compared to the positive control.

3.2.3 Biofilm quantification

Paper I-IV:

The biofilm producing ability of the isolates was determined by a semi-quantitative assay (37, 139). Briefly, overnight cultures were diluted 1:100 in Tryptic Soy Broth (TSB, Becton Dickinson, Puls AS, Norway) with 1% glucose and incubated for 24 hours at 37°C in polystyrene microtiter plates (Nunclon, Roskilde, Denmark). The biofilm was washed, fixed and stained with crystal violet. Before detection the stain was dissolved with an ethanol/acetone mixture. Semiquantitive determination of the biofilm mass was performed by measurement of the optical density in an ELISA reader.

3.2.4 Foreign body implant model

Paper IV:

Pre colonized silicone implants measuring 5x5 mm were cut from silicon tubes with a thickness of 2 mm (Ole Dich, Denmark) and inserted into the intraperitoneal cavity of mice in order to mimic a foreign body infection. The mouse implant infection model used was established by Christensen *et al.* [172, 173].

Preparation of implants: Inoculum was adjusted to an optical density (OD_{600}) of 2 McFarland in 0.9% NaCl. Biofilm growth was induced by using TSB with 1 % glucose. The implant model required several steps of optimisation, concerning implant material and inoculation time. A thick adherent biofilm was obtained by culturing the bacteria on the implants for 24 hours, and transferring the implants to a new flask containing new media (TSB with 1% glucose) every 24 hours, for a total of five days. We did not manage to establish a biofilm on the implants by direct injections of bacterial culture into the peritoneal cavity. No differences in biofilm formation were observed when using implants pre –incubated in mouse plasma or not.

Treatment. SAMP Ltx 21 concentrations of 0.5 mg/kg, 2mg/kg, 10mg/kg or 20mg/kg were tested in a pilot study. The final treatment dose of 10mg/kg was chosen due to animal welfare and due to no apparent concentration dependent effect between the chosen dosage and higher doses. Vancomycin 20 mg/kg (Sandoz) was given according to previous studies.

Collection of blood and peritoneal fluid. Mice were sedated by sub cutaneous injections of Hypnorm, Midazolam (2:1) in sterile water (Roche). Blood was collected by cardiac puncture of the deeply sedated mice. The blood was drawn in syringes containing heparin or lepirudin (Refludan®), depending on further analyses. Blood used for complement analyses was added lepirudin, and blood for FACS was added heparin. Blood for FACS analyses was stored on ice until further analyses (on the same day). Blood for complement and analyses was centrifuged, and plasma was kept at -70ºC until further analysis. Peritoneal fluid was collected by injecting 5ml of PBS, followed by gently massaging of the abdomen. The fluid was then re-collected and stored on ice/-70ºC until further analysis.

3.2.5 Complement, cytokine and FACS analysis

Paper IV:

Complement: Levels of C5a and C3a were measured by using the enzyme-linked immunosorbent assay kits C5a (E90399Mu), C3a (E90387Mu) (USCN life Science Inc.). The measurements were performed in plasma according to the manufacturer`s description.

Cytokines: Levels of interleukin 1β (LUM401), TNF-α (LUM410), G-CSF, (LUM415B), MIP-2 (LUM452) and MCP-1 (LUM479) were measured in plasma and peritoneal fluid using Fluorokine ® MAP (cat.no LUM000) from R&D systems in combination with a dual laser, flow based sorting and detection analyser (Luminex Corporation), according to manufacturer's description.

Blood cell counts: Total leucocyte counts and fraction of granulocytes and macrophages in blood and peritoneal lavage fluid was estimated according to a modification of Jensen *et al*. (130).

3.2.6 Microscopy

Paper III and IV.

Biofilm imaging was performed using an inverted confocal laser scanning microscope (Leica TCS SP5). Biofilms were dyed using LIVE/DEAD® dye containing SYTO 9 and propidium iodide (Invitrogene). SYTO 9 was detected using the argon laser at wavelength 488 nm, and PI was detected using the 561 nm line. The fluorescent signals were collected sequentially at 400 Hz. Biofilm thickness was measured using the z-stack mode, creating an overlay of several images.

