The transition between sessile and motile bacterial lifestyles

Tsompanidou, Eleni

THE TRANSITION BETWEEN SESSILE AND MOTILE BACTERIAL LIFESTYLES

Eleni Tsompanidou

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1. The *agr* system plays a decisive role in the choice of *S. aureus* cells between a sessile and a motile lifestyle (this thesis).

2. Mutation of the *srtA* gene locus leads to a hyper-spreading phenotype both in *S. aureus* and *S. epidermidis* (this thesis).

3. PSM peptides not only allow *S. aureus* cells to spread to new surfaces that might be richer in nutrients, but they also allow *S. aureus* to compete with other bacterial species that colonize the same surfaces (this thesis).

4. Cells that form strong biofilms are poor spreaders and *vice versa* (this thesis).

5. PSM-mediated spreading plays a major role in the movement of *S. aureus* over biotic surfaces (this thesis).

6. Drugs that target the PSM peptides in combination with cell-surface exposed proteins, such as FnbpA, FnbpB, ClfA and ClfB might decrease the survival of *S. aureus* in the host and limit their transmission both in the community and within a hospital setting (this thesis).

7. The results you obtain with *S. aureus* are seemingly dependent on the part of the world in which you conduct your experiments.

8. Cell wall-associated factors that promote a sessile lifestyle of *S. aureus* antagonize this bacterium's colony spreading motility.

9. Working with *S. aureus* makes you feel like a speeder. You have to be the first one to reach the editors office before 'the others' do.

10. Working with PSMs and trying to get my work published was like a battle between a woman and men.
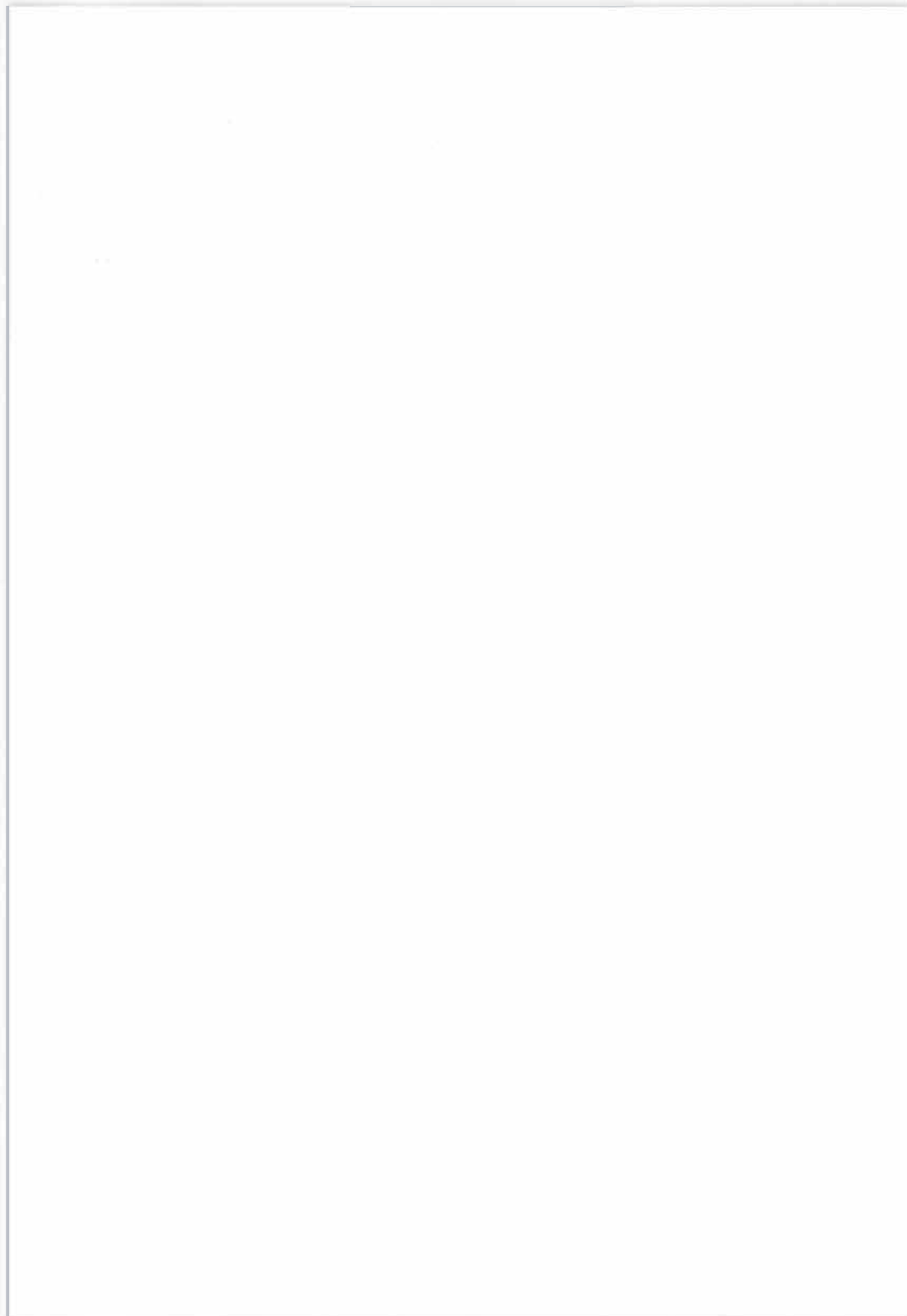
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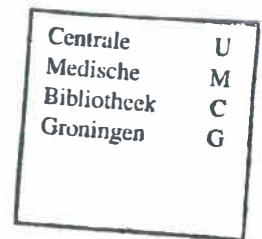
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Για τους γονείς μου

