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STUDIES ON STAPHYLOCOCCUS EPIDERMIDIS BIOFILM FORMATION AND THE BACTERIAL INTERACTION WITH THE HUMAN CATHELICIDIN ANTIMICROBIAL PEPTIDE LL-37

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Studies on Staphylococcus epidermidis biofilm formation and the bacterial interaction with the human cathelicidin antimicrobial peptide LL-37

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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“Share your knowledge. It’s a way to achieve immortality.”
/*Dalai Lama*

To all of you...

Abstract

The long-term use of central venous catheters for delivering nutrients and drugs in preterm neonates has been related to nosocomial infections. The majority of late-onset sepsis in very preterm infants (<28 gestational weeks) are caused by Gram positive bacteria. Coagulase-negative staphylococci (CoNS) are responsible for almost the half of these cases. *Staphylococcus epidermidis* is the most prevalent bacteria identified from CoNS bacteraemia and biofilm production is found to be the main determinant of persistent infection.

The major host defense peptide LL-37 is the only cathelicidin antimicrobial peptide that exists in humans. The peptide is broadly distributed in the human body and possesses several additional functions related to host defense. As a cathionic peptide, it interacts with the negatively charged bacterial surface. LL-37 is shown to inhibit biofilm formation and regulates biofilm-associated gene expression in *Pseudomonas aeruginosa in vitro.*

In *Paper I*, we showed that *S. epidermidis* strains obtained from bloodstream infection in preterm infants had different characteristics than the skin strains isolated from healthy term neonates. The blood isolates were equipped with an invasive genetic element IS256 and showed higher antimicrobial resistance compared with the skin isolates. However, vancomycin resistance was not detected among any of the strains. We also observed short and long filament-like structures on the cell surface of *S. epidermidis*. These filaments were involved in the attachment to the catheter surface and also in cell to cell attachment and/or communication.

Our in vitro studies in *Paper II* and *Paper III*, revealed that physiological LL-37 peptide concentrations, below those that kill or inhibit growth of the free-floating bacteria, inhibited *S. epidermidis* attachment and biofilm formation on abiotic surfaces. In *Paper III*, we observed that the peptide regulates genes involved in the biofilm formation.

In *Paper IV*, we found that the circulating serum level of hCAP18/LL-37 was similar in preterm and term neonates at birth and both the inactive protein and the active peptide were detectable independent of the gestational time. We observed positive correlation between maternal and infant peptide concentration. This may indicate that the peptide passes over early during pregnancy.

In summary, our work revealed that *S. epidermidis* strains that cause bloodstream infection in preterm infants are more virulent compared with skin strains in term neonates. Physiological concentration of the human cathionic peptide LL-37 had inhibitory effect on *S. epidermidis* biofilm formation by regulating biofilm genes. The similar LL-37 peptide concentration in preterm and term infants' blood might suggest that these neonate's vulnerability is not connected to the lower antimicrobial peptide level at birth.

LIST OF PUBLICATIONS

I. Hell, E., Giske, CG., Hultenby, K., Marchini, G., Gemzell Danielsson, K. **Attachment and biofilm forming capabilities of *Staphylococcus epidermidis* strains isolated from preterm infants.** *Current Microbiology (2013)67(6):712-7*

II. Hell, E., Giske, CG., Nelson, A., Romling, U., Marchini, G. **Human cathelicidin peptide LL-37 inhibits both attachment capability and biofilm formation of *Staphylococcus epidermidis*.** *Letters in Appl Microbiol (2010), 50:211-215*

III. Hell, E., Ujvari, D., Hultenby, K., Rohde, H., Gemzell Danielsson, K., Marchini, G. **Biofilm-associated gene regulation by the human cathelicidin LL-37 in *Staphylococcus epidermidis*.** *Manuscript*

IV. Hell, E., Dubicke, A., Fransson, E., Gemzell Danielsson, K., Marchini, G. **Circulating cathelicidin antimicrobial peptide LL-37 levels in newborn infants in relation to gestational time.** *Manuscript*

CONTENTS

LIST OF ABBREVIATIONS

NICUs	neonatal intensive care units
CVC	central venous catheter
VLWB	very low birth weight
CoNS	coagulase-negative staphylococci
S. epidermidis	*Staphylococcus epidermidis*
S. aureus	*Staphylococcus aureus*
P. aeruginosa	*Pseudomonas aeruginosa*
STs	sequence types
CC2	clonal complex 2
IS256	insertion sequence 256
SEM	scanning electron microscopy
MSCRAMMs	microbial surface components recognizing adhesive matrix molecules
Fbe (SdrG)	fibrinogen binding protein
Embp	extracellular matrix binding protein
GehD	collagen binding protein
AtlE	autolysin-E
Aap/Aae	accumulation-associated protein
Bhp	Biofilm-associated protein homolog protein
PIA	polysaccharide intercellular adhesin
PNAG	poly-N-acetyl-glucosamine
AMPs	antimicrobial proteins/peptides
CAMPs	cationic antimicrobial proteins/peptides
hCAP-18	human cathelicidin protein-18
PSMs	phenol-soluble modulins
Agr	accessory gene regulator
TLRs	Toll-like receptors
PAMPs	pathogen-associated molecular patterns
NETs	neutrophil extracellular traps
BHI	brain heart infusion

TSB	tryptic soy broth
PBS	phosphate buffered saline
MIC	minimal inhibitory concentration
CFU	colony forming unit
OD	optical density
SD	standard deviation
PCR	polymerase chain reaction
qRT-PCR	quantitative real time polymerase chain reaction
mRNA	messenger RNA
PFGE	pulsed-field gel electrophoresis
UPGMA	unweight pair group method with arithmetic mean
Ig	immuno-globulin

1 BACKGROUND

Newborn infants in neonatal care units (NICUs) are at risk of infection (Group, 2010) (Power Coombs *et al.*, 2013), due to their low gestational age, their low birth weight, long-term use of central venous catheters (CVCs) as well as ventilator support, and also a prolonged necessity of parenteral nutrition (Stoll *et al.*, 2002). The long-term use of CVCs for delivering nutrients and drugs in preterm neonates has been related to nosocomial infections (Klein and Shahrivar, 1992; Garland *et al.*, 2001). The most common source of the colonization is the skin insertion site as well as the hub of the catheter. Thus, the microorganisms may enter the bloodstream and cause acute late-onset sepsis infection (Garland *et al.*, 2001). Almost half of the preterm infants born before the gestational weeks 25 get late-onset sepsis (Stoll *et al.*, 2002), while term neonates are rarely infected (Isaacs and Australasian Study Group for Neonatal, 2003; Carrieri *et al.*, 2003).The majority of late-onset sepsis in very low birth weight (VLBW) newborn infants are caused by Gram positive organisms and coagulase-negative staphylococci (CoNS) are responsible for almost the half of these cases (Stoll *et al.*, 2002; Lim *et al.*, 2012). *Staphylococcus epidermidis* is the most prevalent bacteria identified from CoNS bacteraemia and biofilm production is found to be the main determinant of persistent infection (Dimitriou *et al.*, 2011). Noteworthy, *S. epidermidis* infections are commonly nosocomial and only develop after surgery or in immuno compromised patients. These infections are always sub-acute compared with the acute diseases caused by *Staphylococcus aureus* (Vuong and Otto, 2002). Despite the fact that CoNS infections can often be cleared within a few days by intravenous antibiotics, they induce inflammatory responses which often result in long-term harm to the newborn, including potential cerebral injury (Group, 2010; Stoll *et al.*, 2004) and these events are also responsible for significant healthcare costs (Payne *et al.*, 2004).

1.1 *STAPHYLOCOCCUS EPIDERMIDIS*

1.1.1 The first colonizer after birth and the nosocomial pathogen

S. epidermidis, belonging to the coagulase-negative staphylococci, is a commensal bacterium of the skin and mucous surfaces of humans (Roth and James, 1988). Their lack of the coagulase enzyme differentiates them from the coagulase-positive staphylococci such as *S. aureus*.

Healthy newborn infants become colonized with *S. epidermidis* within the first days after birth (Marchini *et al.*, 2005), in direct connection to birth and to environmental adaptation. However, preterm infants, due to their longer stay in NICU, very often become colonized with the hospital acquired bacterial strains (Botelho *et al.*, 2012). These strains are often multidrug resistant (D'angio *et al.*, 1989) and thought to be acquired by health care workers (Milisavljevic *et al.*, 2005). Colonization with the commensal *S. epidermidis* plays an important role in balancing the human epithelial microbiota and preventing the overgrowth of the more aggressive pathogen *S. aureus*. It was recently reported, that a subset of *S. epidermidis* in the nasal cavity produces a serine protease (Esp), which inhibited *S. aureus* biofilm formation *in vitro* (Iwase *et al.*, 2010). This mechanism of bacterial interference might have an important probiotic function by limiting *S. aureus* nasal colonization and infection, although there is no evidence that *S. epidermidis* produces such inhibitory factors *in vivo* (Otto, 2014).