3.2.7 Molecular epidemiological typing

Paper I and II.

PFGE after restriction digestion by SmaI, was performed on all isolates used in paper I and II, by applying a previously described method [174]. PFGE patterns were analysed using Gel Compar software version 2.5 (Applied Maths, Ghent, Belgium).

Paper II.

Multi locus sequence typing (MLST): MLST was performed using the newly designed *S. haemolyticus* MLST scheme, including the genes; *arcC, cfxE, hemH, leuB,* Ribose ABC, SH 1200 and SH 1431. The selection of the final genes applied in the newly designed MLST scheme was based on a selection of the most discriminatory genes tested. The initial 18 genes tested were selected based on previously published MLST schemes for *S. aureus* and S. *epidermidis*, in addition to genes found to confer more diversity is *S. aureus* and genes exclusively found in *S. haemolyticus*. The genes initially tested were dispersed throughout the genome of *S. haemolyticus*. We designed primers for the selected genes based on the fully sequenced genome of *S. haemolyticus* JCSC 1435 (230). Sequence types were assigned based on allelic variation, using the fully sequenced JCSC 1435 as the default sequence.

Multi locus variable number of tandem repeats fingerprinting (MLVF): Tandem repeat regions were detected in the published genome of JSCS 1435 (accession number AP006716) using the tandem repeats finder (http://tandem.bu.edu/trf/trf.html) (Benson, 1999). The number of putative target genes was in total 45. Nine of them contained tandem repeats and were selected for the assay. Nine PCR primer pairs targeting conserved flanking regions of repeat containing genes (orfs SH 0324, SH 0326, SH 0326b, SH 0040, SH 0040b SH 0999, SH 01184, SH 1645 and SH 2426) were designed using Jellyfish (version 1.3 Biowire). PCR products were separated agarose gels, and the number of bands was visually inspected.

3.2.8 Phylogenetic analysis

Paper I.

PFGE: The Dice band-based similarity coefficient was calculated with a band position tolerance of 1.0%. The overall genetic relationship was determined creating a dendrogram by the unweighted pair group method with arithmetic means (UPGMA) logarithm. The isolates were assigned to different groups, where groups were defined as two or more isolates with >80% similarity [175].

eBURST: Each of the selected isolates was defined by a seven digit allelic profile where each unique allelic profile defines a sequence type (MLST) or a repeat type (MLVF). eBURST V3 (http://eburst.mlst.net) was used to determine the most putative relationship between isolates [176, 177]. eBURST was performed both for the MLST and the MLVA data.

Molecular Evolutionary Genetics Analysis (MEGA): Neighbor joining dendrogram for the individual MLST loci were created. Maximum likelihood phylogenetic trees were constructed for the concatenated MLST sequences of six of the seven loci (*cfxE*, *hemH*, *leuB*, Ribose ABC, SH 1200 and SH 1431) using the general time reversible model with 2000 bootstrap resampling replications [178]. All analyses were performed using Molecular Evolutionary Genetics Analysis 4 [179].

3.2.9 DNA isolation and quantification

Paper I and II.

DNA used for PCR was isolated by the boiling method (104). Full genome sequencing required DNA concentrations of 20 ng/µl. DNA was isolated by using phenol/chloroform extraction, and precipitation by NaAc according to Saulnier (30). The DNA concentration was measured by use of Quant-iT™ Pico green® ds DNA (Invitrogen P7589) according to the manufacturer's instructions.

3.2.10 Sequencing

Paper I.

Sequencing of the MLST PCR products was obtained by Sanger dideoxy terminator sequencing (Applied Biosystems 3130xl Genetic Analyzers).

Paper II.

Full genome sequencing was performed by the Illumina sequencing technology as previously described (20).

3.2.11 Bioinformatics analysis

Paper I.

Sequences were aligned and analysed by using BioEdit version 7.0.9.0 (99).

Paper II.

