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VIRULENCE FACTORS OF THE COAGULASE-NEGATIVE STAPHYLOCOCCI

Michael Otto

Rocky Mountain Laboratories, Laboratory of Human Bacterial Pathogenesis, National Institute of Allergy and Infectious Diseases, The National Institutes of Health, 903 S 4th Street, Hamilton, MT 59840

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1. ABSTRACT

Coagulase-negative staphylococci (CNS) have gained substantial interest as pathogens involved in nosocomial, particularly catheter-related infections. The pathogenic potential of CNS is mainly due to their capacity to form biofilms on indwelling medical devices. In a biofilm, the bacteria are protected against antibiotics and

from attacks by the immune system. The factors contributing to biofilm formation are among the best-studied virulence factors of CNS and comprise factors involved in the adhesion to a catheter surface and in cell accumulation. CNS usually persist in the host in relative silence, but may cause sepsis, for which the recently found inflammatory peptides called phenol-soluble modulins are prime candidates. Many CNS also produce several lipases, proteases, and other exoenzymes, which possibly contribute to the persistence of CNS in the host and may degrade host tissue. We are also beginning to understand how regulators of virulence trigger the expression of virulence factors in CNS. A better conception of the mechanisms underlying the pathogenicity and the frequently encountered antibiotic resistance of CNS may help to develop novel, efficient anti-staphylococcal therapeutics.

2. INTRODUCTION

Staphylococci are Gram-positive, AT-rich cocci, which often stick together in grape-like clusters. The name *Staphylococcus* is derived from the Greek word for grape (staphylos). The genus can be separated into two groups, based on the ability to produce coagulase, an enzyme that causes clotting of blood plasma. Coagulase-positive staphylococci comprise the main species *Staphylococcus aureus*, while *S. epidermidis* is the most frequently found member of the coagulase-negative staphylococci (CNS) in humans (1).

CNS are normal and abundant colonizers of humans and become pathogenic only in certain situations. Whereas *S. aureus* has been a well-known human pathogen for years, the medical interest in CNS has only recently developed. This has mainly been due to the strongly increasing rate of opportunistic infections and infections on medical devices caused by CNS (2). Two main reasons for the increasing rate of CNS infections are the spreading antibiotic resistance among CNS and the increasing use of medical devices over the recent years. *S. epidermidis* is believed to account for most of the infections caused by CNS, but often the specific infecting CNS species remains undetermined in the hospital. CNS also comprise species that colonize and infect animals, and less pathogenic or non-pathogenic species, of which some are used for biotechnology purposes. This article will describe the virulence factors of CNS, with a focus on human infection and the most important and best described species, *S. epidermidis*.

3. VIRULENCE FACTORS OF THE COAGULASE-NEGATIVE STAPHYLOCOCCI

3.1. Infections and epidemiology

CNS found in humans colonize different parts of the human skin and mucous membranes, with each species having a certain predominance on specific parts of the body (1). Generally CNS have a benign relationship with their host and develop from commensals to pathogens only after damage of a natural barrier such as the skin, which can occur by trauma, inoculation, or implantation of medical devices. Every species of CNS that has been characterized as a resident of the human body (*S. epidermidis*, *S. capitis*, *S. hominis*, *S. haemolyticus*, *S. saccharolyticus*, *S. warneri*, *S. lugdunensis*, *S. saprophyticus*, *S. cohnii*) has also at least once been connected to an infection. The specific sites and frequency of infection seem to be related to the site and frequency of normal colonization. *S. saprophyticus* for example is often found in the inguinal and perineal areas and is a common cause of urinary tract infections (1). In general, the factors that CNS need to survive in their habitat in or on the human body are likely the same that they need to efficiently colonize tissue during an infection. Success as a pathogen in these cases depends on adhesion factors, evasion of the host's immune system, and the production of factors harmful to host tissue, such as toxins and degradative exoenzymes. However, the latter class of virulence factors is less frequently found in CNS compared to *S. aureus*, which makes infections with CNS more silent than those with *S. aureus*. According to the National Nosocomial Surveillance System Report 1998 (Centers for Disease Control and Prevention, Atlanta, GA), CNS are the most predominant cause of nosocomial bloodstream infections, cardiovascular infections, and infections of the eye, ear, nose, and throat. Except for opportunistic infections in immuno- or otherwise compromised patients like premature newborns, AIDS patients, or drug abusers, infections caused by *S. epidermidis* and many other CNS are usually non-pyogenic, with one exception being native valve endocarditis. For all CNS species, the most common group of infections are infections on foreign bodies such as any indwelling catheter, implanted device, or contact lenses. Biofilm formation is the cause for the difficulty to eradicate the infection and the impaired effectiveness of antibiotics in these cases.

3.2. Virulence factors of *Staphylococcus epidermidis*

The genome of *S. epidermidis* RP62A has been sequenced by TIGR (The Institute for Genomic Research, Rockvile, MD) and the sequence has been made public. However, the annotation of the *S. epidermidis* genome has not been published yet. Therefore this review will mostly focus on factors, for which evidence is available from biochemical or molecular biology experiments, or from epidemiological data. Still some very interesting putative new virulence factors found in the genome will be mentioned. Nevertheless it should be kept in mind that we do not know yet if these factors are produced and play a role in *S. epidermidis*. An overview of known virulence factors of *S. epidermidis* is given in Table 1.

3.2.1. Adaptation to a specific habitat

CNS have to deal with the specific character of the human skin and mucous membranes. They must adapt for example to high osmolarity, relatively low pH, mechanical stress, and varying moisture and temperature. A high concentration of blood vessels and sweat glands on the skin means that plenty of nutrients are available and consequently, more staphylococci are found in these regions, such as the scalp, ears, or nares (1). Most importantly perhaps for being the predominant species on the human skin, *S. epidermidis* is known to withstand very high salt concentrations (3). This is reflected by the finding that the genome of *S. epidermidis* RP62A has a high

Table 1. Virulence factors of *Staphylococcus epidermidis*

Name	Genetic locus identified (Reference)	Protein identification/Characterization (Reference)	Function
Biofilm factors			
Attachment to hydrophobic surfaces			
• AtlE	(17)	(46)	Autolysin/adhesin: attachment to polystyrene, vitronectin binding
• SSP1, SSP2	No	(25)	Attachment to polystyrene
• Delta-toxin	(112)	(91),	Inhibits binding to polystyrene
Attachment to to host matrix proteins			
• Fbe/SdrG	(30, 33)	(30, 33)	Fibrinogen binding, inhibition of phagocytosis
• Embp	(37)	(37)	Fibronectin binding
• GehD	(80)	(49)	Collagen binding
Accumulation			
• PIA	*ica* (17)	(10, 17, 56)	Exopolysaccharide: cell-cell adhesion, haemagglutination
• AAP	N-terminal sequence identified	(75)	Accumulation
Other factors			
Exoenzymes			
• Lipases	*gehC* (79)	GehC (79)	Persistence in fatty secretions
• Lipases	*gehD* (80)	GehD (80)	Persistence in fatty secretions
• Cysteine protease	N-terminal sequence identified	(85)	Unknown, possibly tissue damage
• Metalloprotease Sep-P1	(84)	(84)	Lipase maturation, possibly tissue damage
• Serine protease GluSE	(86)	(86, 87)	Biofilm formation (?), degradation of fibrinogen and complement factor C5
• Fatty acid-modifying enzyme (FAME)	No	(97)	Detoxification of host-produced bactericidal fatty acids
Regulators of virulence			
• agr	(112)	(83)	Affects lipase and protease production
• sar	(123)		
• sigB	(129)	(60)	Affects biofilm formation
Others			
• Phenol-soluble modulin (PSM)	(77)	(77, 89)	Several inflammatory effects
• Staphyloferrin A and B	No	(101)	Siderophores: iron uptake
• SitABC	(102)	(102)	Possibly involved in iron uptake
• Lantibiotics (epidermin, Pep5)	(199, 200)	Reviewed in (167)	Bacterial interference (?)

number of genes involved in osmoregulation, such as genes with similarity to Na^+/H^+- and glycine betaine transporters.

The production of adhesion factors can be interpreted as another adaptation to a habitat, as the bacteria need those to efficiently colonize human tissue surfaces. Adhesion also constitutes the first step of the infection process, in which the interaction with the host's immune system represents a further factor that determines the bacterium's ability to survive. As colonizers of the human skin and mucous membranes, CNS mainly have to defend themselves against antibacterial secreted peptides (4). During a systemic infection they have to be prepared to encounter many more defense mechanisms of innate and adapted immunity. The most important mechanism of CNS to escape from the host's immune system during infection seems to simply hide behind a layer of extracellular matrix in relative silence. However, more recent findings show that CNS also produce factors that cause an inflammatory response and, in some cases, exotoxins.

3.2.2. Factors involved in biofilm formation

Biofilms are surface-attached, multi-cell agglomerations with a unique physiology, gene expression, and structure. Of the genes specifically expressed in a biofilm some are needed for the altered metabolism in a biofilm compared to planktonic growth. Some others code for factors that are directly linked to the biofilm structure, such as genes coding for the expression of extracellular macromolecules like binding proteins and polysaccharides. The advantages of bacterial life in a biofilm comprise an increased resistance to antibiotics, to attacks by the immune system, and to mere mechanical force (5). The formation of a biofilm is therefore an immense contribution to the successful colonization of host tissues and indwelling medical devices.