On the other hand, *S. epidermidis* is the most frequent causative agent of device-related infections, particularly when venous catheters are being used (Bouza *et al.*, 2007). There is a high level of diversity among the *S. epidermidis* strains. Several different sequence types (STs) (Miragaia *et al.*, 2007) has been identified. One of them is the most frequently isolated ST2 which belongs to the clonal complex 2 (CC2) (Miragaia *et al.*, 2007). Most of the ST2 isolates form biofilm *in vitro* (Li *et al.*, 2009), whereas, all of them are equipped with virulence determinants, which will be mentioned later. These features together correlate with *S.*

epidermidis invasiveness and contribute to the successful spread of ST2 (Gu *et al.*, 2005; Kozitskaya *et al.*, 2004; Yao *et al.*, 2005).

1.1.2 Virulence determinants

Several studies have aimed to determine virulence factors which can discriminate between invasive and skin commensal *S. epidermidis* strains (Gu *et al.*, 2005; Kozitskaya *et al.*, 2004; Yao *et al.*, 2005; Rohde *et al.*, 2004). There is evidence indicating that most virulence factors have their original role in the commensal lifestyle of this species. For instance, different factors involved in biofilm formation all have such original roles in establishing growth and allowing survival in microbial agglomerations on the skin (Otto, 2009), however, those determinants are also thought to have importance in the invasiveness of *S. epidermidis*.

The insertion sequence element IS256 is a mobile genetic element that often exists in multiple copies on the *S. epidermidis* chromosome (Kozitskaya *et al.*, 2004). Thus, inserted into the host chromosome, it may affect the expression of different genes associated with pathogenesis, such as biofilm formation (Hennig and Ziebuhr, 2010) and antimicrobial resistance (Kozitskaya *et al.*, 2004) and may thus increase the fitness of *S. epidermidis* in the hospital settings (Gu *et al.*, 2005). All of the ST2 isolates are equipped with the IS256 and with the *ica*-gene (Li *et al.*, 2009).

1.2 *S. EPIDERMIDIS* BIOFILM FORMATION

Early scanning electron microscopy (SEM) studies on medical devices observed that *S. epidermidis* was embedded into bacterial cells derived extracellular matrix and formed a multilayer bacterial community (Marrie *et al.*, 1982; Peters *et al.*, 1982). This special growth of mode was mentioned as slime production at that time, but today it is termed as biofilm formation (Hall-Stoodley *et al.*, 2004).

The biofilm-mode of growth provides a protection for *S. epidermidis* because it prevents bacteria from being washed away from a surface and increases bacterial resistance to both antibiotics and multiple host defense mechanisms (Von Eiff *et al.*, 2002). Furthermore, it increases survival under environmental changes for both the colonizing and the infectious bacteria (Otto, 2013). Similarly to other bacteria, gene expression in *S. epidermidis* biofilms is showing suppression of several active cell processes as well as adaptation of metabolic activity to fermentative growth (Yao *et al.*, 2005).

S. epidermidis establishes biofilms through a well-defined process, in which the bacteria adhere to the surface (primary attachment), and then organize a three dimensional, mature biofilm community (biofilm maturation) (Christner *et al.*, 2012) (Figure 1). Detachment of biofilm bacteria (Figure 1) is also important in re-structuring the biofilm and also in dissemination of infection.

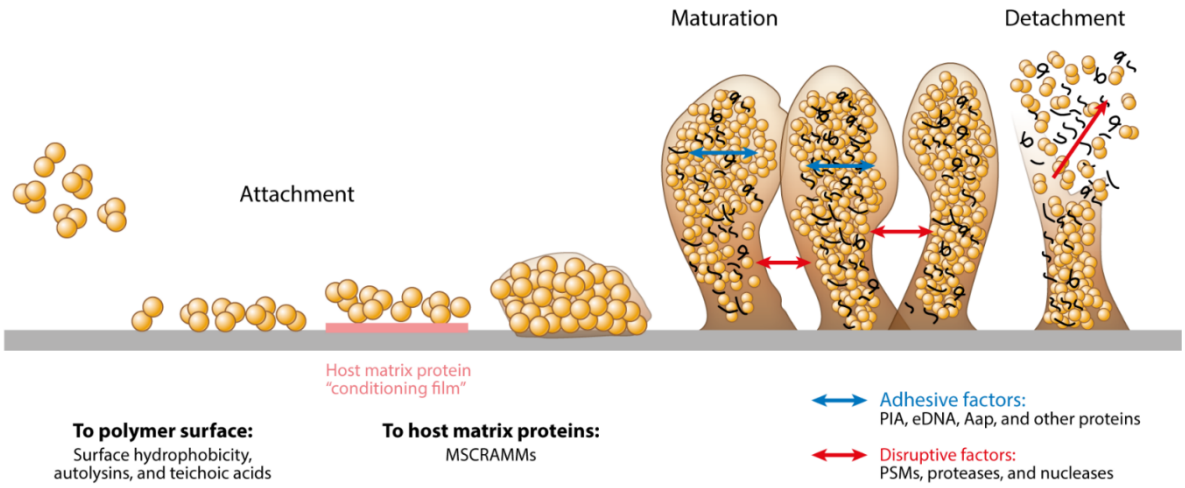

Figure 1. Biofilm development in *S. epidermidis*. (*Reprinted from Otto, Annual review of Medicine 2013 with the license from Annual Reviews* (Otto, 2013).

1.2.1 Adhesion, initial attachment

Initial attachment occurs to biotic (tissue) or abiotic surfaces (medical indwelling devices) (Figure 1). After insertion of medical devices into the human body, the device surfaces are soon covered with human matrix proteins (Heilmann *et al.*, 1997) (Figure 1). The adhesion to tissues or matrix proteins is mediated by the specific protein adhesins (MSCRAMMs) (Bowden *et al.*, 2005) (Figure 3a). These adhesins can bind to fibrinogen (SdrG/Fbe) (Davis *et al.*, 2001; Hartford *et al.*, 2001), fibronectin (Embp) (Williams *et al.*, 2002), vitronectin (AtlE, Aae) (Heilmann *et al.*, 1997; Heilmann *et al.*, 2003) and collagen (GehD) (Bowden *et al.*, 2002). Aap proteins aggregate through G5 domains and form filament-like structures. G5 domain also binds to N-acetylglucosamine and can interact with PNAG (PIA). The amino terminal region (A repeats and globular α/β domain) of Aap can be proteolitically removed to gain the function in intercellular aggregation (Joo and Otto, 2012) (Figure 3a). The attachment to an abiotic surface, such as a plastic indwelling device *in vitro*, is dependent of the physicochemical characteristics of the device and the bacterial surfaces. Thus, this mechanism is less specific and is driven by the bacterial surface located adhesins, such as Bhp (Cucarella *et al.*, 2001) and AtlE (Rupp *et al.*, 2001).

1.2.2 Biofilm maturation

During accumulative phase bacterial cells attach to each other by expressing intercellular adhesins (Figure 1.), like the the most important polysaccharide intercellular adhesin (PIA), also named poly-N-acetyl-glucosamine (PNAG) due to its chemical composition (Mack *et al.*, 1996) (Figure 3). Products of the *ica* gene locus are responsible for the synthesis, the export and modification of PIA (Figure 3b). The *ica* gene locus comprises the *icaA*, *icaD*, *icaB* and *icaC* genes (Heilmann *et al.*, 1996). IcaA, IcaD and IcaC proteins are anchored in the bacterial membrane, while IcaB is a surface-located enzyme (Vuong *et al.*, 2004b). IcaA and IcaD is responsible for the elongation of the polysaccharide chain, which is then exported by the IcaC. IcaB complete the process by partial de-acetylation, thus giving positive charges to the originally neutral polymer (Vuong *et al.*, 2004b; Rohde *et al.*, 2010). Due to this positive charge, PIA/PNAG is important in surface binding and has also been shown to protect *S. epidermidis* from cationic antimicrobial peptides (AMPs) and neutrophil killing, the complement as well as the immuno-globulin deposition (Vuong *et al.*, 2004c). However, it has recently been recognized, that PIA/PNAG is not produced in all *S. epidermidis* strains due to

the lack of the *ica* genes (Kogan *et al.*, 2006). In those strains, the biofilm formation may be mediated by surface proteins such as the accumulation-associated protein (Aap) (Hussain *et al.*, 1997) (Figure 3a), the Bap homolog protein (Bhp) (Cucarella *et al.*, 2001), or the extracellular matrix binding protein (Embp) (Christner *et al.*, 2010). The mechanism of protein-mediated biofilm formation is still not fully known, but large fibrils, such as in the case of Aap (Banner *et al.*, 2007; Gruszka *et al.*, 2012) (Figure 3a) or PSMs (Schwartz *et al.*, 2012), are thought to be involved in the cell-cell adhesion (Figure 2).