Each genome was assembled individually using VELVET (258) and contigs > 500 bp were ordered relative to the complete reference genome sequence (Accession number: AP006716) using ABACAS (10). Protein coding sequences (CDSs) were predicted using Glimmer 3 (53). Functional annotations were transferred from the fully sequenced *S. haemolyticus* JCSC 1435 by using annotation_update.pl (Sanger Institute script)

Clustering of homologous genes was performed using OrthoMCL (152) on the translated protein sequences of all predicted genes.

The phylogenetic relationship between the isolates was studied using two different strategies. I) phylogeny was built based on gene content. Pairwise distances were calculated using an approach similar to the one proposed by Snel *et al* (21). II). Second, isolate relationship was built from variable sites in the form of single nucleotide polymorphism (SNP) in the core genome using Gubbins (46).

3.2.12 Statistical analysis

Paper III.

Pearson's two-tailed correlation between the Alamar blue and crystal violet methods was calculated on averaged data. Statistical analysis was performed with SPSS for Windows software version 14.0.

Paper IV.

Two-way ANOVA test with Bonferroni multiple tests were used to compare complement response, cytokine secretion, blood cell count fractions and CFU between the three different treatment groups using the GraphPad Prism version 5 (GraphPad Software, Inc., San Diego, CA, USA) software, or by using non parametric Mann-Whitney U tests using the IMB SPSS Statistics 19 software, when comparing two groups.

3.2.13 Ethical considerations

Paper I and II.

The regional committee for medical research ethics and the Norwegian Social Science Data approved the retrospective collection and analysis of patient data. All patient data was made unidentifiable.

Paper IV.

The animal studies were carried out in accordance to the European Convention and Directive for the Protection of Vertebrate animals used for Experimental and Other Scientific Purposes and the Danish law on animal experimentation. All animal experiments were authorized and approved by the National Animal Ethics Committee, Denmark.

4 Summary of main results

Paper I: Core genome conservation of *Staphylococcus haemolyticus* limits sequence based population structure analysis.

- Thirty eight separate PFGE types were defined among the 45 *S. haemolyticus* isolates. Among these 38 PFGE types there were six groups (A–F). The largest group (B) contained three isolates from Switzerland. The remaining five groups contained two isolates each. The remaining isolates (n=32) were considered unrelated when using an 80% cut-off value.

- MLST analysis resulted in 17 unique STs. eBURST grouped the isolates in one major group or clonal complex (CC), two minor CCs and six singletons. There was a good discrimination between isolates of human and animal origin, but not between isolates of different geographical origin.

- Visual categorization of band patterns from the MLVF analysis, defined fourteen unique repeat types (RTs) among the isolates. eBURST grouped all isolates, except one of the veterinary isolates in one CC. Sixteen isolates originating from the UK, Norway, Switzerland, Japan and Greece shared the same RT.

- PFGE had a higher discriminatory power than MLST and MLVF. The overall concordance of the different typing methods was low. The highest concordance was found between MLST and MLVF. The directional agreement between PFGE and MLST as well as between PFGE and MLVF was low. Also the probability of MLST to predict MLVF type and vice versa was very low.

- The new sequence based typing methods (MLST and MLVF) did not resolve the population structure of *S. haemolyticus* but rather suggested the presence of a conserved core genome.

Paper II: Whole genome sequencing reveals clonal expansion of multi-resistant *Staphylococcus haemolyticus* in European hospitals

- Phylogenetic reconstruction based on core genome SNPs of 134 sequenced isolates divided the isolates in one minor (8) and one major group (126 isolates). SNP analyses revealed hot spots of recombination throughout the genome associated to specific locations. A high degree of recombination was observed around the *oriC* environ. In general, isolates in the minor group carried less genes encoding antimicrobial resistance compared to the isolates forming the major group and most of them (7/8) were *mecA* negative.

- The 126 isolates forming the major group were further divided into 7 clades (A-G). Five of them (A-E) were quite homogenous while two (F-G) were composed of more divergent isolates. The observed similarity between isolates within Clades A-E suggested an expansion of a successful common ancestor in these groups.