At a time when CNS were still only regarded as innocuous commensals of human skin, the first reports on a possible role of *S. epidermidis* in catheter-related infection came from several groups, which found *S. epidermidis* as the most frequently isolated species from indwelling catheters (6-8). Soon afterwards it became clear that the virulence of *S. epidermidis* in the course of catheter-related infections is linked to the formation of a biofilm on the catheter surface. The extracellular matrix between the cells in a biofilm in those times was called "slime" (8). Since then, several components involved in "slime" formation and in adhesion to foreign bodies have been proposed and identified. Relatively simple methods based on the adherence to microtiter plates have been used to identify biofilm factors by genetic approaches (9). A model of biofilm formation has been developed, which mainly distinguishes between the initial attachment to a surface

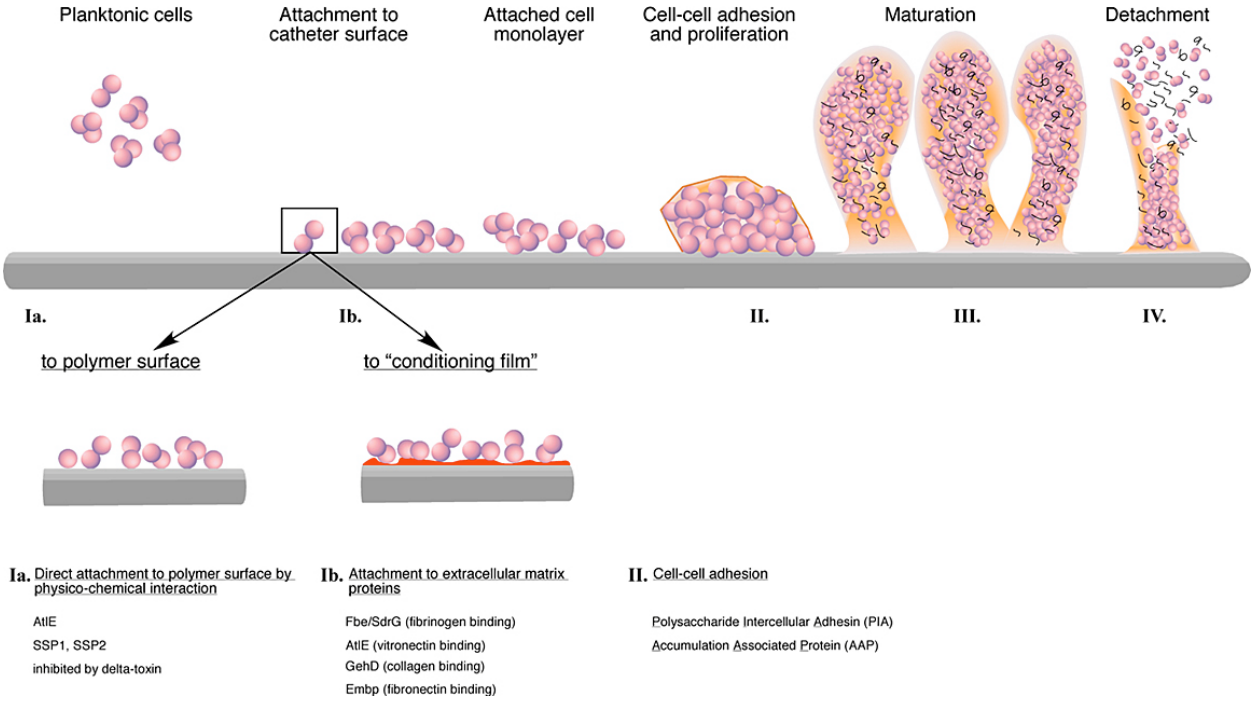

Figure 1. Model of biofilm formation on a catheter surface in *Staphylococcus epidermidis*. Biofilm formation is shown as a four-step process involving initial attachment, accumulation, maturation, and detachment. Several factors involved in the attachment and accumulation phases have been described and are noted on the bottom of the figure. Factors involved in maturation and detachment have not been identified yet. Initial attachment can occur as direct adhesion to the polymer surface or depend on the interaction of dedicated bacterial binding proteins with host matrix proteins that cover the catheter as a "conditioning film".

and the proliferation of cells to a multi-layered biofilm (10) (Figure 1). More recently, the investigation of biofilms of various bacterial species grown in biofilm reactors for longer periods of time and the visualization of biofilm structure by methods such as confocal scanning laser microscopy led to the description of a matured biofilm as a more complicated 3-dimensional structure, which includes water channels and mushroom-shaped cell agglomerations (11). Furthermore, the importance of the detachment of cells or cell clusters from a biofilm was recognized. This mechanism is believed to be crucial for the spread of an infection. Pioneering work on the 3-dimensional shape of biofilms has been performed using *Pseudomonas aeruginosa* as a standard biofilm-forming organism. Much of what we know about biofilm structure is based on investigation with this Gram-negative bacterium. However, more recent investigation using genetically defined mutants of other biofilm-forming organisms demonstrated that big differences may exist between the species with respect to biofilm factors and their regulation.

3.2.2.1. Initial attachment

With regard to the initial attachment of bacteria to a surface, one has to distinguish between the attachment to host tissues and to foreign bodies, or between biotic and abiotic surfaces. Whereas adhesion to abiotic surfaces is thought to be determined by the physico-chemical properties of the bacterial and the abiotic surface, the interaction of bacteria with receptors on host tissue involves specific protein interaction. Among abiotic surfaces, most important for CNS infections are hydrophobic surfaces of plastic materials as used for catheters and other indwelling devices. The extent of adhesion of *S. epidermidis* to plastic can vary with respect to the specific polymer material used (12). However, CNS are able to colonize virtually any type of plastic (13). Every foreign material in the human body soon becomes covered by host matrix proteins after insertion. The host material that covers the foreign body has also been called "conditioning film". The adhesion of bacteria to foreign bodies already covered by host proteins is similar to the adhesion to tissues, as it depends on the interaction of bacterial surface proteins with host factors. Thus, bacteria can colonize indwelling devices by either direct adhesion to plastic or by specific binding proteins interacting with the host matrix (14). These two mechanisms also reflect different scenarios of an infection. Bacteria might directly stick on a catheter surface before or during the insertion process, or colonize the pre-coated catheter surface by gradually proliferating into the damaged tissue starting from the skin surface.

3.2.2.1.1. Initial attachment to plastic surfaces

Direct binding to a plastic surface involves hydrophobic interaction, van-der-Waals forces, and possibly electrostatic interaction (14). Although it has been shown that CNS can adhere to plastic materials of varying composition *in vitro*, the importance of this in human infections is still unclear. Strains show highly variable surface hydrophobicity, which can be correlated with the

ability to stick to plastic surfaces *in vitro* (15). However, no clear correlation between surface hydrophobicity and infectiveness has been shown (16). The fact that surface polymers and proteins can interact in many ways makes it very difficult to distinguish between direct interaction with a surface and secondary effects. For example, it is hardly conceivable that a highly charged polymer such as teichoic acids, which have been linked to the attachment to plastic (see 3.2.2.1.1.2) directly interacts with a polymer surface. It is much more likely that teichoic acids bind proteins, which in turn interact with the plastic by hydrophobic interaction. Unfortunately, an interaction of a protein or polysaccharide of CNS with a surface has never been shown on a molecular level. We therefore do not know of any of the factors mentioned below, if they directly bind to plastic or serve as "bridging molecules" in a complicated network of extracellular matrix molecules.

3.2.2.1.1.1. AtlE

Staphylococcal autolysins of the Atl type are a family of cell wall-lytic enzymes with adhesive properties. All members of this family – including AtlE - have been described to mediate binding to host matrix proteins (see 3.2.2.1.2.4). The autolysin AtlE of *S. epidermidis* is the only member of this family reportedly also involved in the direct attachment to hydrophobic surfaces. Evidence for the involvement of AtlE in the adhesion to plastic has been derived from a transposon mutant devoid of AtlE production in *S. epidermidis* that lacked the ability to adhere to polystyrene microtiter plates (17). The research on the homologous autolysins AtlC of *S. caprae* and Aas of *S. saprophyticus* has not focused on the adhesion to plastic. Therefore we do not know if adhesion to plastic is a common feature of staphylococcal autolysins or is restricted to AtlE in *S. epidermidis* and a possibly specific situation. AtlE and its degradation products represent the major portion of surface-attached proteins in *S. epidermidis* and the decreased ability to stick to plastic in the *atlE* mutant might therefore be caused by the general lack of surface protein rather than by an AtlE-specific feature. In general, the role of the direct attachment of staphylococci to plastic in infections of indwelling medical devices is a controversial issue. With respect to AtlE, the finding that the *atlE* mutant showed a somewhat reduced virulence in a rat model of catheter-related chronic infection does not necessarily support the hypothesis that AtlE-mediated direct attachment is important *in vivo* (18). It is possible that the reported vitronectin-binding capacity of AtlE (see below) alone accounts for the difference in virulence seen in the mutant. Therefore convincing proof for the importance of AtlE-mediated direct attachment to hydrophobic surface during infection remains to be given.

3.2.2.1.1.2. Teichoic acids

Teichoic acids (TA) are polymers of alternating phosphate and ribitol- or glycerol moieties on the cell surface of Gram-positive bacteria. They can either be linked to the cell wall (wall teichoic acids, WTA) or bound on the cell membrane by a membrane-spanning lipid anchor (lipoteichoic acids, LTA). A very strong biofilm former, *S. epidermidis* RP62A, has been reported to contain unusually high amounts of teichoic acids (19). Therefore teichoic acids have been considered an important part of the "slime" on the staphylococcal cell surface. However, the involvement of TA in biofilm formation has only been demonstrated recently in *S. aureus* (20). A mutant of the *S. aureus dlt* system, devoid of the ability to D-alanylate the phosphate group in TA, showed a decreased capacity to attach to polystyrene. Usually, a certain portion of TA are D-alanylated, which decreases the negative charge of the TA polymer, as esterification with D-alanine introduces one positive charge per D-alanine residue. The mechanism, by which altered D-alanylation influences attachment to polystyrene is not clear. One possible explanation could be altered binding of autolysins that possibly mediate attachment to plastic surface as discussed above. There is some evidence that autolysins use teichoic acids as a binding receptor on the bacterial surface (21, 22). Teichoic acids also bind several other non-covalently attached surface proteins in different Gram-positive bacteria (23). In *S. aureus*, D-alanylation is important for efficient binding of several surface proteins, suggesting that the charge of TA plays an important role in surface protein binding (C. Vuong, M. Otto, unpublished). The altered charge of non-D-alanylated TA also is responsible for the decreased resistance of the *S. aureus dlt* mutant against cationic anti-microbial peptides, which will be discussed in section 3.2.6.2.

Although these results have been achieved in *S. aureus*, and not in CNS, we can assume that the same mechanisms exist in CNS, as TA are a common feature of both and chemically similar. Interestingly, an additional LTA-like molecule named lipid S, which has a shortened chain length compared to usual LTA, has recently been found in *S. epidermidis* (24). Its role in virulence is not clear yet.