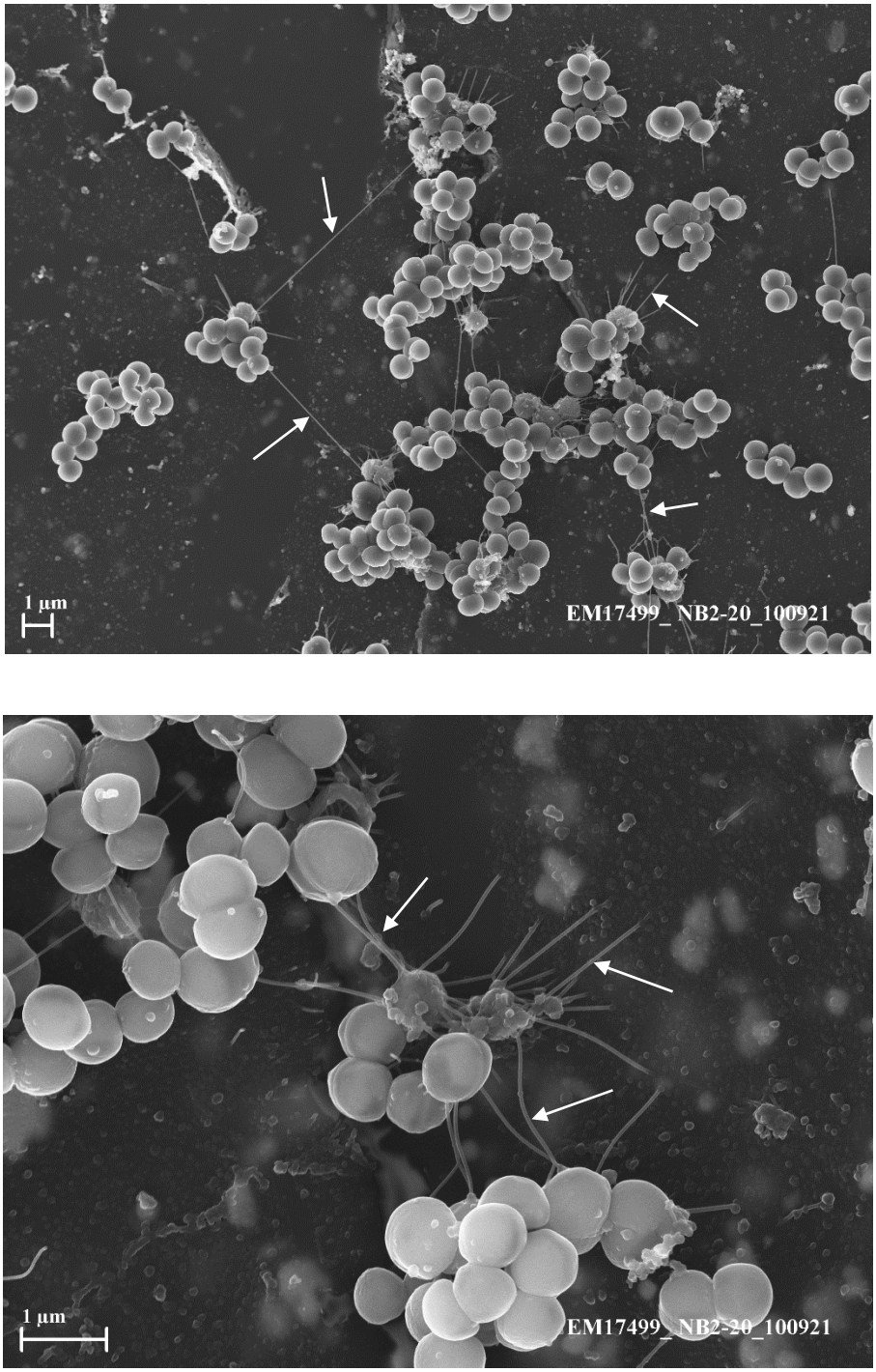

Figure 2. Short and long filament-like structures (white arrows) between *S. epidermidis* cells or between bacteria and the catheter surface. (*Unpublished SEM image, obtained from an experiment where catheter pieces were incubated with S. epidermidis ATCC35984 stain cultured in TSBg for 24 h under shaking conditions*).

In addition, teichoic-acids have also been shown to be involved in biofilm formation (Gross *et al.*, 2001; Sadovskaya *et al.*, 2005). They might interact with other surface polymers, such as PNAG, via electrostatic interactions (Otto, 2013).

1.2.3 Biofilm detachment and regulation

Disruptive determinants of the biofilm are important for structuring and re-modeling biofilm architecture. Detachment and dissemination of cell clusters might be a presumed starting point for the spreading of biofilm-associated infections. In staphylococci, phenol-soluble modulins (PSMs), proteases and nucleases promote these disruptive functions *in vitro* (Figure 1), and are believed to facilitate dissemination of biofilm-associated infection, in humans (Otto, 2013). PSMs in *S. epidermidis* are described as a complex of three peptides named PSMα, PSMβ and PSMγ, latter also known as δ-toxin (Mckevitt *et al.*, 1990). PSMs are coded in the core genom. They have pronounced surfactant-like features due to their amphipathic α-helical structure (Cheung and Otto, 2010; Wang *et al.*, 2007), which indicates their role in spreading on surfaces (Tsompanidou *et al.*, 2013). The PSMβ peptides are produced in large amounts in *S. epidermidis*, especially during the biofilm-mode of growth (Wang *et al.*, 2011) which lead to restructuring the biofilm.

The accessory gene regulator (Agr) quorum sensing system is the main regulator of biofilm formation in *S. epidermidis*. The *agr* gene expression in the outer layers of the biofilm leads to detachment and regrowth (Yarwood and Schlievert, 2003), however, expression of *agr* in deeper layers promotes channel formation (Periasamy *et al.*, 2012). Generally, the Agr-system up-regulates the degradative exoenzymes, such as proteases, and down-regulates several surface adhesion protein expression (Otto, 2013). The AgrA, one of the Agr-system components, binds to two promoters which activate Agr operon (as an auto-regulation) as well as the RNAIII operon (the intracellular effector of the system). Additionally, AgrA can directly bind to operons encoding PSMs (Vuong *et al.*, 2004a) (Figure 3a).

However, transcription of *ica* genes is not regulated by Agr. The *icaA* promoter or the regulatory IcaR protein regulates directly the expression of the *icaADBC* operon and these regulatory functions are under control of other virulence regulators (Mack *et al.*, 2000; Tormo *et al.*, 2005; Handke *et al.*, 2007; Xu *et al.*, 2006) (Figure 3b). The insertion and excision of the insertion element IS256 can also turn on/off PIA/PNAG expression (Ziebuhr *et al.*, 1999).

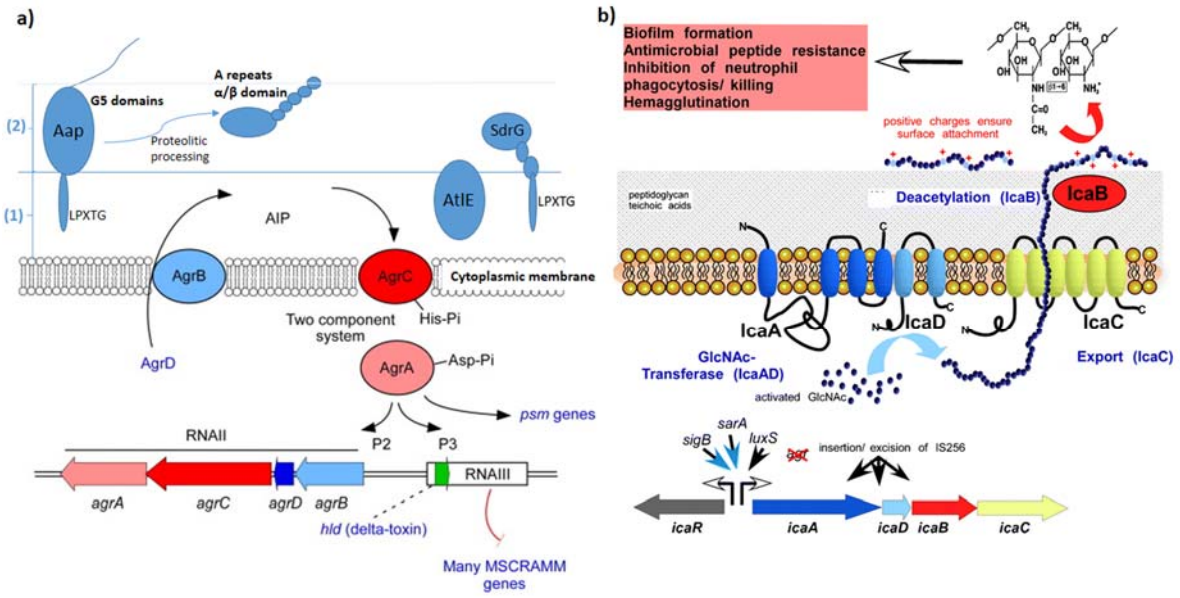

Figure 3. *Staphylococcus epidermidis* cell surface structure and the regulatory systems.

a) MSCRAMMS proteins such as SdrG (Fbp) and Aap can be attached to the cell surface covalently. They carry an LPXTG motif which is linked to peptidoglycan. AtlE is a bifunctional adhesion and autolysin. It is non-covalently attached to peptidoglycan. (1) Peptidoglycan and teichoic acid, (2) PNAG/PIA. *Psm* genes and many of the MSCRAMMs genes are regulated by the Agr system. (*Adapted from Joo and Otto, Chemistry and Biology 2012 with the license from Elsevier, and completed with MSCRAMMs porteins and the cell surface structures (Joo and Otto, 2012).*
b) PNAG is synthesized by the gen products of *ica* operon. The *ica* operon is regulated by global regulators, but not Agr. IcaB is responsible for de-acetylation of pre-PIA at the cell surface, thereby giving positive charges to PIA, which is important for surface location and multiple function of PIA. (*Reprinted from Otto 2012, Springer Seminars in Immunopathology with the license from Springer (Otto, 2012).*