- Most isolates showed multiresistance to antimicrobial agents and overall there was a good concordance between phenotypic observations and the presence of cognate resistance genes. However, 5 isolates resistant to erythromycin did not contain any of the known genes encoding macrolide resistance. From the genomic sequences a novel Erythromycin Resistance Putative gene Cluster (EPRC) was identified in a total of 37 erythromycin resistant isolates.

- The frequent acquisition of resistance genes might possibly be due to the low prevalence of CRISPR elements and host restriction systems.

Paper III: High in vitro antimicrobial activity of synthetic antimicrobial peptidomimetics against staphylococcal biofilms.

- Detection of metabolic activity in biofilms by Alamar Blue (AB) proved to be a highly sensitive method, revealing a strong correlation between biofilm quantification by crystal violet staining and measured metabolic activity. The AB method was able to detect dose-dependent differences in the effect of antibiotics and SAMPs.

- Ltx SAMPs were clearly more effective in reducing metabolic activity in staphylococcal biofilms at low concentrations compared with rifampicin, linezolid, tetracycline and vancomycin.

- SAMPs were frequently able to suppress metabolic activity completely, indicating effective killing.

- Based on confocal images, the mode of action is believed to be by lysis of staphylococci when these agents are used above a certain concentration. Apparently Ltx SAMPs cause damage of the bacterial cell membranes even in slow growing or dormant bacteria embedded in a biofilm.

- Ltx SAMPs are potential new therapeutic agents in biofilm-associated infections.

Paper IV: Efficacy of synthetic antimicrobial peptide against *Staphylococcus epidermidis* peritonitis in a device related murine infection model

- The *S. epidermidis* (SE) device related murine infection model enabled the study of effects of two different treatment regimens and the host innate immune response towards a mature biofilm.

- Treatments with neither vancomycin nor Ltx 21 resulted in complete clearance of bacteria from the implants. Mice treated with vancomycin had a significantly lower reduction in CFU counts on their implants compared to the Ltx 21 and placebo treated groups on day 3, 6 and 8.Ltx 21 treatment of mature SE biofilms *in vivo* did not correspond to the efficacy observed *in vitro*.

- Ltx 21 treatment of mature SE biofilms *in vivo* did not correspond to the efficacy observed *in vitro*.

- Mice treated with vancomycin had a significantly stronger reduction in CFU counts on their implants compared to the Ltx 21 and placebo treated groups on day 3, 6 and 8

- Elevated levels of leucocytes in the peritoneal fluid from mice with pre-colonised implants, receiving treatment (Ltx 21, vancomycin or placebo) were observed throughout day 3, 6 and 8 when compared to animals with sterile implants.

- Significantly increased values of GM-CSF in blood were observed in animals with sterile implants compared to animals without surgery on day 3, 6 and 8.Mice with sterile implants showed significantly elevated levels of MCP-1 in peritoneal fluid on day 3, 6 and 8 compared to mice with pre-colonised implants.

5 General discussion

5.1 Answers sought-questions found

The questions we sought to answer when this work was initiated were many. The studies included in this thesis, aim to answer questions about; i) what is the genetic relationship between isolates of *S. haemolyticus* and what is the genetic basis for their multi resistance (Paper I and II), and ii) can staphylococcal biofilms be treated by novel SAMPs *in vitro* and *in vivo*, and what are the effects of SAMPs compared to vancomycin on *S. epidermidis* biofilms. We also wanted to investigate factors contributing to successful persistence of biofilm associated infections, if success is defined as survival of both host and bacteria (Paper III and IV).