3.2.2.1.1.3. Other factors

Some protein factors other than AtlE have been linked to the attachment to plastic, namely two very large proteins of molecular weights higher than 200 kDa, SSP1 and SSP2 of *S. epidermidis*, with one very likely representing a degradation product of the other (25). However, their molecular nature is not clear. The Bap protein of *S. aureus*, which has been reported to impact initial attachment and accumulation, and its homolog in *S. epidermidis*, Bhp, are described below (see 3.2.2.1.2.2). The detergent-like peptide delta-toxin, which is produced by *S. aureus* and many CNS, influences binding to plastic in a negative way, as it presumably reduces hydrophobic interaction (26).

3.2.2.1.2. Initial attachment to host matrix proteins

CNS can attach to host matrix proteins (e.g. fibrinogen, fibronectin, collagen, vitronectin) on host tissues or in a conditioning film around foreign bodies. Bacterial proteins that bind to host matrix proteins are called MSCRAMMS (microbial surface components recognizing adhesive matrix molecules). MSCRAMMs are important virulence factors as attachment constitutes a crucial step during bacterial colonization. Many MSCRAMMs are covalently bound to the bacterial cell wall by an enzyme called sortase (27). Sortase is present in

most Gram-positive bacteria including CNS. A defect in sortase function can be expected to result in an almost complete loss of host cell binding, as only some MSCRAMMs are linked to the cell surface by other, non-sortase catalyzed mechanisms. In fact, a deletion mutant of sortase in *S. aureus* showed dramatically reduced virulence in an animal infection model (28). Often, several MSCRAMMS with overlapping binding capacity are produced in one strain (29). Therefore, the loss of one binding protein does not necessarily mean the loss of the capacity to bind to a specific matrix protein. Thus, the bacteria seem to take high precautions to maintain the ability to bind to host matrix proteins, suggesting that binding of these proteins is of enormous importance to the bacteria.

3.2.2.1.2.1. SD-repeat proteins

Much work has been performed with respect to the fibrinogen-binding protein (Fbe) of *S. epidermidis*. It is present in most *S. epidermidis* strains, has a size of 119 kDa, and is similar to the fibrinogen-binding proteins of *S. aureus* called clumping factors (ClfA, ClfB) (30). Antibodies against Fbe can block adhesion to fibrinogen-coated surface and implanted catheters, demonstrating that this protein is a major factor mediating adhesion to fibrinogen in *S. epidermidis* (31, 32). A second protein with 95% similarity to Fbe is found in another *S. epidermidis* strain, which has been called SdrG, and also contributes to fibrinogen binding (33). Both proteins belong to a family of binding proteins with serine/aspartate (SD) repeats. As only one open reading frame with similarity to SdrG and Fbe is found in the *S. epidermidis* RP62A genome, Fbe, SdrG, and the Fbe/SdrG homolog in strain RP62A most likely represent the same protein, which varies from strain to strain. SdrG and Fbe bind to the beta chain of fibrinogen, like ClfB of *S. aureus*, but in contrast to ClfA of *S. aureus*, which binds to the gamma chain. In-depth biochemical studies of SdrG binding to fibrinogen revealed that SdrG binds to a peptide near the thrombin cleavage site in fibrinogen (33). SdrG inhibits thrombin-induced clotting of fibrinogen by interfering with the release of fibrinopeptide B. As fibrinopeptide B functions as a chemotactic molecule, SdrG binding might reduce the influx of phagocytic neutrophils to the infection site. SdrG might therefore have two tasks to help bacterial survival in the host: it can serve as a binding molecule and reduces phagocytic elimination. A further protein with SD repeats, SdrF, is only present in some strains of *S. epidermidis* (34). In addition to SdrG and SdrF, there is a gene coding for a third SD repeat protein in the *S. epidermidis* RP62A genome, named SdrH (34). This SD repeat protein lacks an LPXTG motif. Nothing is known about a potential binding function of SdrH or SdrF.

3.2.2.1.2.2. Biofilm-associated protein

The *S. epidermidis* RP62A genome harbors two open reading frames with similarity to the gene encoding Bap (biofilm-associated protein) of *S. aureus*. One of these open reading frames (Genbank Acc. No. AY028618) has been called *bhp* (coding for Bap-homologous protein). The 239 kDa surface-associated Bap of *S. aureus* has been demonstrated to promote attachment to abiotic surfaces and intercellular adhsion *in vitro* (35), but to hinder several other aspects of colonization and infectivity, like adherence to immobilized fibronectin and fibrinogen, adherence to mammary gland tissue *ex vivo*, internalization by epithelial cells, and infection of mammary glands (36). The precise function of Bap in colonization therefore needs further investigation. The role of Bhp and of the putative other Bap-like gene product in *S. epidermidis* is unknown.

3.2.2.1.2.3. Fibronectin-binding protein

Recently, a fibronectin-binding protein of *S. epidermidis* has been identified and has been named Embp (37). Interestingly, recombinant proteins comprising the fibronectin-binding part of Embp of *S. epidermidis*, or of the fibronectin-binding protein FnBP of *S. aureus*, respectively, were not able to inhibit binding of cells of the other species to fibronectin-coated microtiter plates (38). This demonstrates that fibronectin-binding by these two proteins uses a different mechanism and possibly a different binding domain in fibronectin. In *S. aureus*, fibronectin binding is a crucial step not only for attachment but also for the internalization process in epithelial cells (38), which will be described as a virulence factor in section 3.2.7. In *S. epidermidis*, fibronectin binding proteins have not yet been linked to internalization, but are a prime candidate for the binding step preceding internalization. Except for Fbe, SdrG, and Embp, there is lack of experimental evidence so far in *S. epidermidis* and other CNS with respect to specific covalently bound MSCRAMMs. However, several non-covalently bound proteins have also been demonstrated to mediate attachment to host matrix proteins.

3.2.2.1.2.4. Non-covalently bound MSCRAMMs

Autolysins are cell wall-degrading enzymes involved in cell separation. The Atl type of staphylococcal autolysins consists of a glucosaminidase and an amidase part. Among CNS, the autolysins AtlE of *S. epidermidis*, AtlC of *S. caprae* (39), and Aas of *S. saprophyticus* (40) have been identified and characterized. The *S. aureus* homolog is called Atl (41). All these enzymes have signal peptide and propeptide sequences and lack a typical cell wall-binding domain with an LPXTG motif necessary for the covalent anchoring to the Gram-positive cell wall (42). The non-covalent nature of cell wall binding is demonstrated by the fact that boiling with 1% to 4% sodium dodecyl sulfate or treatment with 1 M to 3.5 M lithium chloride releases the surface-bound autolysin from the cell (43, 44). The autolysins harbor repetitive sequences of about 150 amino acids each, which are repeated 3 times. The repetitive sequences have been demonstrated to direct the enzyme to its site of action, the cell separation site (45). Aas of *S. saprophyticus* has 7 additional short repetitive sequences of unknown function between the propeptide region and the amidase part of the protein (Figure 2).

Staphylococcal autolysins represent one example of a growing family of proteins with both cell wall-lytic and binding function, which has been called autolysin/adhesin family (23). AtlE shows vitronectin binding and Aas and AtlC have been reported to bind to fibronectin (39, 40, 46). Aas has also been demonstrated to

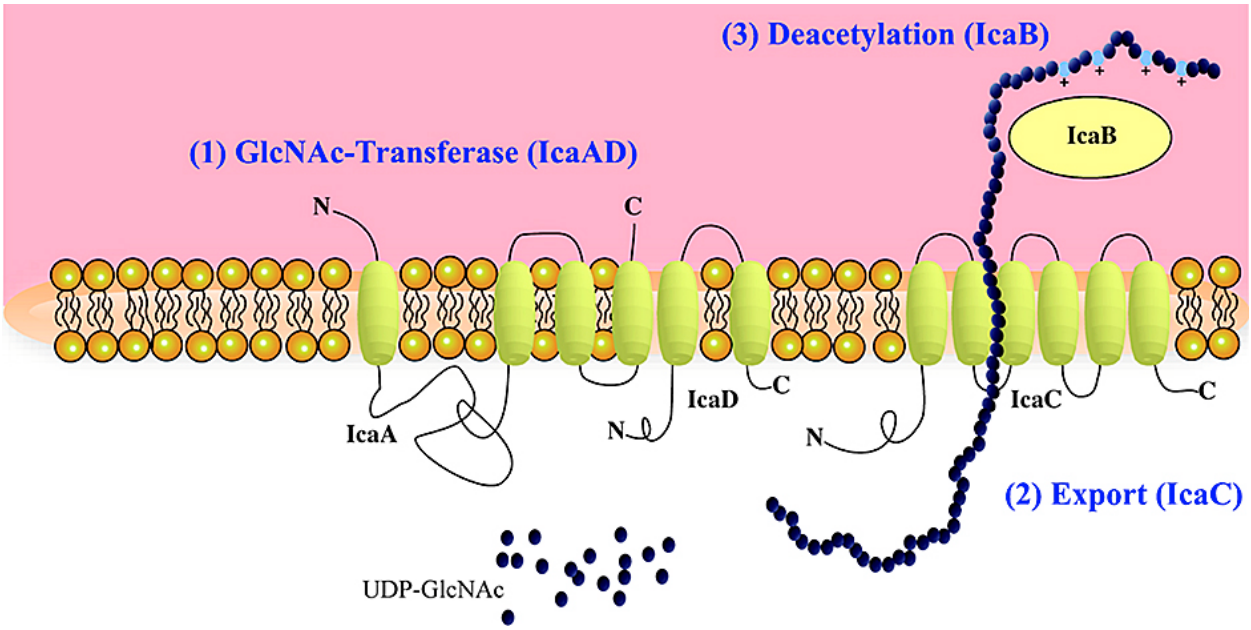

Figure 2. Comparison of staphylococcal autolysins. The structures of AtlE of *S. epidermidis* (1335 amino acids), Aas of *S. saprophyticus* (1463 amino acids), Atl of *S. aureus* (1256 amino acids), and AtlC of *S. caprae* (1395 amino acids) are shown. S, signal peptide; P, propeptide; AM, N-acetylmuramoyl-L-alanine amidase domain; R1, R2, R3, repeat domains; GL, endo-beta-acetylglucosaminidase domain; N, short repetitive domains of Aas.

have haemagglutinating function (47). In the similar autolysin Ami of *Listeria monocytogenes*, multiple diaminoacyl sequences of glycine and tryptophan (GW motifs) are responsible for the adhesive function (48). These also occur within the repetitive sequences of the staphylococcal autolysins.