1.3 ANTIMICROBIAL SUSCEPTIBILITY OF *S. EPIDERMIDIS*

The primary treatment for staphylococcal infections is the use of antibiotics which is associated with the development of antibiotic resistance. Furthermore, it may alter the host microbiota composition, thus influencing the local microbial diversity (Power Coombs *et al.*, 2013). The prevalence of methicillin resistance has been reported to range from 75% to 90% among hospital *S. epidermidis* isolates (Diekema *et al.*, 2001). Methicillin resistance is encoded in staphylococcal mobile genetic elements which carry the *mecA* gene (Miragaia *et al.*, 2005). In addition, resistance to several other antibiotics such as aminoglycosides (gentamicin), macrolides, tetracycline, chloramphenicol and clindamycin are also frequently observed (Rogers *et al.*, 2009). However, high-level resistance to vancomycin has not been reported yet in *S. epidermidis* isolates (Otto, 2012).
Besides the specific resistance genes, the biofilm matrix also serve a non-specific tolerance for *S. epidermidis* due to the limited diffusion of several antibiotics. However, vancomycin, rifampin and daptomycin has been shown to diffuse easily to *S. epidermidis* biofilm (Leite *et al.*, 2011; Dunne *et al.*, 1993). *S. epidermidis* is also an optimal carrier and reservoir for antibiotic resistance genes due to its ubiquity and may thereby spread these genes within microbial communities (Cheung and Otto, 2010).

1.4 THE NEONATAL INNATE IMMUNITY

The innate immune responses at birth are mediated by neutrophils, macrophages, monocytes and dendritic cells. However, skin and mucosal epithelia are also involved (Levy, 2007). The neonatal immune system is highly dependent on the first bacterial colonization on the skin and the gastrointestinal epithelia. Toll-like receptors (TLRs) of the innate immune cells are recognizing the pathogen-associated molecular patterns (PAMPs) on the microbes' surface. TLR activation then leads to phagocytosis and cytokine release (Heine and Ulmer, 2005). Whereas, the most important mechanism by which the innate immune system kills microbes is via antimicrobial protein and peptide expression as well as mobilization (Levy, 2000). Furthermore, AMPs play an important role as a link between innate and adaptive immunity. However, neonatal umbilical cord plasma contains more adenosine, a purine metabolite that inhibits TLR-mediated response, compared with adults (Levy *et al.*, 2006). Moreover, neutrophils in newborn infants show lower level of phagocytic and chemotactic ability (Filias *et al.*, 2011; Bjorkqvist *et al.*, 2004) as well as lower production of neutrophil extracellular traps (NETs) that leads to impaired distribution of AMPs (Yost *et al.*, 2009). The observed impairments of the innate immune components may explain why neonates are more susceptible to infections.

1.5 ANTIMICROBIAL PEPTIDES

Antimicrobial proteins and peptides are the key molecules of the innate immune system (Sorensen, Borregaard 2008). There are three major antimicrobial peptide classes in humans: the cathelicidins, defensins, and thrombocidins. All these AMPs are cationic (CAMPs), a feature believed to have evolved to interact with the negatively charged bacterial surface, thus altering both fluidity of the phospholipid bilayer and bacterial cell surface composition (Otto, 2009).

1.5.1 The human cathelicidin peptide LL-37

The major host defense peptide LL-37, is the only cathelicidin antimicrobial peptide that exists in humans (Agerberth *et al.*, 1995; Durr *et al.*, 2006). The active LL-37 was first identified in blood (Agerberth *et al.*, 1995). The inactive hCAP18 has been detected in metamyelocytes in the bone marrow and is also present in granules of neutrophils as well as in subpopulations of lymphocytes and in monocytes (Sorensen *et al.*, 1997a; Sorensen *et al.*, 2001). The 37-amino-acid peptide LL-37 becomes activated when the C-terminal domain of the hCAP18 protein is cleaved off extracellularly by proteinase 3 (Figure 4b) (Sorensen *et al.*, 2001).

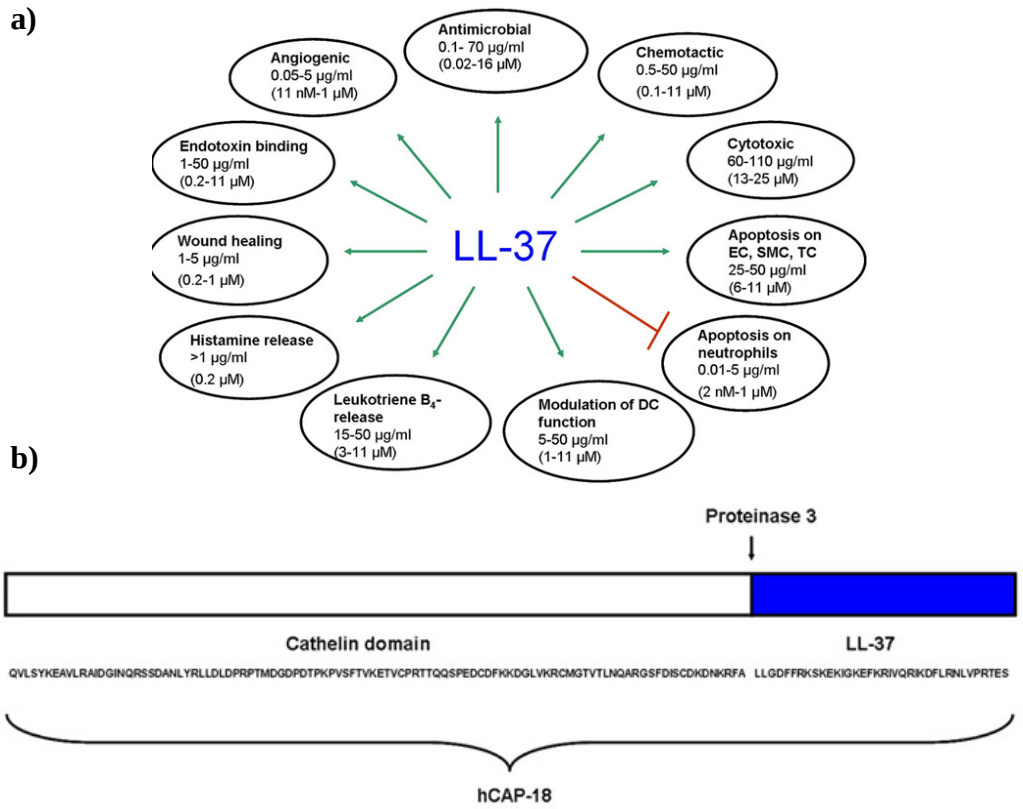

Figure 4. a) Concentrations of different activities of LL-37. EC: Epithelial cells; SMC: Smooth muscle cells; TC: T-cells; DC: Dendritic cells. **b)** The structure of hCAP-18 with the cathelin domain and the C-terminal antimicrobial domain (LL-37). *Reprinted from Kai-Larsen and Agerberth; Frontiers in Bioscience 2008, with a permission from Frontiers in Bioscience (Kai-Larsen and Agerberth, 2008).*

The peptide is broadly distributed in the human body (Durr *et al.*, 2006). The expression of LL-37 is tissue-specific and different level of expression has been shown in leukocyte precursors in the bone marrow (Storici and Zanetti, 1993), in epithelial tissues (Hase *et al.*, 2002), in lung (Bals *et al.*, 1998) and also in seminal fluids (Malm *et al.*, 2000). The peptide is produced by keratinocytes after injury on the skin (Dorschner *et al.*, 2001). LL-37 inhibits biofilm formation *in vitro* (Overhage *et al.*, 2008) and affects the composition and growth of commensal flora (Putsep *et al.*, 2002). Also, it actively provides protection against a broad range of bacteria, virus and fungi (Chromek *et al.*, 2006). LL-37 may destroy the cell membrane (direct killing) or may act on the internal cellular targets (genes, proteins) (Hancock and Sahl, 2006) (Figure 5.).

Apart from exhibiting a broad antimicrobial spectra (Strempel *et al.*, 2013), LL-37 possesses several additional functions related to host defense, such as chemotactic, endotoxin neutralizing, angiogenesis and wound healing capabilities, as well as being a mediator between innate and adaptive immunity (Figure 4a) (Kai-Larsen and Agerberth, 2008).