To date study of the molecular epidemiology of *S. haemolyticus* has been limited to a few studies applying pulsed field gel electrophoresis (PFGE). These studies have identified persistent endemic clones of multi –resistant *S. haemolyticus* in different units (18, 26, 51, 140, 187, 229). The primary question posed, was if there were specific global lineages of *S. haemolyticus* associated with nosocomial infections, biofilm formation and increased resistance? In Paper I, we aimed at developing MLST and MLVF schemes in order to identify possible clonal lineages of *S. haemolyticus*. MLST and MLVF were based on the, until then, only sequenced genome of *S. haemolyticus*, JCSC 1435 (230). This limited the selection of loci, as a comparative analysis of several genomes could have detected genes with more variability. Typing by MLST and MLVF revealed a conserved core genome of *S. haemolyticus*, and did not provide sufficient discriminatory ability. A second MLST scheme was developed by Voronina *et al*. (242) in parallel to the establishment of our MLST scheme. The almost simultaneous publication prevented a comparison of the two typing schemes. Of the 14 genes employed in the two MLST schemes, only, *arcC* was used by both schemes. In addition the Russian MLST scheme included *mvaK, rphE, gtr, tpi* and *aroE*. The tree latter genes were excluded from further analysis in the establishment of our scheme, due to their poor discriminatory power. However in this paper,

published in Russian only, the authors described that MLST had a discriminatory ability dividing 28 strains into 11 genotypes [229].

5.1.1 Typing and re-typing

Application of the newly developed MLST and MLVF schemes for *S. haemolyticus* in paper I, resulted in the finding of a conserved core genome, and neither typing methods provided sufficient discriminatory ability in order to discern possible hospital associated clones. To date there are few MLST and MLVA schemes developed for the epidemiological typing of staphylococci. In addition to the current MLST schemes for *S. haemolyticus*, *S. epidermidis* and *S. aureus*, there is a scheme under development for *S. pseudintermedius* (1). The developed MLVA schemes for *S. epidermidis* and *S. aureus* confer good discrimination, comparable to MLST and PFGE (80, 81).

It was challenging to select the most "discriminatory genes" in the development the new *S. haemolyticus* MLST-scheme (Paper I) as only one fully sequenced isolate was published (230). Initially we tested several genes in geographic diverse isolates, but low levels of polymorphism were found. In comparison, in the process of the development of the presently used MLST scheme for *S. epidermidis*, there were originally three schemes developed (Wang, Wisplinghof and one unpublished scheme by Peacock *et al.*), (250, 254). The application of these initial schemes did not manage to resolve the population structure of geographically and clinically diverse *S. epidermidis* isolates. The scheme currently in use, was developed by a comparative study of the three previously designed schemes (233). The initial difficulties in establishing a MLST scheme with sufficient discriminatory ability for *S. epidermidis* could reflect core genome conservation also found in this species. This was supported by a previous study, where the presence of conserved housekeeping genes in *S. epidermidis* was observed (179). Population structure analysis of *S. epidermidis* by MLST resulted in the proposal of a clonal population structure of clinical isolates, with evidence of recombination. However there seems to be a higher degree of variation in *S. epidermidis* compared to *S. aureus*. Distribution of *S. aureus* inferred by MLST, seems to be dominated by a few successful clonal lineages which have acquired increased virulence and resistance (105).

As the developed MLST and MLVF schemes did not answer our questions regarding the *S. haemolyticus* population structure we decided to perform full genome sequencing on 134 *S. haemolyticus* isolates (Paper II**),** in order to further understand the genomic composition.

Full genome sequencing provides molecular epidemiological data with a high discriminatory power, enabling resolution of relationships between closely related isolates, not obtainable by MLST. Comparative analyses based on full genome sequencing have been employed for epidemiologic analyses of closely related MRSA isolates, both for the investigation of intercontinental spread of isolates belonging to the same clone, outbreak situations and for the studies of microevolution within isolates subsequently sampled from one patient (105, 146, 175). In *S. aureus* full genome sequencing has proved to be a valuable tool, resulting in detailed insight into transmission events, factors responsible for host adaption and has also proved to be a useful tool in the discovery of new drug-resistance mechanisms (87).