Another example of a protein with a dual adhesive and enzymatic function is the collagen-binding lipase GehD of *S. epidermidis* (49). The structure of GehD is much different from that of Cna, the collagen-binding adhesin in *S. aureus*, but resembles a mammalian cell-surface collagen-binding receptor, namely the alpha1 integrin I domain. Recombinant GehD and anti-GehD antibodies can inhibit binding of *S. epidermidis* to immobilized collagen.

In *S. aureus*, a huge MSCRAMM of a predicted molecular weight of 1.1 MDa has recently been shown to bind to fibronectin and has been named Ebh (extracellular matrix protein-binding protein homolog) (50). It is also associated to the cell surface by a non-covalent mechanism and its production is repressed by the quorum sensing system *agr*. A deletion mutant in *ebh* did not show altered virulence in a mouse model of infection, presumably as *S. aureus* has other fibronectin-binding proteins to compensate for this loss. Ebh is a homolog of Emb, the major adhesin of *Streptococcus defectivus* (51). It is also homologous to two other predicted proteins in *S. aureus*, Mrp and FmtB. In two *S. aureus* strains with completely published genomes, N315 and Mu50, the open reading frame for *ebh* is split into two predicted genes named *ebhA* and *ebhB*. If EbhA and/or EbhB in *S. aureus* have fibronectin-binding activity is not known. A homolog of *ebh* is present in *S. epidermidis* and is also split into *ebhA* and *ebhB*. No experimental data exist on the expression and function of EbhA and EbhB in *S. epidermidis*.

Clearly, it is a prerequisite for all these proteins not only to interact with the host matrix protein, but also to efficiently bind to the bacterial surface in order to serve as MSCRAMMs. Not much is known about the mechanism by which these proteins bind to the bacterial surface except for some evidence that teichoic acids might serve as a receptor for autolysins (see 3.2.2.1.1.2). Much experimental work still has to be performed in the field of binding proteins of CNS to complete our understanding of the initial colonization process during infection.

3.2.2.2. Cell cluster formation

The second step of biofilm formation consists of the accumulation of bacteria in multi-layered structures. The factors involved in this process generally are different from those involved in the initial attachment to a surface. The main factor responsible for the formation of cell clusters is an exopolysaccharide of *S. epidermidis* named PIA for polysaccharide intercellular adhesin (52). The specific involvement in the second phase of biofilm formation is often demonstrated by a factor's capacity to enhance cell clustering without the need for a surface. This phenomenon can be observed for example by selective over-expression of the exopolysaccharide PIA in a heterologous host (10). Some protein factors have also been suggested to be involved in the accumulation phase. Furthermore, a look at the *S. epidermidis* genome reveals the presence of a gene locus responsible for the production of an extracellular polymer that might potentially contribute to cell cluster formation, namely the genes

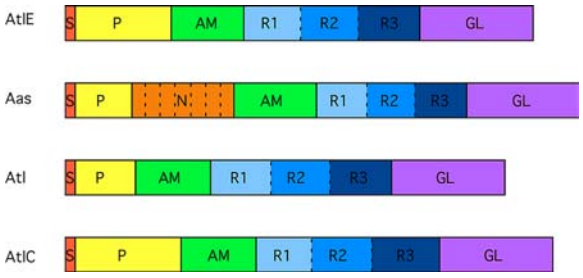

Figure 3. Model of PIA biosynthesis. The exopolysaccharide PIA (polysaccharide intercellular adhesin) is produced by the gene products organized in the *ica* locus. IcaA, IcaD, and IcaC are integral membrane proteins. IcaA and IcaD form an N-acetylglucosamine transferase, which uses UDP-glucosamine (UDP-GlcNAc) to build unbranched glucosamine (GlcNAc) homopolymers up to a chain length of 15 to 20 residues. IcaC is needed for the production of longer chains and is thought to be involved in export. IcaB is believed to de-acetylate some of the GlcNAc residues, which renders the formerly neutral polymer cationic if in an environment with a pH value lower than about 6.

encoding the polyphosphate kinase and polyphosphatase genes needed for the production and degradation of polyphosphate. Polyphosphate has been shown to influence cell clustering and biofilm formation in *Pseudomonas aeruginosa* (53). The role of this gene locus in *S. epidermidis* remains to be investigated.

3.2.2.2.1. PIA

PIA is an unbranched homopolymer of beta-1,6-linked N-acetylglucosamine residues, of which 15-20% are de-acetylated (54). It is produced by the *ica* operon consisting of *icaA*, *icaD*, *icaB*, and *icaC*. The regulatory gene *icaR* is located upstream of the other genes and transcribed in the opposite direction (10). The *ica* gene locus was first characterized in *S. epidermidis*, but has also been found in *S. aureus* (55), and among other CNS in *S. caprae* (39). IcaA and the smaller IcaD are integral membrane proteins. IcaA is the N-acetylglucosaminyltransferase, with IcaD having accessory function, as full transferase activity is only seen in the presence of IcaD. IcaC is needed for the production of higher polymers. In the absence of IcaC, IcaAD only produce oligomers of up to 15-20 units (56). IcaC is also an integral membrane protein and the lack of production of longer chains in an *icaC* mutant is believed to be caused by the lack of coupled transferase and export activity. Therefore, it is assumed that IcaC may be involved in the export of the growing poly-N-acetylglucosamine chain. By similarity, IcaB seems to be an extracellular deacetylase, which is responsible for the deacetylation of PIA. However, no experimental evidence exists for the deacetylase function (Figure 3).

Expression of *ica* is regulated by a variety of conditions and factors. IcaR is a transcriptional repressor of the *icaADBC* operon (57). Activation of the *icaADBC* operon by ethanol, which has been reported, is *icaR*-dependent (58). In contrast, activation of the *ica* operon by NaCl and glucose is not *icaR*-dependent. Being a substrate for the synthesis of PIA, glucose, N-acetylglucosamine, and glucosamine have been shown to increase PIA production (56, 59). PIA expression in *S. epidermidis* and *S. aureus* is not regulated by quorum sensing (26)(C. Vuong, M. Otto, unpublished), but seems to be influenced by the alternative sigma factor SigB in *S. epidermidis* (60), and by oxygen concentration in *S. epidermidis* and *S. aureus* (61). However, PIA production has been reported to be induced by anaerobic conditions in *S. aureus* while being induced by aerobic conditions in *S. epidermidis*, reflecting that PIA production might be regulated by different regulatory effects in the two species (62). Insertion of an IS element, IS256, also influences PIA expression (63). IS256 has been found in laboratory strains and in clinical isolates at preferred sites within the *ica* operon. Whether regulation by IS256 insertion and excision constitutes a general method of regulation of PIA expression *in vivo* is not clear. In a study with 41 *S. epidermidis* strains isolated from urinary tract infections, 7 strains harbored the *ica* genes, but were biofilm-negative. In only one of these strains insertion of IS256 was responsible for the observed phenotype (64). Another study also found IS256-dependent lack of biofilm formation in *ica*-positive strains only in one case (65). Subinhibitory concentrations of some antibiotics (tetracycline, quinupristin-dalfopristin) have been reported to stimulate PIA expression *in vitro* (66), whereas subinhibitory concentrations of quinolones reduced the number of bacteria adhered to catheters *in vivo* in another study (67).

Haemagglutination of erythrocytes is common among *S. epidermidis* strains. PIA has been shown to increase haemagglutination and an *ica* mutant has been reported to be devoid of the ability to agglutinate erythrocytes (68, 69). However, PIA alone cannot itself mediate haemagglutination. The importance of PIA as a virulence factor has been demonstrated by animal models of infection. An *ica* mutant was less virulent in a mouse model of subcutaneous infection (70) and in a rat model of central venous catheter-associated infection (71). In an epidemiological study, *S. epidermidis* strains isolated from patients with nosocomial catheter-related infection much more often harbored the *ica* genes and formed biofilms *in vitro* than strains from healthy individuals (72).

Several other exopolysaccharides of *S. epidermidis* have been described. SAA (slime-associated antigen) (73) has now also been found to be produced by the *ica* locus and to be identical to PIA. Another reported product of the *ica* locus, poly-N-acetylglucosamine (PNAG) produced by *S. aureus* (74), differs from PIA only in chain length and, most likely as a consequence, in immunogenicity. However, as these differences might only be due to the purification procedures used, it is now believed to represent chemically the same molecule.

3.2.2.2.2. Accumulation-associated protein

Protein factors might also be involved in the accumulation phase. The 140 kDa extracellular AAP (accumulation-associated protein) enhances accumulative