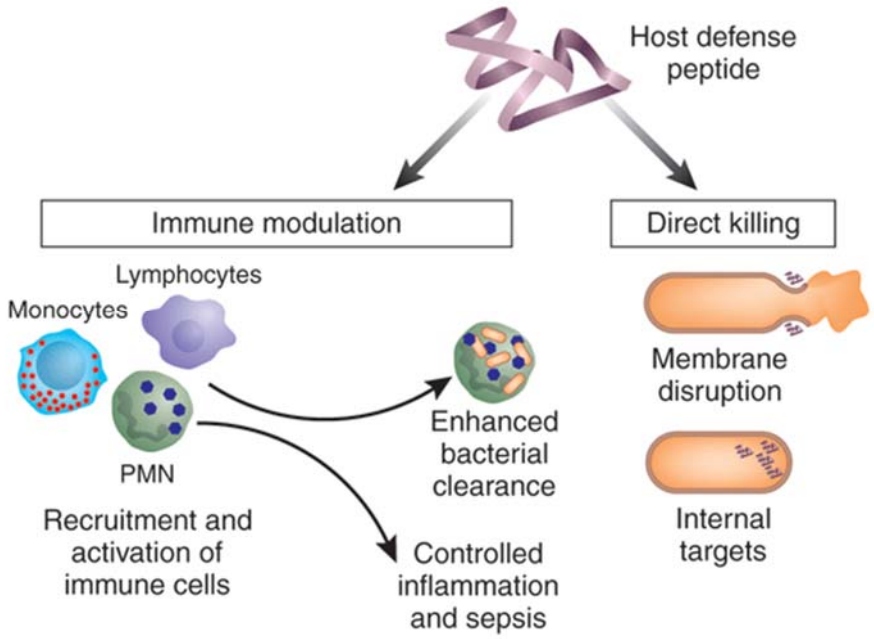

Figure 5. Antimicrobial killing and innate immune modulation of host defense peptides. *Reprinted from Hancock and Sahl; Nature Biotechnology 2006, with a permission from Frontiers in Bioscience* (Hancock and Sahl, 2006).

Sorensen *et al.* has described that the inactive hCAP18 protein is cleaved off and possessed extracellularly to the active antimicrobial peptide LL-37 by a serine protease (proteinase 3) found in specific azurophil granules of neutrophils (Sorensen *et al.*, 2001). The concentration of LL-37 has been shown to be increased due to neutrophil degranulation (Sorensen *et al.*, 2001). It was also recently shown that the delivery mode plays an important role in activating several immune mechanisms in the fetus/infants. Neonates have an increased number of natural killer cells and polymononuclear cells (PMNs) after vaginal delivery (Herson *et al.*, 1992). Also, the level of LL-37 in plasma as well as in the cytoplasm of PMNs is higher in normal deliveries compared with elective C-section (Mandic Havelka *et al.*, 2010). However, preterm infants have lower number of granulocytes (neutrophils) than term neonates (Hartel *et al.*, 2008).

Blood levels of AMPs including the peptide LL-37 were found to be reduced in preterm infants (Strunk *et al.*, 2009), whereas in term neonates they were comparable to corresponding maternal levels (Mandic Havelka et al., 2010). Also, lower levels of the peptide LL-37 were measured in the newborn lung tissue (Starner *et al.*, 2005). However, the perinatal skin showed increased LL-37 expression in comparison to the adult skin (Dorschner *et al.*, 2003). Thus, lower AMP levels in the blood could be a possible contributing factor to the increased susceptibility of preterm infants to nosocomial *S. epidermidis* infection (Cheung and Otto, 2010).

2 AIMS OF THE THESIS

The overall aim of the thesis was to study the underlying nature of a commensal bacteria causing late-onset sepsis in preterm infants, as well as to study the effect of the major human host defense peptide LL-37 on that bacteria.

Our specific aims were:

- To describe the characteristics of *Staphylococcus epidermidis* strains isolated from bloodstream infection in preterm infants and also of commensal isolates obtained from healthy newborns, regarding their attachment- and biofilm formation capability, antimicrobial susceptibility as well as their clonal relationship.

- To study the effect of human cathelicidin antimicrobial peptide LL-37 on the initial attachment to a solid surface as well as the biofilm formation of *S. epidermidis* ATCC35984 strain.

- To further study, whether the peptide also affects the expression of biofilm-associated genes comparing planktonic and sessile bacteria and the effect on the production of the cell surface protein Aap.

- To evaluate the maternal and umbilical cord serum level of peptide LL-37 in relation to gestational time. We also addressed the question as to whether the active form of the peptide was detectable in preterm and term infants at birth.

3 MATERIALS AND METHODS

3.1 PATIENT MATERIALS

All participants gave their written consent. The study was approved by the Regional Ethical Review Board at Karolinska Institutet (*Paper I*: 2007/674-31/1; *Paper IV*: 04-637/4 and 2011/48-32).

Paper I

Blood cultures obtained from preterm infant and clinical bacterial isolates derived from the skin of healthy term infants were sampled at different time points during an 18 month interval, 2007-2009. Ten consecutive blood isolates (B1-10) were obtained from extremely preterm infants born before 28 gestational weeks, admitted to the NICU of the tertiary public Karolinska University Hospital, Solna, Sweden, and diagnosed with growth of coagulase-negative staphylococci (CoNS) in their blood culture. Bacterial strains were isolated from the skin of healthy infants born at term (NS1-16) after uncomplicated pregnancy and delivery.

Paper IV

The recruitment of participants took place during 2006 and 2009 at Karolinska University Hospital, Solna, Sweden. Women with delivery after 24-36 completed weeks of gestation (preterm group) or after 37-42 weeks of gestation (term group) were included in the study. Exclusion criteria were fetal malformation, multiple gestation, preeclampsia, diabetes or other systematic disease during pregnancy. Maternal venous blood was obtained in labor. Blood samples from the umbilical cord were obtained according to the hospital routine.

3.2 BACTERIA, PEPTIDE, GROWTH MEDIA AND BLOOD SAMPLES

3.2.1 Bacterial strains

Blood (B1-10) and skin (NS1-16) isolates were identified as *Staphylococcus epidermidis* by VITEK2 (bioMérieux, Marcy l'Etoile, France).

Two *S. epidermidis* strains were used as reference strains: ATCC35984 (also referred as RP62A) as a biofilm-positive strain, first isolated from bloodstream infection and ATCC12228 strain as first isolated from the skin (Wei *et al.*, 2006).

Staphylococcus aureus CCUG41582 reference strain was used in the pulsed-field gel electrophoresis experiments.

3.2.2 Human cathelicidin peptide LL-37

Peptide LL-37 was purchased from Innovagen (Lund, Sweden). Distilled water contained 0.05% acetic acid was used for the dilutions of LL-37 in order to prevent precipitation of the peptide (Nelson *et al.*, 2009b).

3.2.3 Growth media and bacterial culture conditions

Bacteria were grown in sterile brain heart infusion (BHI) broth at 37°C (Sigma-Aldrich Chemical Co.) for overnight (OD600 ~1.0), then the cultures were adjusted to an OD600 of 1.0 for the initial attachment and diluted to 1: 1000 for biofilm formation, in fresh sterile tryptic soy broth supplemented with 1% glucose (TSBg)(Eftekhar and Speert, 2009).

Combined media were used in the growth experiment and in the initial attachment as well as in the biofilm assays with the peptide LL-37, allowing bacteria to form a biofilm as well as maintaining and keeping the activity of the peptide. The combined growth media were the following: one part of LL-37 solution in sterile distilled water (0.05% acetic acid) and one part of bacterial culture in TSBg (0.05% acetic acid).

3.2.4 Blood samples

Venous blood samples obtained from mothers and from the umbilical cord were allowed to clot, then centrifuged at 2400 g for 10 min at 4°C. Serum samples were stored at -20°C for further analysis.

3.3 GROWTH EXPERIMENT (PAPER II)

Growth of planktonic cells was measured photometrically at 600 nm in liquid, combined media (see above) supplemented with 8 and 16 mg/L of the peptide under shaking conditions at 37 ºC. Measurements were taken hourly for 7 hours.

3.4 CULTURE PLATE ASSAYS

3.4.1 Initial attachment and biofilm formation in polystyrene microtiter plates (Paper I)

Determination of initial attachment and biofilm production was adapted from the method of Banner et al.(Banner *et al.*, 2007). Diluted bacterial cultures were inoculated in 4 parallel wells in a 96-well polystyrene microtiter plate (Corning Incorporated Life Sciences, Lowell, MA). After incubation at 37 °C for i) 2 h and ii) 20 h, respectively, attached cells and biofilm were stained by crystal violet as previously described by Nelson *et al.* (Nelson *et al.*, 2009a). The optical density of the adherent cells/biofilm was measured at 590 nm. Negative control of sterile TSBg without bacteria was included and the assay was independently repeated three times.

Comparative analysis was performed based on the mean absorbance values of bacterial biofilms, using the lowest value as negative control (Stepanovic *et al.*, 2007). Briefly, the cut-off value for the optical density (ODc) was established as three standard deviations (SD) above the mean OD of the negative control. Final OD value of a tested strain is expressed as average OD value of the strain reduced by ODc value. Isolates were classified as no biofilm former (OD<ODc), weak producer (ODc<OD<2xODc), intermediate producer (2xODc<OD<4xODc) or strong biofilm producer (4xODc<OD).

3.4.2 Initial attachment and biofilm formation in the presence of peptide LL-37 in polystyrene microtiter plates (Paper II)

The same experimental settings were applied as mentioned above, using the combined growth media and different concentrations of the peptide LL-37 (0, 1, 4, 8 and 16 mg/L). The 32 mg/L concentration was also tested in the biofilm formation assay. This concentration is the minimal inhibitory concentration (MIC) of this particular strain (Nelson *et al.*, 2009b). Experiments were done in triplicate and six independent experiments were performed.