Phylogenetic reconstruction based on core genome SNPs was performed on 134 isolates and clustered them into two groups; one major group comprising 126 isolates and a minor group of 8 isolates. The major group comprising 126 isolates revealed evidence of possible clonal expansion of five groups, associated with European hospitals. Within the seven clades further sub-grouping of isolates was possible. These sub-groups were associated both with geographic origin and year of isolation. Isolates from a Norwegian neonatal unit, isolated from 1991-2001, were clustered together. This indicated the expansion of a particularly well adapted clone, in line with findings from a previous study where these isolates were clustered in one single PFGE type (140). In addition to the clonal groups, two clusters of more diverse isolates formed within the major group, and 8 isolates forming the minor group, representing more diverse *S. haemolyticus* isolates were detected. In order to answer our initial question; there seems to be a correlation between hospital adaption and clonal expansion of particular successful clones in *S. haemolyticus*.

In several studies *S. haemolyticus* is ranked as the most antibiotic resistant CoNS species (49, 86, 116, 142) . The genetic basis for multi resistance was investigated by a search for genes known to encode antimicrobial resistance in staphylococci. There was a high prevalence of resistance genes detected in the sequenced isolates. Resistance genes were detected both located on plasmids,

transposons and chromosomally integrated. Systems known to restrict the acquisitions of foreign DNA in staphylococci were only detected in a few *S. haemolyticus* isolates, which could explain the fluid acquisition of foreign DNA observed in the majority of the collection (paper II) As systems restricting uptake of foreign DNA are themselves found on mobile elements (256), they might have been lost as an adaptive response, due to high selective pressure. In *S. aureus* the host restriction systems Sau1, has proven to efficiently reduce horizontal gene transfer between different *S. aureus* lineages (246), which is hypothesised to have contributed to acquisition of e.g. SCC*mec* and *vanA*, only in a few lineages. In *S. epidermidis*, prevention of plasmid conjugation by the CRISPR system has been demonstrated in one of the two full genome sequenced isolates. The absence of these host restriction systems in *S. haemolyticus* and the observation of several recombinases associated with SCC*mec* indicate that mobile genetic elements play an important role in *S. haemolyticus* adaption. It could be tempting to speculate that the evolutionary refinement of *S. haemolyticus* and possibly other CoNS has not yet resulted in the specialisation of few lineages better adapted to a hospital environment, as observed in *S. aureus*. This could reflect the recent introduction of CoNS as opportunistic pathogens.

However one should bear in mind that inferring population structure mainly based on a biased (selected) collection of isolates causing disease possibly could lead to wrong assumptions. Additional isolates of community and carriage origin should be included, in order to correctly infer the population structure of *S. haemolyticus*. Thus, it would be interesting to study the frequency of resistance and systems restricting uptake of foreign DNA in community associated *S. haemolyticus*.

5.1.2 Survival of the fittest by the employment of modules

Bacterial survival is based on evolutionary adaption and horizontal acquisition of genes in response to rapid external alterations. The ability to fight change with change drives evolution (177). Frequent uptake and integration of antibiotic resistance mechanisms are associated with increased cost and reduced fitness (5), however in *S. haemolyticus* the presence of resistance genes seems to be prevalent. The stabilisation of resistance in bacterial populations can be due to mechanisms restoring host fitness in spite of the presence of resistance genes such as

compensatory mutations, coselection of resistance genes or introduction of cost free mutations have been detected (5). The concerted action of these mechanisms, and the fact that a selective pressure can be exerted by low concentrations of antibiotics, results in maintenance of resistance in bacterial populations (118).

Baquero (12) proposes modularization as one of the mechanisms bacteria can employ to circumvent the costly and possibly deleterious effects of adaptive variation. By sectioning the genome in primary and secondary modules, essential housekeeping/maintenance genes are more protected from variation as they are located in second order modules. Genetic variation is mainly located in modules of the first order. This is proposed to create a mosaic pattern composed of modules shaping the genome. Modularisation is frequently enhanced by IS elements (13). In *S. haemolyticus* JSCS 1435, several intact IS elements, enhancing genomic flexibility and high variability in the *oriC* environ was observed (230). Non-random distribution of genomic variability was also observed in the current sequenced S. *haemolyticus* collection. SNP analyses showed the presence of areas of conservation interspersed with areas of variation, revealing a non-random mosaic SNP structure. Areas of variation seemed to be associated with specific genes/sequences in the majority of the isolates. SNP analysed confirmed the presence of a higher frequency of variation in the *oriC* environ. This was further supported by the presence of a high degree of allotypes of the staphylococcal cassette chromosome recombinase genes, which integrates by site specific integration in the *oriC* environ.