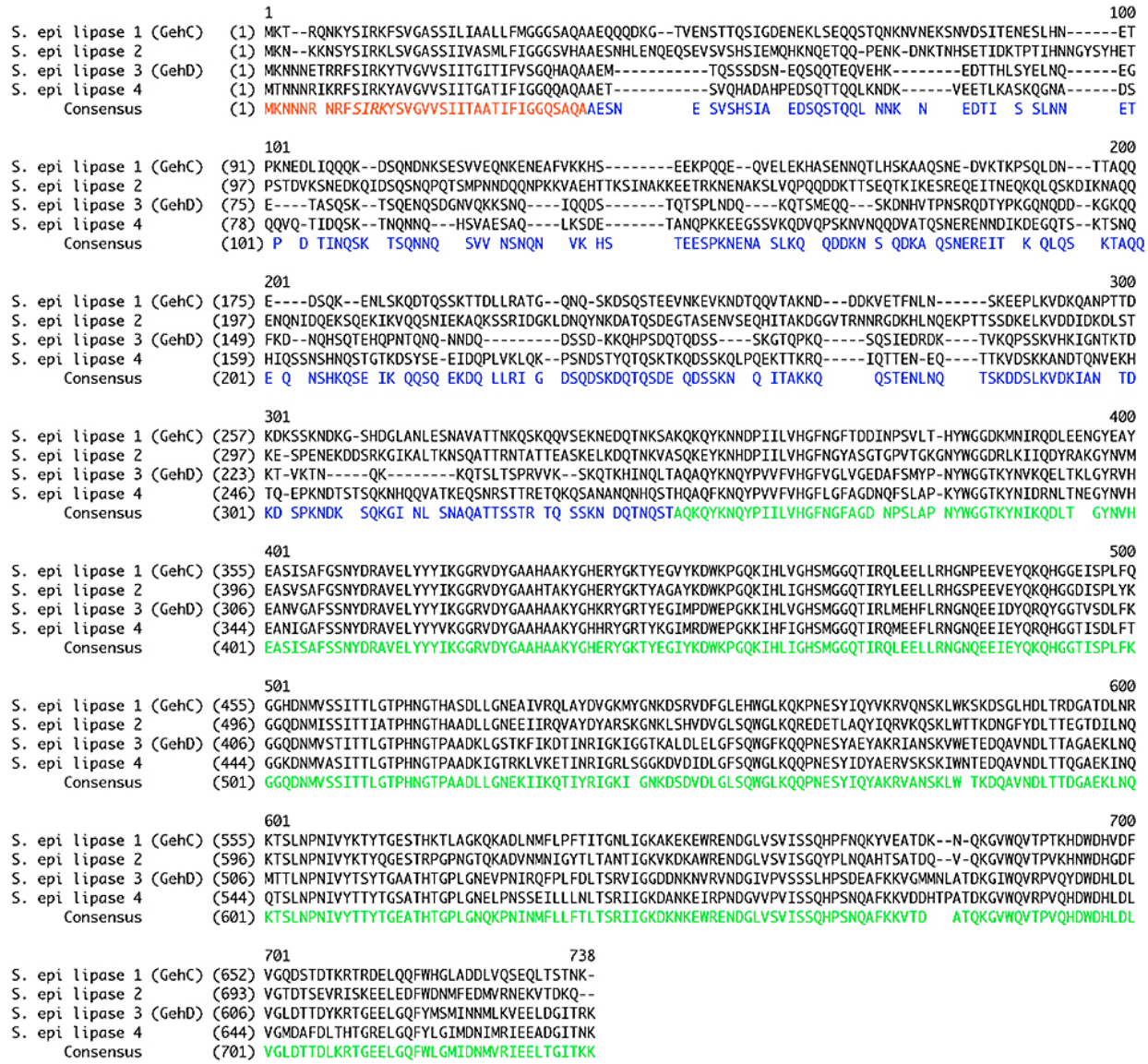

Figure 4. Lipases of *Staphylococcus epidermidis*. Four open reading frames coding for highly similar lipases are found in the genome sequence of *S. epidermidis* RP62A and are shown in the figure. The lipases GehC and GehD have been characterized. Geh-1 is a lipase almost identical to GehC and likely represents the same lipase showing strain to strain variation. If the two additional lipase genes are expressed is not known yet. All lipases are organized as prepro-enzymes. The consensus sequence of the signal peptide part is shown in red, that of the propeptide part in blue, and that of the mature enzyme in green. The conserved "SIRK" sequence in the signal peptide is shown in italics.

growth in microtiter plates (75). One can speculate that the role of this protein might be to link PIA to the bacterial cell surface. A specific mechanism, which links PIA to the bacteria is not known yet. PIA might interact with proteins in a similar fashion as teichoic acids, contributing to a very heterogenous meshwork of polysaccharides and proteins involved in cell-cell adhesion.

3.2.2.3. Biofilm maturation

Microscopic visualization of the structure of mature biofilms of several bacteria showed that mature biofilms are not only an amorphous agglomeration of cells. They show a defined structure with channels between mushroom-shaped cell communities (11). The fluid-filled channels are believed to deliver nutrients to the bacteria. Obviously, regulatory processes are needed for the building of such a structure. We know from other bacteria that quorum sensing regulators play a role (76), but there is no information about the involvement of *agr*, the quorum sensing system of staphylococci (see 3.2.5.1), or of any other regulator in this process in staphylococci so far. The investigation of the processes in biofilm maturation in staphylococci clearly need to be intensified and will hopefully benefit from modern microscopic methods.

3.2.2.4. Biofilm detachment

Very little is known about the detachment phase in staphylococci. The detergent-like peptide delta-toxin has been shown to abolish initial attachment (26), but it is not clear if it also might promote detachment from plastic surfaces *in vivo*. Expression of delta-toxin is cell density-controlled, as delta-toxin is encoded within the regulatory molecule of the quorum sensing system *agr*, RNAIII (see 3.2.5.1). The biological significance of the controlled co-expression of delta-toxin with RNAIII in many staphylococci is unclear.

In addition to physico-chemical detachment processes, by a detergent-mediated mechanism or by mechanical forces, one can think of dedicated enzymes that degrade principal constituents of the extracellular biofilm matrix, such as enzymes cleaving the exopolysaccharide PIA. However, no such enzymes have been found in CNS so far. In many cases, the genetic locus that encodes a polymerase for the production of a biofilm polymer also encodes a degrading enzyme. In *Pseudomonas aeruginosa* for example, the polyphosphate kinase responsible for the production of the biofilm factor polyphosphate, is encoded next to a dedicated polyphosphatase (53). The locus encoding a putative polyphosphate production system in *S. epidermidis* shows the same organization. The *ica* locus, in contrast, does not encode a protein to which a PIA-degrading activity could be attributed by similarity.

3.2.3. Exoenzymes and toxins

CNS do not produce many factors that would cause a severe sepsis. TA and peptidoglycan have a much lower inflammatory effect than lipopolysaccharide, which is restricted to Gram-negative bacteria. CNS also do not usually produce molecules with superantigenic activity. However, there are some factors that might contribute to tissue degradation and an inflammatory response. Information about host interaction mostly is derived from analogy to *S. aureus*, where more work has been performed. This field is only beginning to be investigated in CNS. The most interesting new discovery is the finding that a complex of amphiphilic peptides found in *S. epidermidis*, called PSM for phenol soluble modulin, has a considerable inflammatory activity, which is higher than that reported for LTA or peptidoglycan (77, 78).

3.2.3.1. Lipase

S. epidermidis has been reported to produce three lipases, GehC, GehD, and Geh-1 (79-81). Four open reading frames that code for highly similar lipases are found in the RP62A genome (Figure 4). They are all organized as prepro-enzymes. The reason for the presence of four similar lipase genes might consist in a possibly differing substrate specificity. The signal peptide region and the mature lipase region are very highly conserved among the four putative proteins. The pro-peptide region, which is believed to assist in the correct folding of the mature enzyme, shows less similarity. A conserved "SIRK" sequence has been shown, which might be responsible for efficient export. Two of the reported lipases, GehC and Geh1, are highly similar to each other and most likely do not represent products of different genes, but represent strain variations. Lipases from *S. haemolyticus, S. hyicus, S. warneri,* and *S. xylosus* have also been described (for a review on lipases see ref. (82)). Lipase production in *S. epidermidis* is regulated by the quorum sensing system *agr* by regulation of the proteolytic processing to the mature and active form (83). Lipases might contribute to virulence by enabling the bacteria to persist in the fatty secretions of the human or mammalian skin, and possibly by interfering with phagocytosis. The recent finding that the lipase GehD of *S. epidermidis* can bind to collagen (49) might constitute a novel role for lipase in virulence.

3.2.3.2. Protease

Three proteases have been described in *S. epidermidis* with different substrate specificities and mechanisms of action: a Zn^{2+}-dependent 32 kDa metalloprotease (84), a 41 kDa staphopain-like cysteine protease (85) and a 27 kDa serine protease (86). The serine protease shows a high preference for cleavage after glutamate and has recently been named GluSE. This protease is preferentially expressed in adherent culture, suggesting a possible role in biofilm formation (86). In another study, most likely the same protease has been reported to be able to degrade fibrinogen, complement protein C5, and several other proteins, suggesting a role in the escape from the immune defense system (87). Homologs of the metalloprotease exist in *S. hyicus* (88) and probably in *S. chromogenes*. The metalloprotease is regulated by the *agr* system and is most likely responsible for the maturation step of the above mentioned lipase in *S. epidermidis* (83). It is not known if the *S. epidermidis* proteases contribute to virulence by degrading host tissues, however this is to be expected.

3.2.3.3. Phenol-soluble modulin

Phenol-soluble modulin (PSM) is a complex of (at least) three secreted amphiphilic peptides with inflammatory properties found in *S. epidermidis* (77, 78). PSM activates the HIV-1 LTR in cells of macrophage lineage and induces cytokine release (TNF-alpha, IL-1beta, IL-6) and NF-kappaB production in certain cell types. It causes degranulation, enhanced respiratory burst activity, and inhibition of spontaneous apoptosis in human neutrophils (89). It also functions as a chemoattractant for neutrophils and monocytes. The induction of cytokine release by PSM is stronger than that promoted by peptidoglycan or lipoteichoic acid (LTA), which in Gram-positives is believed to be the equivalent of the cytokine release inducer lipopolysaccharide of Gram-negative bacteria. PSM constitutes one of the first examples of a strong sepsis-inducing factor of CNS and might force us to reconsider the notion of CNS as bacteria that merely remain in "relative silence" during an infection.

PSM consists of the peptides PSMalpha, PSMbeta, and PSMgamma, and potentially further components (77). It is not clear yet if the PSM complex has a higher inflammatory activity than its single components and how the single components contribute to the biological activity. PSMgamma is identical to delta-toxin, PSMalpha is a delta-toxin-similar peptide with a length of 22 amino acids, and PSMbeta is a 44 amino acid peptide with

similarity to the slush peptides of *S. lugdunensis* and the gonococcal growth inhibitor peptide from *S. haemolyticus*. Synergistic hemolysis, which is typical for PSM-like peptides, occurs in most CNS (90). Therefore, production of PSM-like peptides is likely to be widespread among CNS. Delta-toxin-like peptides have been found in *S. epidermidis* (91), *S. saprophyticus*, and S. *haemolyticus* (92), and the *hld* gene necessary for the production of delta-toxin has been detected in even more species (93). PSMbeta-like peptides occur in *S. epidermidis*, *S. haemolyticus* , and *S. lugdunensis* (94).

In earlier reports on neonatal necrotizing enterocolitis caused by *S. epidermidis* it has already been suspected that delta-toxin contributes to the symptoms of the disease (95). We now know that delta-toxin as a component of PSM is an important virulence factor. It is likely that the inflammatory properties of PSM are involved in the symptoms of neonatal necrotizing enterocolitis.

Many PSM-like peptides are known to insert into membranes, form pores, and disturb membrane integrity due to their extremely amphiphilic nature. Delta-toxin has often been used as a model substance for a pore-forming peptide. We do not know if the bacteria that produce PSM-like peptides need a dedicated transporter to export these peptides out of the cell. It is conceivable that PSMs can easily diffuse through the membrane layer, which would be in accordance with their physico-chemical properties. More recently, it has been demonstrated that insertion of delta-toxin into phospholipid vesicles is dependent on the peptide-to-lipid ratio and that no stable pore is formed, but that delta-toxin rapidly crosses the membrane (96). These findings are clearly in favor of a model, in which delta-toxin and similar peptides can cross the membrane without the need for a dedicated exporter.