3.4.3 Biofilm formation in the presence of peptide LL-37 in 6-well culture plate (Paper III)

To investigate possible effects of peptide LL-37 on bacterial gene- and protein expression, we cultivated bacterial cells in 6-well polystyrene culture plates (Corning Incorporated Life sciences, Lowell, MA, USA). Gene expression in planktonic versus sessile bacteria was investigated after 24 h incubation. Overnight bacterial culture (OD600=1.0) was diluted 1:1000

in combined media (described above) using 0, 0.01 and 1 mg/L concentrations of LL-37 solution. Plates were incubated at 37 °C for 24 h under static condition. Three independent experiments were performed.

3.5 ELECTRON MICROSCOPY

Diluted bacterial culture of *S. epidermidis* ATCC35984 and a piece of a sterile central venous polyurethan catheter, generally used for long-term access in preterm infants (1 Fr/28G Premicath, Vygon, Ecouen, France), were incubated in plastic tubes at 37 °C under shaking conditions.

3.5.1 Transmission electron microscopy (TEM) (Paper I)

Attachment of bacterial cells and biofilm formation for 2 h and 20 h, respectively, were investigated. After incubation, catheter pieces were transferred to fixative solution (2% glutaraldehyd, 0.5% paraformaldehyd in 0.1 M Phosphate buffer). Samples were embedded, sectioned and examined in a Tecnai FEI 10 electron microscope at 80 kV.

3.5.2 Scanning electron microscopy (SEM) (Paper III)

Biofilm formation in the presence of peptide LL-37 (1 mg/L concentration) was studied, using the combined media. After 24 h incubation, catheter pieces were placed into fixation solution (2% glutaraldehyde + 1% Paraformaldehyde in 0.1 M phosphate buffer at pH 7.4) and processed for analysis using an Ultra 55 field emission scanning electron microscope (Zeiss, Oberkochen, Germany) at 3 kV. Digital images were randomly taken and ten fields per each catheter piece were investigated to analyze the bacterial biofilm phenotype.

3.6 DNA- AND RNA BASED ANALYSIS

The clinical isolates (B1-10 and NS1-16) as well as the reference strains were the subject of the polymerase chain reaction (PCR) and the pulsed-field gel electrophoresis (PFGE) analysis, while the *S. epidermidis* ATCC35984 strain was subjected to the quantitative real time PCR (qRT-PCR) analysis.

3.6.1 PCR (Paper I)

The presence of the following biofim-associated genes: *ica* (Eftekhar and Speert, 2009), *bhp* (Bowden *et al.*, 2005), *fbe* (Nilsson *et al.*, 1998), *atlE* (Vandecasteele *et al.*, 2003), *embp* (Williams *et al.*, 2002), *aap* (Nelson *et al.*, 2009a) as well as the insertion element IS256 (Gu *et al.*, 2005) were screened with PCR. Genomic DNA from each bacterial strain was extracted using QIAmp DNA minikit (Qiagen, Hilden, Germany) with the addition of 200 µg/ml lysostaphin (Sigma-Aldrich Chemical Co.) at the lysis step. The PCR mixtures and conditions were adapted from Bowden *et al*. (Bowden *et al.*, 2005) and Gu *et al*. (Gu *et al.*, 2005) in the case of IS256. Reaction mixture without DNA as negative control was included in each PCR run. The 16S rRNA (Gazzola and Cocconcelli, 2008) gene was used as an internal control. PCR products were visualized by 1.5% agarose gel electrophoresis.

3.6.2 PFGE (Paper I)

SmaI (Life Technologies, Inchinnan, UK) digests of whole-genome DNA followed by PFGE was used to calculate the genetic similarity between the *S. epidermidis* isolates. The GelCompar software (Applied Maths BVBA, Kortrijk, Belgium) was used in the comparisons of the band patterns, where a dendrogram was generated by the UPGMA clustering method (unweight pair group method with arithmetic mean) using the Dice co-efficient. A similarity co-efficient (Dice) of 80% corresponds to around 6 band differences, and was used for defining potential

clonal relatedness (Tenover *et al.*, 1995). A tolerance of 3% was applied during the comparison of patterns (Supplementory figure). The reference strain used in the normalization procedure was *Staphylococcus aureus* CCUG41582.

3.6.3 RNA extraction, reverse transcription and qRT-PCR (Paper III)

Gene expression in free-floating (planktonic) versus biofilm (sessile) bacteria was investigated after 24 h incubation exposed to the peptide LL-37, when bacterial biofilm were grown in a 6-well culture plate.

For planktonic cells, the culture media was removed from the culture plate wells and bacterial cells were pelleted by centrifugation. Culture media was discarded and cells were frozen in liquid nitrogen followed by mechanical disruption. Finally, Trizol reagent (Ambion, Life Technologies Corporation) was added to the cells. Total RNA was extracted using PureLink RNA Micro Kit (Invitrogen, Life Technologies Corporation), followed by treatment with PureLink DNase (Invitrogen, Life Technologies Corporation) according to the manufacturer's instructions. The A260/280 ratio for the samples was measured (NanoDrop^{TM} 1000 Spectrophotometer) and the concentration of the total RNA was calculated. For sessile cells, after removal of the culture media, biofilm was washed with sterile PBS (pH 7.4, Gibco, Life Technologies Corporation). Trizol reagent (Ambion, Life Technologies Corporation) was added for removing the cells from the surface then the cell solution was transferred to eppendorf tubes. After freezing, cell disruption and RNA isolation were performed as above.

RNA was reverse transcribed using Super Script Vilo (Invitrogen, Life Technologies Corporation) according to the manufacturer's instructions.

Biofilm-associated gene expression was determined by real-time PCR. Oligonucleotide primers for the detection of *aap* gene (coding the accumulation-associated protein Aap), *atlE* gene (coding the AtlE cell wall autolysin), *icaB* gene (coding the IcaB enzyme), psmB1 gene (coding the beta type phenol-solubil modulin) and the *rna*III gene (coding the RNAIII protein) were designed based on the genomic sequence of *S. epidermidis* ATCC35894 (PubMed accession number NC_002976.3) using the Primes3 software (Rozen and Skaletsky, 2000). RT-PCR was performed using SYBR Green Master mix (Applied Biosystems, Foster City, CA, USA). Ct values were normalized against the 16S ribosomal gene, as a housekeeping gene assumed to be evenly expressed in all cases.

The efficacy of the PCR reaction was between 90-100% for each gene. Relative fold change of specific mRNA transcripts were calculated using $2^{-\Delta\Delta Ct}$ method where ΔCt= Ct(target gene)-Ct(housekeeping gene). Data analysis was based on three parallel measurements. Median fold changes were calculated using data from three independent RNA extractions.

3.7 PROTEIN ANALYSIS

3.7.1 Immuno dot-blot analysis (Paper III)

S. epidermidis surface protein Aap was investigated in planktonic cells. Planktonic cells were used from the culture plate settings, as described above.

The culture media was removed from the culture plate wells and the bacterial cells were pelleted by centrifugation. The pellet was washed with sterile ice-cold sterile PBS (pH 7.4, Gibco, Life Technologies Corporation), then centrifuged again. The pellet was re-suspended in ice-cold sterile PBS and adjusted to identical cell densities by absorbance measurement at 600 nm (OD600). Serial dilutions (5 µl) were spotted onto PVDF membrane (Immobilon-FL,

Millipore Corporation, Billerica, USA), then immersed in blocking buffer (LI-COR, Inc., Nebraska, USA) and developed by standard immune-blotting procedures. Expression of Aap was detected by using a polyclonal rabbit anti-rAap antiserum raised against Aap domain B (Rohde *et al.*, 2005) diluted 1:20000. Bound antibodies were detected with IRDye 800CW conjugated goat anti-rabbit IgG (LI-COR, Inc., Nebraska, USA) diluted 1:15000 and visualized by CLx Odyssey Infrared Imaging System (LI-COR, Inc., Nebraska, USA). Signal intensities were calculated by the Image Studio 3.1 software (LI-COR, Inc., Nebraska, USA).

3.7.2 ELISA (Paper IV)

Level of hCAP18/LL-37 were determined by a commercial ELISA kit (Hycult Biotechnology, Netherlands) according to the instructions of the manufacturers.

3.7.3 Immunoprecipitation and western blot analysis (Paper IV)

The presence of different LL-37 isoforms was studied in serum samples. Since the concentration of the peptide is low in serum (ng/mL), immunoprecipitation was performed prior to the Western Blot analysis, to be able to detect the peptide.

Serum samples (n=6 from each infants group and n=2 from each maternal group) were proceeded for immunoprecipitation using Dynabeads Protein G Kit (Life Technologies, CA, USA). The beads were incubated with 3 µg of rabbit polyclonal anti human hCAP18/LL-37 antibody (Innovagen, Lund, Sweden) then the precipitation was preformed according to the manufacturer's instruction.