5.2 Biofilm, treatment and immune evasion

Important survival strategies of nosocomial CoNS are multi resistance and biofilm formation. If the aim is to maintain peaceful co-existence with its host, the presence of few other virulence factors is beneficial. Due to the low pathogenic potential of CoNS, Otto describes *S. epidermidis* as the "accidental pathogen" (191). Massey *et al*. demonstrates, by using a mathematical model, that the combined mode of transmission and high prevalence of *S. epidermidis* does not encourage high levels of virulence, as increased virulence would result in reduced transmissibility due to host immobility (174). In Paper III the effects of a novel SAMP on staphylococcal biofilms *in vitr*o is described. In Paper IV the effects of a SAMP compared to vancomycin is studied *in vivo*, together with the immune response to the biofilm infection.

The value of animal models was clearly demonstrated when comparing effects of treatment *in vitro* versus *in vivo* (**Paper III-IV**). In paper III an efficient reduction in the number of viable bacteria was observed, when SAMPs were compared to conventionally used antimicrobial agents. However, the superior effect of SAMPs observed *in vitro* did not correlate to the effects observed *in vivo*. Neither vancomycin, nor SAMP (Ltx 21) demonstrated efficient clearance of biofilm embedded bacteria on silicone implants. There are several possible explanations of the discrepancies between the *in vitro* and the *in vivo* results. First, the biofilm used in the *in vivo* model was more mature than the biofilm studied *in vitro*. Second, factors associated with the host, such as protein binding in peritoneal fluid might reduce the available concentrations of the SAMP. Third, in retrospect the mice model might have benefitted from an even longer study period, in order to investigate if the infection eventually would have been cleared. Finally, treatment optimisation using different drug concentrations could also have affected the outcome (**Paper IV**).

In the co evolution with the human host several bacterial response mechanisms towards host cationic peptides have evolved. Mechanisms such as active efflux, proteolytic activity, alteration of surface charge and biofilm formation has been demonstrated in *S. epidermidis* (147, 155, 243). Ltx 21 is a peptide-derived substance and it might therefore induce the bacterial peptide sensing response mechanisms. It has been demonstrated that Daptomycin and vancomycin cause up

regulation of bacterial defence mechanisms, resulting in alterations of cell wall composition (27). Mishra *et al*. demonstrated reduced susceptibility to host defence peptides after treatment failure with the peptide antibiotic daptomycin in *S. aureus* (180). The complex interplay of biofilm, treatment and host innate immune responses is not possible to measure *in vitro*. This reflects the necessity of good animal models which allows parallel measurement of several variables.

5.2.1 Hide and seek

Reduced cytokine secretion and a significant complement activation in response to PIA producing SE 1457 in an *ex vivo* full blood model was previously reported by our group (2). The *in vivo* murine model used in paper IV enables further study of the complex interplay of the various components of the innate immune response towards biofilm embedded bacteria. We were not able to demonstrate *in vivo,* complement activation as previously observed in the *ex vivo* full blood model. Still, we found reduced levels of cytokines in response to the *S. epidermidis* PIA biofilm. The incongruence observed regarding complement activation probably reflects the different models employed. Besides the obvious difference between an *in vivo* and an *ex vivo* model, the *ex vivo* model only allow short term observations, with complement activation being observed 30 min post infection. It is worth noticing that cytokine and complement measurements were performed at different time points, and thus are not completely comparable.