3.2.3.4. Fatty acid-modifying enzyme

Fatty-acid modifying enzyme (FAME) - first identified in *S. aureus* - has also been found in 88% of *S. epidermidis* strains and some other CNS (97). FAME inactivates bactericidal fatty acids by esterifying them to cholesterol. The extracellular enzyme may provide protection for *S. epidermidis* by inactivating these bactericidal lipids present on the skin. The molecular nature of FAME and the gene locus responsible for its expression are unknown.

3.2.4. Iron acquisition

As almost all bacteria, *Staphylococcus* is dependent on iron acquisition for growth. The free iron concentration in extracellular body fluids is very low (10^{-18}M) due to the presence of high-affinity iron binding proteins such as transferrin and lactoferrin. Bacteria have invented two principal mechanisms to deal with this problem. The first involves high-affinity iron-binding molecules called siderophores and specific import systems, to which siderophore/iron complexes bind. *S . epidermidis* produces two siderophores called staphyloferrin A and staphyloferrin B, of 481 and 448 Da, respectively (98-100), that were first described in *S. hyicus*. The staphyloferrins have been linked to virulence in CNS, as they are produced more frequently by strains isolated from infection (101). The second mechanism depends on direct binding of transferrin to a membrane-bound bacterial receptor. An iron-regulated ABC transporter of *S. epidermidis*, SitABC, is believed to be involved in iron uptake (102).

3.2.5. Regulators of virulence

Bacteria respond to changing environmental conditions by altering gene expression, which enables them for example to deal with a different supply of nutrients, altered oxygen pressure, and the switch from planktonic growth to life in a biofilm. Regulatory systems involved in such basic changes are called global regulators, which also often control the expression of virulence factors. We know of several global regulators of virulence in CNS, such as the quorum sensing system *agr*, the *sar* family of transcriptional regulators, and the alternative sigma factor *sigB*. Except for *agr*, on which considerable research has also been performed in *S. epidermidis* (103), most of what we know about regulators of virulence in staphylococci has been investigated in *S. aureus*. Specifically, the regulation of biofilm formation by global regulatory systems is an evolving field of research, in which a stronger focus will be on CNS as well. Mutants in regulatory systems have not yet been tested in animal models of infection in CNS.

3.2.5.1. *agr*

A quorum sensing system regulates gene expression in response to cell density. The *agr* quorum sensing system was first recognized as a regulator of virulence (104), but has now been shown to also regulate metabolism (105). *agr* generally up-regulates the expression of exoenzymes and toxins and down-regulates the expression of surface proteins like MSCRAMMS at the onset of post-exponential growth phase. Regulating colonization factors, toxins, and exoenzymes by cell density makes sense during an infection, as colonization factors are needed at low cell density during the beginning of an infection, and toxins and degradative factors are needed at high cell density when there is a lack of nutrients and an activated immune response. From the fact that *agr* is found in every *Staphylococcus* species including species that are considered to be non-pathogenic (93), one can deduce that the regulation of virulence factors in pathogenic species is probably not the genuine task of the *agr* quorum sensing system. It is more likely that it was advantageous for the bacteria to put virulence factors under quorum sensing control after they were acquired. In *S. aureus*, the importance of *agr* for virulence has been shown in many models of animal infection (106-108). A recent investigation has raised some doubt about the importance of *agr* for the virulence of *S. aureus in vivo*, based on microarray studies of *in vivo* expression (109). Further investigation will be needed to address this question.

agr constitutes a classic quorum sensing system, which uses auto-regulatory feedback and a peptide-based pheromone (104, 110, 111). The *agr* locus produces a two-component system for signal transduction (AgrA/AgrC), a prepheromone peptide (AgrD) and an enzyme most likely needed for prepheromone maturation and export (AgrB). It also harbors the gene for a regulatory RNA, RNAIII, which

regulates the expression of target genes in a mostly unknown fashion. RNAIII also comprises the *hld* gene, which encodes delta-toxin. Varying pheromone sequences define subgroups among one species and are responsible for the phenomenon of cross-inhibition (see 3.2.9.1). Three *agr* subgroups have been detected in *S. epidermidis* (93, 112). The structure and function of thiolactone-containing *agr* pheromones has been proven by chemical synthesis and investigation of the biological activity of the *S. epidermidis* pheromone of subgroup 1 (112). The pheromone structures of the two other *S. epidermidis agr* subgroups are not clear yet. Also, the relative distribution of *S. epidermidis agr* subgroups in the population and in specific infections must still be investigated. We know about the prepheromone (AgrD) sequences of many CNS, but lack information – as in the case of *S. epidermidis* subgroups 2 and 3 – about the precise pheromone structure, because the N-terminal "tail" adjacent to the C-terminal thiolactone ring can vary in length and therefore can not be clearly predicted from the DNA sequence alone.

Most of the above mentioned results on *agr* have been achieved in *S. aureus*. However, the system is found in every staphylococcal species and the investigation of a deletion mutant of *agr* in *S. epidermidis* and of the *S. epidermidis agr* system in general have shown that it also has the role of a regulator of virulence in this CNS species (83). We have shown recently that expression of the autolysin AtlE, which is involved in primary adhesion to hydrophobic surfaces (see 3.2.2.1.1.1), is regulated by *agr* in *S. epidermidis* (C. Vuong, M.Otto, unpublished), whereas the homologous Atl is not *agr*-regulated in *S. aureus* (105, 113). We should therefore keep in mind that regulation of the targets of the regulatory systems might well differ between the species, even if the systems appear very similar.

3.2.5.2. *sar*

The dimeric DNA-binding protein SarA regulates many virulence factors acting as a transcriptional regulator (114, 115). It may act directly on the transcription of target genes or also via regulation of the *agr* system (116-118). Three differently regulated promoters drive transcription of *sarA* itself, one of which is under control of the alternative sigma factor *sigB*. (119). The *sigB*/*sarA*/*agr* system is a very complicated hierarchical regulatory network that we are only beginning to understand. More recently, several SarA homologs have been found in *S. aureus* that act as transcriptional regulators and/or repressors (120-122). The *S. aureus* genome encodes a dozen *sar* homologs and the *S. epidermidis* genome seven; however, these data are dependent on the stringency of similarity comparisons. The *S. epidermidis* homolog of SarA has been described (123). For CNS, no data on *sar* regulation exist up to now, but we can speculate that SarA and its homologs also regulate virulence factors in CNS and that the reason for the abundance of similar regulatory proteins might be to very finely trigger the expression of virulence factors and other targets in response to environmental factors. The order of the three promoters in front of *sarA* is not the same in *S. epidermidis* as in *S. aureus*, which might reflect different regulation. For an in-depth review of the Sar protein family see Cheung and Zhang (124).

3.2.5.3. *sigB*

The alternative sigma factor SigB controls several virulence factors in *S. aureus* and influences the other global regulators *sarA* and *agr* by acting on one of the promoters of *sarA* (125-127). Mutants in *sigB* in *S. aureus* do not show a loss of virulence comparable to *sar* and *agr* mutants in animal models (128). SigB is also present in *S. epidermidis* and controls biofilm formation and the expression of lipase and protease production (60, 129).

3.2.6. Antimicrobial resistance

3.2.6.1. Resistance against antibiotics

Increasing resistance against antibiotics in staphylococci is an enormous problem for the public health system and one of the primary reasons for the in-depth investigation of staphylococcal pathogenicity and resistance factors. Methicillin is an antibiotic of first choice against staphylococci. In nosocomial infections, the most important type of infection linked to *S. epidermidis* and other CNS, about 80% of strains are resistant to methicillin and many strains are resistant to other antibiotics as well (National Nosocomial Surveillance System Report 1998). In addition to the problem of widespread specific antibiotic resistance genes, biofilm formation contributes enormously to the resistance against antibiotics. Slime-producing strains are more often resistant to a variety of antibiotics, such as penicillin, teicoplanin, erythromycin, and kanamycin, than non-slime producers (130). To many antibiotics, a biofilm constitutes an almost impermeable barrier. However, several antibiotics can easily diffuse through a biofilm layer. The mechanism of increased resistance in a biofilm in these cases is believed to be due to the altered physiological state of the bacteria in a biofilm compared to other forms of growth (131).

CNS most likely can easily acquire resistance from *S. aureus* and transfer to other species of the genus. For the *mec* element, which confers resistance against methicillin, transfer from CNS to *S. aureus* has been shown to have occurred even in a patient during an infection (132).

Glycopeptide antibiotics like vancomycin and teicoplanin are antibiotics of last resort against many multi-resistant staphylococcal strains. Intermediate resistance against glycopeptide antibiotics occurs frequently. It is due to alterations of the target of the antibiotic, the peptide part of peptidoglycan. This includes an increased molar proportion of the major monomeric muropeptide containing intact D-alanyl-D-alanine termini as well as serine or alanine substitutions of glycine residues in the pentapeptide cross-bridge (133). The very recent alarming finding that high level resistance against vancomycin has been transferred from enterococci to *S. aureus* (134), very likely means that we will face highly vancomycin-resistant CNS in the very near future.

Among the other CNS, *S. haemolyticus* has been reported to have a tendency to acquire antibiotic resistance comparable to *S. epidermidis*, showing variable resistance to beta-lactam antibiotics, erythromycin, and tetracycline (135). Multiple resistance is also not uncommon.

Intermediate resistance against vancomycin has been shown (136). CNS other than *S. epidermidis* and *S. haemolyticus* have not (yet) acquired antibiotic resistance to a comparable extent.

3.2.6.2. Resistance against factors of the immune system

The host's primary anti-staphylococcal defense comprises the respiratory burst inside polymorphonuclear leukocytes (PMNs), phospholipase A2, platelet microbicidal proteins, and defensins (137). We do not know much about the interference of CNS with the host's immune system, with the exception of the above mentioned PSMs. However, the factors responsible for the acute inflammatory host response are TA and peptidoglycan, which are the same in CNS as in *S. aureus*.