After elution, 5 µl of precipitated peptides were loaded to NuPAGE Bis-Tris 5-12% Mini Gel (Life Technologies, CA, USA) using LDS sample buffer (Life Technologies, CA, USA). The separation was performed under non-reducing conditions. Peptides were blotted onto PVDF membrane (Immobilon-FL, Millipore Corporation, Billerica, USA), then immersed in blocking buffer (LI-COR, Inc., Nebraska, USA) and developed by standard immune-blotting procedures. The different isoforms of the peptide were detected by using a mouse monoclonal anti human hCAP18/LL-37 antibody (Hycult Biotechnology, Netherlands) diluted 1:250. Bound antibodies were detected with IRDye 800CW conjugated goat anti-mouse IgG (LI-COR, Inc., Nebraska, USA) diluted 1:15000 and visualized by CLx Odyssey Infrared Imaging System (LI-COR, Inc., Nebraska, USA).

In order to assess the unspecific precipitation, the procedure was tested on a serum sample without using the polyclonal antibody. Standard LL-37 solution (Hycult, Biotechnology, Netherlands) diluted to 1 ng/mL was also immunoprecipitated to use as a positive control.

3.8 ANTIMICROBIAL SUSCEPTIBILITY TESTING (PAPER I)

The clinical isolates (B1-10 and NS1-16) as well as the reference strains were tested for susceptibility to gentamicin and cefoxitin (marker of methicillin resistance) using the disk-diffusion method, and vancomycin resistance was tested with the gradient MIC method (Etest, bioMérieux, Marcy l'Etoile, France). Disk diffusion was performed with EUCAST methodology (http://www.eucast.org/antimicrobial_susceptibility_testing/disk_diffusion_methodology/, last accessed November 28 2012), whereas the gradient test was carried out according to the manufacturer's description. In brief, colonies were suspended in sterile saline to a density of McFarland × 0.5, corresponding to 10^8 CFU/ml. Prepared inocula were applied to agar plates by cotton swabs before antibiotic disks or gradient strips were placed on the surface. Plates were incubated at 35°C and read after 18 h incubation. Results were interpreted according to EUCAST breakpoints (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/Breakpoint_table_v_4.0.xls).

4 RESULTS AND DISCUSSION

4.1 CHARACTERISTICS OF *STAPHYLOCOCCUS EPIDERMIDIS* STAINS ISOLATED FROM NEONATES (PAPER I)

4.1.1 Primary attachment and biofilm formation

We performed microtiter plate assays, in which bacterial cells were allowed to attach to the polystyrene surface as well as to form biofilm in a glucose rich growth media. Although, the glucose rich media does not reflect the conditions of the skin or human blood, it is a good system to study the potential differences among bacterial strains. Two different time points were used, 2 h incubation to study the primary attachment and 24 h incubation to study the biofilm formation. Since the assay is semi-quantitative, we can only assume whether the investigated strain is weak/intermediate/strong biofilm producer or non-biofilm producer (Stepanovic *et al.*, 2007). Under these experimental conditions, we found that skin isolates more likely formed biofilm (10 out of 16, 62.5%) than blood isolates (5 out of 10, 50%). However, the statistical analysis did not show significant difference, which might be due to the low sample size. Other studies observed various proportions of biofilm positive *S. epidermidis* strains among the isolates obtained from catheter-related infections: 87% shown by Ziebuhr *et al.* (Ziebuhr *et al.*, 1997) and 48% reported by Agricola *et al.* (Arciola *et al.*, 2001). Thus, indicating that mechanisms, other than biofilm formation may also be important in bloodstream infection.

The early bacterial attachment and the biofilm ultrastructure on a medical device surface were also studied with TEM, under the same conditions as in microtiter plate assays. Four strains with different characteristics were selected for this experiment: a biofilm and *ica*-positive skin isolate; a biofilm and *ica*-positive blood isolate; a biofilm and *ica*-negative blood isolate; as well as a biofilm and *ica*-positive reference strain. Attached bacterial cells were detected on the surface after 2 h incubation. Biofilm as a composition of bacterial cells and of the extracellular matrix was visible in the case of the biofilm-positive strains, but only single cells were seen close to the catheter surface when investigating the biofilm-negative isolate. The microscopic observation also revealed that the biofilm-forming *S. epidermidis* expressed short and long membrane filaments. Similar filaments were shown on the cell surface of *S. epidermidis* skin isolates (Nelson *et al.*, 2009a). We detected short filaments between the surface and the cells, and long filaments were seen between cells. This findings suggest that membrane structures may be important in initial adhesion process as well as in the cell-to-cell attachment and/or communication of the bacteria.

4.1.2 Prevalence of the biofilm-associated genes

We did not find significant difference in the presence of biofilm-associated genes (*ica*, *aap*, *atlE*, *embp*, *fbp*, *bhp*) between skin or blood isolates, in line with other findings investigating *S. epidermidis* isolates from NICU patients (Bradford *et al.*, 2006; De Silva *et al.*, 2002; Eftekhar and Speert, 2009). These genes and their products are involved in the primary attachment as well as in the biofilm maturation. However, *S. epidermidis* strains carry different genes with similar functions, which allow the bacteria to form biofilm, thus, provide survival under different conditions.

4.1.3 Antimicrobial susceptibility, IS256 and clonal relationship

IS256 is part of a transposon (*Tn4001*), inserted into the host chromosome in multiple free copies, may lead to the expression of different genes associated with pathogenesis, such as biofilm formation (Hennig and Ziebuhr, 2010) and antimicrobial resistance (Kozitskaya *et al.*, 2004). IS256 was shown to mediate gentamicin resistance by the aminoglycoside-modifying

enzyme (Kozitskaya *et al.*, 2004). Thus, it might increase the fitness of *S. epidermidis* in the hospital settings (Gu *et al.*, 2005). We observed that the presence of the insertion element IS256 was always associated with methicillin and gentamicin resistance in blood isolates, similarly to the control strain ATCC35984. In contrast, all skin isolates were susceptible to both methicillin and gentamicin, and lacked IS256, as did the laboratory strain ATCC12228, too. We did not find vancomycin resistant strains among the isolates, in line with a recent publication (Klingenberg *et al.*, 2007). Our PFGE analysis showed that the blood isolates are not clonally related to the skin isolates, except the strain B4 which has the closest relation to the skin isolate NS16.

4.2 INTERACTION BETWEEN *S. EPIDERMIDIS* AND THE HUMAN CATHELICIDIN PEPTIDE LL-37, *IN VITRO* STUDIES (PAPER II AND III)

4.2.1 Effect of the peptide on primary attachment and biofilm formation

Overhage *et al.* published recently that the human cathelicidin peptide LL-37 prevents *Pseudomonas aeruginosa* biofilm formation (Overhage *et al.*, 2008). We addressed the same question in our study, whereas we investigated the attachment and the biofilm formation of *Staphylococcus epidermidis* ATCC35984 strain in the presence of the LL-37 using microtiter plate assays (*Paper II*). It was shown, that the composition of the artificial growth media influences the antimicrobial activity of LL-37 (Nijnik and Hancock, 2009; Turner *et al.*, 1998) by affecting its cationic and amphiphilic feature (Hancock and Sahl, 2006). However, the particular *S. epidermidis* ATCC35984 strain did not form biofilm in a minimal media (Overhage *et al.*, 2008) and LL-37 did not inhibit biofilm formation in the reach TSBg (Nelson *et al.*, 2009b). Therefore, we performed the assays in a modified TSBg containing 0.05% acetic acid. The inhibitory effect was clear and good biofilm formation was observed in this growth media. However, it is not fully understood how the acetic acid influences the peptide's effect.

We found that already 1 mg/L of the peptide inhibited the attachment of the bacterial cells and reduced the biofilm mass significantly. However, the growth of free-floating (planktonic) bacteria was not inhibited either at 8 mg/L or 16 mg/L concentrations. The 1 mg/L concentration corresponds with 1/32 MIC and also with the normal level of LL-37-precursor (1.18 mg/L) found in the plasma of human adults (Sorensen *et al.*, 1997b). (*Paper II*)

Pieces of sterile CVC (commonly used for long-term access in preterm infants) were also incubated with *S. epidermidis* ATCC35984 cells, in the absence or presence of LL-37 (1 mg/L) for 24 h (*Paper III*). The catheter pieces were then analysed by SEM. We detected a diminished amount of cell aggregates on the catheter surface, when exposed to the peptide.

It is known, that biofilm matrix as a barrier might protect the bacteria against several antimicrobial peptides and antibiotics (Mah and O'toole, 2001). However, some other antibiotics, such as vancomycin and rifampin, were shown to diffuse easily into the biofilm (Dunne *et al.*, 1993). Our results suggest that the peptide LL-37 might penetrate into the biofilm and interact with the bacterial cells. Thus, LL-37 may destroy the cell membrane (direct killing) or may act on the internal cellular targets (genes, proteins) (Hancock and Sahl, 2006). Whereas, both mechanisms can lead to a reduced biofilm mass.

4.2.2 Effect of the peptide on biofilm-associated gene expression

We further investigated the effect of LL-37. Our question was, whether the peptide LL-37 influences the transcription of genes involved in biofilm formation, as it was observed in *P. aeruginosa* studies (Overhage *et al.*, 2008; Strempel *et al.*, 2013). The expression of biofilm-associated genes (*aap*, *atlE*, *icaB*, *psmB1* and *rnaIII*) was studied in sessile (biofilm) vs. planktonic (free-floating) bacteria when exposed to the peptide for 24 h (*Paper III*). Static

biofilm assays were performed using the same combined media as in the previous study. Two different physiological concentrations of the peptide were used: 0.01 mg/L that refers as the circulating blood concentration in healthy newborn infants and adults (Mandic Havelka et al., 2010) and 1 mg/L that corresponds to the average level found in tissues and mucosal surfaces in humans (Overhage *et al.*, 2008).