Schommer *et al.* demonstrated reduced uptake of biofilm embedded bacteria by macrophages and reduced production of IL-1β *in vitro* (216). A recent *in vivo* study by Thurlow *et al.* examined the immune response towards *S. aureus* biofilm implant infections (234), and demonstrates macrophage death and a significant reduction in IL-1β, TNF-α and MCP-1 production. Results obtained in our model with a *S. epidermidis* biofilm infection are in line with their observations. We also found that most cytokines, including IL-1β were not significantly elevated in response to the biofilm infection. However, the two cytokines found to be elevated, GM-CSF and MCP-1, are both involved in leucocyte recruitment and activation. This concurs with the elevated levels of leucocytes in the peritoneal fluid from mice with pre-colonised implants receiving treatment (Ltx 21, vancomycin or placebo) compared to animals with sterile implants. This clearly demonstrates

that the infection induce a leucocyte response, albeit not able to clear the infection which demonstrates a failure to efficiently phagocytise the biofilm embedded bacteria.

The pathogenic potential of *S. epidermidis* is low compared to *S. aureus*. It is believed that the factors causing immune evasion in conjunction to device related infections, otherwise are primarily involved in colonisation of the skin and mucous membranes. When considering the CoNS as inhabitants of the skin and mucous membrane, they daily encounter host derived peptides secreted in perspiration, in addition to exposure to certain antimicrobial agents secreted in sweat (120). This creates a selection of traits that enables survival, and persistence.

Mice are convenient to use for laboratory experiments, but it should be acknowledged that *S. epidermidis* does not belong to their normal skin flora. Several CoNS are found, but this mainly includes *S. saprophyticus, S. xylosus S. sciuri, S. lentus* and *S. aureus* (232). The infliction of an *S. epidermidis* infection in mice not normally exposed to *S. epidermidis*, could also have affected the outcome of the study. It is important to bear this in mind when interpreting results.

5.2.2 *S. epidermidis* goes undercover

Small colony variants (SCV) are easily overseen due to their loss of pigmentation and slow growth. Interestingly we were able to study the differential growth of SCV at days 6 and 8 in the animal study. The colonies appeared so altered that repeated species determination was demanded, (**Paper IV**). The SCV seemed to be induced both by the host immune response and/or the two treatments administered. SCVs are known to contribute to persistence in *S. aureus*, by intracellular localisation. Increased resistance particularly to aminoglycosides has been demonstrated for *S. aureus* SCV (241). The variable milieu established within the biofilm, created by fluctuating levels of oxygen and nutrient availability, could possibly contribute to the induction of SCV. The role of SCV in persistence in *S. epidermidis* infections and conditions influencing formations of SCV needs further investigation.

6 Summary and conclusion

After several years of research, endless speculations, loads of laboratory work and the contribution from several mice, what is the essence of our results?

Genome analysis of several *S. haemolyticus* isolates reveals the emergence of hospital adapted clones. The presence of a multitude of resistance genes and the absence of mechanisms reducing uptake of foreign DNA reveals an opportunistic pathogen with a high degree of flexibility and capability of rapid adaptation. The molecular epidemiology of *S. haemolyticus* and other members of CoNS, suggests a more diverse population structure, than observed in *S. aureus*, with frequent genomic rearrangements shaping the genomes, particularly in the *oriC* environ.

The combined effects of biofilm formation and a high level of multi resistance in *S. epidermidis* complicate efficient treatment and enables persistence of CoNS infections. We need to search for new effective drugs to combat these infections. However, it is obvious that drugs showing a promising potential in *in vitro* experiments need to be investigated also in *in vivo* models.

7 Future aspects

In order to reduce the spread of resistance genes and to prevent and treat biofilm infection in the future, several studies based on the findings of the results from my thesis could be conducted.

- Further studies should investigate the role of *S. haemolyticus* in the dissemination of resistance genes to more pathogenic staphylococci such as *S. aureus*.

- The novel Erythromycin Resistance Putative Encoding genes conferring putative macrolide resistance warrant further functional studies.

- Full genome sequencing allows search for genes involved in *S. haemolyticus* biofilm formation, this would allow targeted intervention against biofilm formation.

- The effects of the combined treatment of SAMPS and vancomycin towards *S. epidermidis* implant associated biofilm infections should be evaluated in a follow up study.

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Paper 1

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Paper 4

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