Biofilm formation has a very strong influence on the host's capacity to clear a CNS infection. The "slime" substance is thought to play a similar role as the *S. aureus* capsular polysaccharides, i.e. to decrease the phagocytic activity. In fact, "slime" reduces the phagocytic activity of murine peritoneal macrophages (138) and *S. epidermidis* strains isolated from hip prostheses show an enhanced capacity to resist phagocytosis (139). However, investigation about the contribution of specific biofilm factors to this phenomenon has not been performed yet. It must be noted here that *S. epidermidis*, unlike *S. aureus*, harbors the genes necessary for the production of a poly-gamma-glutamate capsule as found first in *Bacillus anthracis* (140). In *B. anthracis*, this capsule plays a similar role as the mentioned polysaccharide capsule, helping the bacteria to "hide" from phagocytic cells.

Recently two novel resistance factors, *dlt* and *mprF*, have been found in *S. aureus*. The *dlt* locus is responsible for the D-alanylation of TA. TA without D-alanylation, as present in a *dlt* mutant, have a higher negative charge and bind cationic peptides such as defensins more efficiently than D-alanylated TA, as present in the wild-type (141). Consequently, *dlt* mutants show a higher sensitivity towards defensins and other antimicrobial cationic peptides. The membrane protein MprF is needed for the incorporation of the unusual phospholipid phosphatidyl-lysine into the *S. aureus* membrane (142). This phospholipid carries an additional positive charge in comparison to other phospholipids. Therefore membranes with phosphatidyl-lysine have a more cationic character and repel cationic antimicrobial peptides more efficiently than those without phosphatidyl-lysine as found in *mprF* mutants. The activity of phospholipase A2 (PLA2), which has highly cationic properties, is also influenced by the presence of D-alanylated TA and MprF (143). Both *dlt* and *mprF* genes are found on the *S. epidermidis* RP62A genome. Experimental evidence about the function of these two factors with regard to resistance against cationic antimicrobial peptides in *S. epidermidis* or other CNS has not been achieved yet.

3.2.7. Intracellular persistence

Intracellular persistence has been reported for *S. aureus* already some time ago (144). Bacteria are believed to persist inside some cell types in a status of reduced metabolism and reduced production of extracellular virulence factors, similar to the status in so-called SCVs (small colony variants) (145). SCVs have been reported in *S. epidermidis* and *S. capitis* in one case (146), but evidence for SCVs in CNS is scarce. Although certainly not a primary habitat for *S. aureus*, intracellular persistence might contribute to the phenomenon of recurring infections. Persistence inside pericatheter macrophages has been shown for *S. epidermidis* (147). *S. epidermidis*, *S. xylosus*, and *S. hyicus* can adhere to and internalize bovine mammary epithelial cells in a receptor-mediated fashion (148). *S. epidermidis* can also be internalized by endothelial cells (149). These mechanisms might be in part responsible for the difficulty to clear CNS infections.

3.2.8. Superantigenic toxins

Superantigens can activate T-cells without the need for proteolytic processing by antigen-presenting cells or for antigen specificity. Furthermore, they bind to the invariant region of the T-cell receptor. As a consequence, the immune response to superantigens is much higher than to normal antigenic molecules. Symptoms include high fever and emesis in the case of enterotoxins (SEs). The production of superantigenic toxins such as SEs A through E, toxic shock syndrome toxin (TSST-1), and exfoliative toxin, is typical of *S. aureus* but is not a usual feature of CNS. In several studies, superantigen production has been found in CNS (150, 151), however, these findings are controversial. In a more recent study with 136 strains isolated from skin lesions, 6.6% of CNS strains had the ability to produce a superantigen, mostly one of the SEs (152). By some cases, SE production in CNS has been made responsible for the outbreaks of food poisoning (153). It is not clear if the respective genes have been acquired by horizontal gene transfer from *S. aureus*, where these genes are abundant and as one might speculate.

3.2.9. Bacterial competition

Bacteria may have a symbiotic relationship with other bacteria in the human or animal body, but they may compete with other bacteria using factors produced to kill or to decrease the expression of other bacteria's toxic products. This phenomenon has been called bacterial competition, or in a broader sense, bacterial interference. Although not a classic virulence factor, bacterial interference might contribute to virulence in a situation where a more virulent strain is able to out-compete less virulent strains. In contrast, if a virulent strain can be reduced in virulence by bacterial interference, the factors underlying the interaction might constitute interesting lead substances for drug development, like in the case of the *agr* pheromones.

3.2.9.1. Cross-inhibiting pheromones

The *agr* pheromone is a thiolactone-containing short peptide, which differs in primary sequence from species to species (93, 112, 154). Among several staphylococcal species, the primary sequence of the pheromone peptide can be used to define different subgroups. Generally the pheromones of different staphylococcal species, and different subgroups among one staphylococcal species, show the phenomenon of cross-

inhibition, which is almost unique among quorum sensing systems to date (112, 154). Inhibition of *agr* results in the decrease of many secreted toxins and exoenzymes (155, 156). *agr* pheromone derivatives have therefore been proposed as potential anti-staphylococcal drugs, which would only suppress virulence but not kill the bacteria, an approach which is believed to minimize the development of bacterial resistance. It is not clear yet if *agr* pheromone-based drugs have therapeutic potential for every kind of infection, as they have been shown to increase biofilm formation (26).

Four subgroups of *agr* have been detected in *S. aureus* and three in *S. epidermidis* (93, 112, 154, 157). The cross-inhibition between *S. epidermidis* subgroup 1 and all *S. aureus* subgroups has been investigated using pure synthetic pheromones (111). Interestingly, *S. epidermidis* subgroup 1 pheromone could inhibit the *agr* response in all *S. aureus* subgroups except in subgroup 4. *S. aureus* subgroup 4 pheromone was also the only one among *S. aureus* pheromones able to inhibit the *agr* response in *S. epidermidis*. *S. aureus* subgroup 4 pheromone is closely related to the pheromone of the most frequently found subgroup 1 – they differ in only one amino acid. Furthermore, *S. aureus* subgroup 4 strains have been correlated with skin infections (157). These findings have led to the speculation that there is bacterial competition between the two species and that *S. aureus* subgroup 4 might have evolved from subgroup 1 by competition with *S. epidermidis*.

3.2.9.2. Lysostaphin

Lysostaphin is an endopeptidase isolated from *S. simulans* biovar. *staphylolyticus* ATCC1362 (158) that cleaves the peptidoglycan pentaglycine interpeptide bridge, which is typical for the staphylococcal cell wall. It is produced as a preproenzyme of 493 amino acids. The propeptide is 211 amino acids in length. 195 amino acids of the propeptide are organized as 15 repeats of 13 amino acids. The propeptide region serves to keep prolysostaphin in a less active form (159). Prolysostaphin is cleaved in the bacterial supernatant to mature and active lysostaphin by a cysteine protease. Lysostaphin and the similar glycylglycine endopeptidase ALE-1 of *S. capitis* (160) belong to a Zn^{2+} protease family with a conserved Zn^{2+}-binding motif. Lysostaphin treatment leads to efficient lysis and fast killing of staphylococcal cells. Resistance against lysostaphin exists in some staphylococcal species, in which some of the glycine residues are substituted for by other amino acids, as for example by serine in *S. epidermidis*. The lysostaphin immunity factor Lif, which is encoded next to the lysostaphin gene on a large plasmid, and the homologous factor Epr encoded next to *ale-1*, also act by increasing the serine/glycine ratio in the interpeptide bridge (159, 161). Lif and Epr are similar to FemA and FemB, cell wall construction enzymes that incorporate the glycine residues 2 to 5 in the interpeptide cross-bridge. Lif and Epr specifically incorporate serine instead of glycine at positions 3 and 5 (162).

Lysostaphin is not only a potential weapon in bacterial interference, but has also been used by molecular biologists for years as a means to lyse staphylococci. Furthermore, it has gained substantial interest more recently as a potential anti-staphylococcal drug. Being an enzyme it does not constitute a prime candidate for a therapeutic substance, however, first experiments are promising with respect to efficiency and the development of resistance (163, 164). Combinations of beta-lactams with lysostaphin have been shown to act against oxacillin-resistant *S. epidermidis* in a synergistic fashion (165).

3.2.9.3. Bacteriocins

Several *S. epidermidis* and *S. aureus* strains that produce lantibiotics have been described (166, 167). Lantibiotics are bacteriocins that contain unusual amino acids with thioether bridges such as lanthionine and methyllanthionine. The genes responsible for the production of lantibiotics are usually clustered and have been found to be encoded on plasmids or on the chromosome. Interestingly, several of the *S. aureus* genomes available to date harbor genes for lantibiotic production, but have not yet been reported to produce an antibacterial activity. We may assume that the ability to produce lantibiotics might be widespread among staphylococcal strains; however, we do not know under which circumstances the biosynthetic genes are expressed, if they are expressed at all. *S. epidermidis* strain Tü3298 produces a lantibiotic called epidermin (168). The structural genes found on the different *S. aureus* chromosomes code for pre-lantibiotics very similar to pre-epidermin (169). Epidermin is active against staphylococci unless resistance genes are present, which are generally encoded in or near the biosynthetic cluster. Resistance factors include an ABC transporter system, which is found in many lantibiotic gene clusters and acts by removing the lantibiotic from the membrane by exporting it to the extracellular medium (170). The mechanism of some other resistance factors, unique to specific clusters, remains unknown (171). Lantibiotics might act as weapons in bacterial competition, against staphylococci and also against other species. However, Gram-negative bacteria are not sensitive against this type of bacteriocins, as they are protected by their outer membrane.

3.3. Virulence factors in other species

We do not know much about most CNS other than *S. epidermidis*, although some of them can cause similarly severe, or even more serious infections (as for example *S. lugdunensis*). Here, only virulence factors specifically found and characterized in the respective species will be dealt with, together with a short description of the single species. It is to be expected that with genome sequencing and functional genomic approaches becoming faster and more common, we will soon gather more information about important pathogens among CNS, and about the distribution of virulence factor in these species.