We observed that planktonic bacterial cells, in their free-floating mode, seem to be more affected by the cationic host defense peptide LL-37, than sessile bacteria. The variability in gene expression (*atlE*, *icaB*, *psmB1* and *rnaIII*) was higher in planktonic bacteria than in sessile bacteria, however, the difference in response between the two cell populations was not significant. We could not detect significant difference in gene expression between treated and untreated cells either in the sessile or the planktonic group.

LL-37 induced an up-regulation of the *icaB* gene in planktonic cells but not in sessile cells. This may indicate that the free-floating bacteria becomes more virulent and more resistant to phagocytosis and further effects of the innate immune components in response to the peptide LL-37. In fact, the *icaB* gene, as part of the *icaADBC* operon, is involved in the synthesis of the cationic exopolymer polysaccharide intercellular adhesin (PIA) (Rohde *et al.*, 2010) (Figure 3b). The IcaB enzyme is responsible for the export and deacetylation of the growing homopolymer, which are crucial for both surface location and biological functions of PIA (Otto, 2014). PIA has been shown to protect *S. epidermidis* from cationic AMPs and neutrophil killing, complement- as well as immunoglobulin deposition (Vuong *et al.*, 2004c).

An increased *atlE* transcript level was found in both planktonic and sessile cells, in a concentration-dependent way. The up-regulation of *atlE* was more pronounced in planktonic bacteria. Our observations might suggest a higher level of cell turn-over connected to higher autolysin AtlE activity in both cell populations. The autolysin AtlE belongs to a group of peptidoglycan-hydrolases that play an important role in the degradation of the bacterial cell wall (Figure 3a). During cell division, these autolysins are responsible for splitting the equatorial septum between two dividing daughter cells (Zoll *et al.*, 2010). AtlE also cause the release of extracellular DNA by *S. epidermidis*, an important component required for initial bacterial attachment to a surface as well as for the early phase of biofilm development, *in vitro* (Qin *et al.*, 2007). A recent study showed that an increased transcript atlE level enhanced cell attachment, extracellular DNA release, cell death and subsequent biofilm formation, in *agr* mutants (Dai *et al.*, 2012).

The accessory gene regulator (*agr*) quorum sensing system is the main regulator of biofilm formation in *S. epidermidis*. AtlE is negatively regulated by the *agr* system which consists of 5 genes including the gene for the effector molecule RNAIII (Dai *et al.*, 2012) (Figure 3a). Higher *rnaIII* gene expression might lead to decreased *altE* transcript level and also reduced biofilm formation. In contrast we found, that *atlE* and *rnaIII* gene were consequently up-regulated and followed the same pattern, in a concentration-dependent way, in both planktonic and sessile cell populations. Possible explanations for this are that the *agr* system is affected by the peptide LL-37 at translational level or that besides *agr*-system other factors are also involved in the regulation of *atlE*.

We detected a strong down-regulation of *psmB1* gene, in a concentration dependent way in the planktonic cell population. We noticed that the baseline gene expression was higher in planktonic than in sessile cells. Despite of the down-regulation induced by the peptide LL-37, the planktonic cells still maintained higher *psmB1* transcript level compared with sessile bacteria, which maybe reflecting that they originate from sessile cells from the biofilm mass. It is in line with findings that an induced expression of the PSM peptides results in biofilm detachment (Wang *et al.*, 2011).

The accumulation-associated protein (Aap) is expressed as a 240 kDa cell surface protein composed of domains A and B in *S. epidermidis*. To become functionally active as an intercellular adhesion, domain A has to be proteolytically cleaved off from full-length Aap, exposing the B domain, which consecutively mediates intercellular adhesion and biofilm formation (Rohde *et al.*, 2005) (Figure 3a). However, Aap of the strain ATCC35984 (RP62A) is not being processed (Banner *et al.*, 2007), suggesting that the protein is in this strain under the conditions used mainly involved in the primary attachment. Therefore, we further investigated whether the peptide LL-37 affected the expression of the Aap protein on the planktonic cell surface, thereby influencing those cells' attachment capability. The expression of domain B was assessed using 24-h whole cell suspension. We observed that there were a slight increase in the Aap protein amount at both peptide concentrations compared with the cells grown in the absence of LL-37. However, we did not find changes in the transcription level of the *aap* gene in planktonic bacteria when exposed to the peptide.

In conclusion, more biological repeats would be needed to obtain consistent results. For better understanding of the mechanism behind the anti-biofilm effect of LL-37, further studies are needed to look at different conditions, more genes and more proteins involved in the biofilm development.

4.2.3 Circulating cathelicidin antimicrobial peptide LL-37 levels in newborn infants in relation to gestational time (Paper IV)

We grouped the study population based on the gestational time: delivery before weeks 28 (Group 1, n=8 mother-infant pairs), delivery between weeks 28 and 36 (Group 2, n=20 mother-infant pairs) and delivery between weeks 37 and 42 (Group 3; n=15 mother-infant pairs + n=12 infant). Circulating serum concentration of the human cathelicidin LL-37 was measured by ELISA as well as the pro- and the active forms of the cathelicidin protein were studied by Western Blot analysis.

Our major finding is that we did not find significant difference in the circulating LL-37 concentration in infants born at different gestational time, in contrast to a recent publication that showed reduced plasma level of the peptide, and also of other AMPs in preterm infants (Strunk *et al.*, 2009). The possible explanation of the differing results can be that it is difficult to obtain sufficient blood sample volume from the umbilical cord of preterm infants. Therefore, the low sample volume might cause unreliable measurements. In addition, the low sample size can also influence the results. We had good sample volume, but rather low sample size (8 infants born before the gestational weeks 28, out of 28 preterm neonates). However, the similar serum LL-37 level in both the preterm and term groups might suggest that the transplacental passage of the peptide LL-37 occurs early during pregnancy.

We detected both the inactive proprotein hCAP18 (18 kDa) and the active LL-37 (4.5 kDa) peptide in newborn infants as well as their mothers at time of birth, independent of gestational time.

We observed a significant positive correlation between the maternal and umbilical cord serum level of human cathelicidin peptide LL-37, independent of the gestational time. Similar positive correlation was recently shown in a term mother-infant group (Mandic Havelka *et al.*, 2010). The correlation between serum LL-37 level and gestational time was also analysed. We found that the concentration of the peptide did not show significant correlation with the gestational time, either in infants' serum or in mothers' serum (p=0.9490 and r=0.0088; p=0.6007 and r=0.082, respectively).

5 GENERAL CONCLUSIONS

- *S. epidermidis* strains obtained from bloodstream infection in preterm infants had different characteristics than the skin isolates of healthy term neonates. The blood isolates were equipped with an invasive genetic element and showed higher antimicrobial resistance compared with the skin isolates. Whereas, vancomycin resistance was not detected among any of the isolates.

- Short and long filament-like structures on the cell surface of *S. epidermidis* seemed to be involved in the attachment to the catheter surface and also in cell-to-cell attachment/communication.

- Physiological LL-37 peptide concentrations, below those that kill or inhibit growth, inhibited *S. epidermidis* attachment and biofilm formation on abiotic surfaces. The peptide regulates genes involved in primary attachment, accumulation and in biofilm regulation.

- The concentration of the circulating hCAP18/LL-37 was not significantly different in preterm and term neonates at birth. There was a positive correlation between maternal and infant peptide concentration. The inactive proprotein hCAP18 and the active LL-37 peptide were present in newborn infants as well as their mothers at time of birth, independent of gestational time. These findings might suggest that preterm infants' vulnerability is not connected to the lower LL-37 peptide level at birth.

6 FUTURE PERSPECTIVES

- The increasing antibiotic resistance among the invasive bacterial strains force us to develop alternative strategies in the treatment or prevention of catheter-related infections. There is a challenging field as to design coated catheters where biomolecules (antimicrobial peptides) or nanoparticles could be used to modify the plastic surface of the device, thereby inhibiting the attachment of biofilm forming bacteria. Drugs could be used to stimulate AMPs' expression or modified antimicrobial peptides might be used as drugs. So far, an increased level of infection control in NICU settings, seems to be a preventive tool in catheter-related infections in preterm infants.

- It is still unclear what components of the bacterial surface form short and long filament-structures. Aap or PSMs could be studied as possible molecules.

- Microarray- and protein analysis at different time points could be performed for better understanding of the anti-biofilm effect of the peptide LL-37. Also, fluorescent microscopy could reveal the capability of the peptide to diffuse into the biofilm. In addition, the anti-biofilm effect of LL-37 in combination with other AMPs could also be studied.

- It is well known that LL-37 plays an important role in the first-line of the immune defense, driving inflammatory responses and activating other innate- as well as adaptive immune components. This study could be the first step in a larger project which could measure the LL-37 concentration in higher number of preterm infants, with a bigger focus on the extreme preterm neonates (born <28 weeks), to confirm our results. It is also an interesting question how the LL-37 level will change after birth and how it can be increased in preterm infants during infection.

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