3.3.1. *Staphylococcus saprophyticus*

S. saprophyticus is considered the second most important urinary tract pathogen (with 10-15% of cases) after *E. coli* (with 80% of cases) (172). Cases of urinary tract infections caused by *S. saprophyticus* are most often linked to young adult women. Prevalence of *S.*

saprophyticus colonization in women is correlated with recent urinary tract infections, recent menstrual period, recent sexual intercourse, or recent infection with *Candida*. *S. saprophyticus* forms biofilms on indwelling medical devices like many other CNS. Biofilm formation is probably also involved in urinary tract infection with *S. saprophyticus*. One factor involved in *S. saprophyticus* biofilm formation is the autolysin Aas, which mediates adherence to fibronectin and promotes haemagglutination. Aas has been discussed in 3.2.2.1.2.4. Another surface-attached protein, Ssp (*S. saprophyticus* surface-associated protein), present in 98% of *S. saprophyticus* isolates, has been found to cause the production of a high amount of surface-associated material, clumping, and binding to uroepithelial cells (174). The protein has an apparent molecular weight of 95 kDa under reducing conditions and aggregates to a complex of about 500 kDa under non-reducing conditions even in the presence of 8 M urea. The genetic locus responsible for the production of Ssp is unknown. We mostly lack information about other virulence factors of *S. saprophyticus* and the reason for its prevalence in urinary tract colonization and infection. One exception is the urease activity that has been found in *S. saprophyticus* (175). Urease is an important virulence factor for *S. saprophyticus* in that it contributes to invasiveness by damaging bladder tissues. Presence of lipase, elastase, and FAME activity has also been reported (reviewed in (176)).

3.3.2. *Staphylococcus haemolyticus*

S. haemolyticus is very similar to many CNS with respect to the infections it can cause in humans (e.g. native valve endocarditis, septicemia, peritonitis, wound and urinary tract infections, and infections of the bones and joints) (reviewed in (176)). Decreased susceptibility to teicoplanin has been reported early in *S. haemolyticus* and is due to the substitution of glycine in peptidoglycan by other amino acids (see 3.2.6.1). *S. haemolyticus* can bind to a variety of host matrix proteins. It produces a peptide similar to the slush peptides of *S. lugdunensis* and to PSMbeta of *S. epidermidis*. This peptide has been named gonococcal growth inhibitor (GGI), as it can cause cytoplasmic leakage of gonococcal cells (177, 178). With the recent finding of the inflammatory activity of PSMs, the anti-gonococcal activity might be regarded as a side-effect compared to the inflammatory properties of all those similar peptides.

3.3.3. *Staphylococcus lugdunensis*

Compared to other CNS, infections with the species *S. lugdunensis* can be more serious. *S. lugdunensis* can cause brain abscesses, sepsis, osteomyelitis, and infective endocarditis (179). The species *S. schleiferi* is similar in this respect. *S. lugdunensis* can bind to a high variety of human matrix proteins, produces FAME, esterase, and lipase, and has been reported to produce an extracellular slime matrix (reviewed in (176)). Interestingly, *S. lugdunensis* does not attach to fibrinogen. *S. lugdunensis* produces three peptides named slush (*Staphylococcus lugdunensis* synergistic haemolysin), which are similar to each other, to PSMbeta of *S. epidermidis*, and to GGI of *S. haemolyticus* (94). The genetic locus responsible for the production of the slush peptides has been characterized. In contrast to the delta-toxin, slush peptides are not encoded in or near the *agr* locus (94). In the meantime more similar peptides, like the PSMs, have been found without a genetic connection to *agr*. It is not known yet if the slush peptides or other similar peptides are regulated by *agr* in the different CNS species.

3.3.4. *Staphylococcus warneri*

S. warneri colonizes human skin in small populations. It can bind to collagen and fibronectin. Protease, lipase, esterase, and urease activity, and glycocalyx production have been reported (reviewed in (176)). *S. warneri* lipase has been investigated in detail (180). It is similar to other staphylococcal lipases, has a molecular weight of 45 kDa in the mature form, and shows a broad substrate specificity. *S. warneri*, like all staphylococci investigated to date, has an *agr* system. Interestingly, RNAIII of *S. warneri* harbors two *hld* genes for delta-toxin that are similar, but non-identical (181). The recently described species *S. pasteuri* (182) is phenotypically similar to *S. warneri*.

3.3.5. *Staphylococcus simulans*

S. simulans can be the cause of a variety of animal as well as human infections, including native valve endocarditis, urinary tract, wound, bone, and joint infections, and septicemia. Lipase, FAME, and urease production have been reported (reviewed in (176)). The strain of *S. simulans*, which produces lysostaphin, harbors a large plasmid with genes for lysostaphin production (*lss*) and a lysostaphin immunity factor (*lif*) (see 3.2.9.2).

3.3.6. *Staphylococcus capitis*

This species has two easily distinguishable subspecies, one that produces urease (ssp. *ureolyticus*), and one that lacks urease production (ssp. *capitis*). *S. capitis* has been reported to cause urinary tract infections, catheter-related bacteremia, endocarditis, necrotizing enterocolitis in neonates, and other infections. It is predominantly found on the scalp. Host matrix proteins, to which *S. capitis* can bind, include laminin, collagen, and fibronectin. Intermediate resistance to vancomycin has been shown in an outbreak in a neonatal care unit. Lipase and FAME activity have been reported (reviewed in (176)). One strain of *S. capitis* (EPK1) produces a plasmid-encoded lysostaphin-like glycylglycine endopeptidase, ALE-1, which also cleaves within the peptidoglycan interpeptide bridge (160). An immunity gene is found next to the *ale-1* gene, *epr*, whose product is believed to act in a fashion similar to the lysostaphin immunity factor Lif (161).

3.3.7. *Staphylococcus hominis*

S. hominis is a frequent colonizer of human skin with low virulence. Binding to vitronectin, laminin, collagen, and fibronectin has been reported. *S. hominis* has esterase, lipase, FAME, and urease activity (reviewed in (176)).

3.3.8. *Staphylococcus cohnii*

S. cohnii ssp. *cohnii* is an exclusive colonizer of humans, whereas ssp. *urealyticum* also occurs in other

primates. *S. cohnii* can bind to vitronectin, laminin, fibronectin, and collagen; lipase and FAME activity have been reported (reviewed in (176)). A lantibiotic similar to epidermin has been found in an *S. cohnii* strain (183).

3.3.9. *Staphylococcus xylosus* and *Staphylococcus carnosus*

S. carnosus and *S. xylosus* are used in sausage and fish fermentation. *S. carnosus* is frequently used as a heterologous host for the expression of virulence factors of other staphylococci and even of foreign factors for basic research and biotechnology projects (184), and is being tested as a potential vaccine delivery system (185). Both species are virtually non-pathogenic, although *S. xylosus* has very rarely been associated with human infection (186, 187).

3.3.10. *Staphylococcus saccharolyticus*

S. saccharolyticus is a fairly undescribed, anaerobic species. It has been reported to cause cases of anaerobic endocarditis and bacteremia (188, 189).

3.3.11. CNS in animals

CNS are also frequently found as colonizers and pathogens of animals. *S. caprae* was originally isolated from goat's milk. It is also usually found in goats, although reports exist that *S. caprae* has been isolated from human infections (190, 191). In these cases, and similarly in cases involving other staphylococcal species predominantly found in animals, the species is thought to have spread to animal care workers or other persons having been in contact with the animals. However, *S. caprae* strains isolated from humans can be distinguished from those found in animals by their PFGE profile and therefore *S. caprae* might be an infrequent, but genuine colonizer of human skin (192). We have discussed the autolysin AtlC of *S. caprae* in 3.2.2.1.2.4. *S. sciuri* is mostly found in rodents. *S. hyicus* is an opportunistic pathogen found in pigs and cattle. The lipase of *S. hyicus* is probably the best characterized lipase among staphylococcal lipases. Specifically, the role of the lipase propeptide has been investigated with this enzyme and has been postulated to function as an "intracellular chaperone" assisting in the correct folding of the active part of the enzyme and preventing full activity before cleavage from the propeptide (82). *S. hyicus* also produces a metalloprotease homologous to the 32 kDa metalloprotease of *S. epidermidis* that is involved in lipase processing (88). *S. gallinarum* is found mainly in poultry, but also in other birds. One *S. gallinarum* strain produces the lantibiotic gallidermin, which is very similar to epidermin of *S. epidermidis* (193, 194). The biosynthetic, immunity, and regulatory genes are encoded on the chromosome (195, 196) and are very similar to those found in the plasmid-encoded epidermin gene cluster and the chromosomally encoded gene clusters of the epidermin-like putative lantibiotic production genes of many *S. aureus* strains. *S. chromogenes* is found in cattle and pigs. It may cause bovine mastitis (197). Recently, a cytotoxic activity has been detected in many strains of *S. chromogenes* that causes cell detachment and cell rounding in many mammalian cell lines (198). The factor is a metalloprotease with a size similar to the metalloproteases from *S. epidermidis* and *S. hyicus*, to which it might be homologous. Of the already discussed species that also often cause human infections, *S. warneri* occurs in prosimians and monkeys. *S. simulans* can be found in a variety of animals and cause abscesses in many species. *S. xylosus* occurs in many mammals (reviewed in (176)).

4. PERSPECTIVES

Some of the most important goals of the research on CNS will be to analyze the data derived from the genome of *S. epidermidis* and to further investigate the mechanisms of virulence factors and the expression of virulence factors under *in vivo* conditions. To demonstrate that *S. epidermidis* is not only a less virulent form of *S. aureus*, but has its own distinguishable mechanisms of virulence, will be another focus. We have seen that *S. epidermidis* harbors putative virulence genes that are not found in *S. aureus*. For example, it remains to be shown if a poly-gamma-glutamate capsule is produced from the *cap* locus in *S. epidermidis* and, if so, if it contributes to virulence. Also, putative polyphosphate production by the *ppk* locus represents a virulence factor unknown from *S. aureus* that might be involved in biofilm formation and evasion from the immune system. A further important field to be investigated relates to the targets regulated by global regulators of virulence and how global regulators affect the expression of virulence factors in specific conditions, like in a biofilm or during catheter infection. Generally, the increasing knowledge about the mechanisms of chronic CNS infection will help to develop novel anti-staphylococcal drugs. These drugs will be less prone to cause rapid development of resistant clones and will efficiently work also against CNS in a biofilm setting. Although research on *S. aureus* has focused on acute virulence, this bacterium can also form biofilms and cause chronic and persistent infections. As *S. aureus* and CNS share many common virulence factors related to the persistence in the human body– in spite of the differences that we have discussed - research on the pathogenicity of CNS will also help to understand chronic *S. aureus* infection.

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Send correspondence to: Michael Otto, Laboratory of Human Bacterial Pathogenesis, Rocky Mountain Laboratories, NIAID, NIH, 903 S 4th Street, Hamilton, MT 59840. Tel: 406-363-9283, Fax: 406-375-9677, E-mail: motto@niaid.nih.gov