Contents of thesis

"Time is a river... and books are boats. Many volumes start down that stream, only to be wrecked and lost beyond recall in its sands. Only a few, a very few, endure the testing of time and live to bless the ages following."

Dan Brown – The Lost Symbol

Chapter 1

General Introduction and
Scope of this Thesis

Chapter 1

Introduction

Single-cellular microorganisms were the first form of life to inhabit the earth three to four billion years ago (9, 64). They can be found everywhere on our planet, ranging from the poles to deserts and from the soil to deep-sea waters. Microorganisms are very diverse and include bacteria, yeasts, archaea and many other species (48, 70, 93). Some microorganims such as bacteria, yeasts and archaea can be found in humans and are known as the normal human microbiota. There are approximately ten times more bacteria than human cells in the human body (66). The vast majority of the human microbiota is commensal, some are beneficial but others are opportunistic pathogens that can cause infectious diseases. One such bacterium is *Staphylococcus aureus* (Figure 1). Once such opportunistic pathogens break through the immune defenses they can infect different organs and tissues in the human body, thereby causing a wide range of diseases that in some cases can be life-threatening. In order to escape the immune defenses and spread to different parts of the human body pathogenic bacteria express a diverse array of virulence factors. These virulence factors can either be exposed at the cell surface or secreted into the extracellular milieu. In both cases they are being produced in the cytoplasm and are then transported to their site of action.
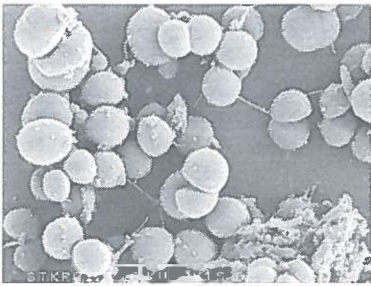
Figure 1. Scanning electron microscopy image of *S. aureus*.

Staphylococcus aureus

S. aureus is a Gram-positive human commensal that can be found in the nose, on the skin and various other sites of our body. Approximately 20% of the human population is persistent carrier of *S. aureus*, whereas 60% carry this bacterium only intermittently (60). For most individuals, *S. aureus* is an apparently harmless commensal. However, once *S. aureus* passes the primary barriers imposed by the human skin or mucosa, it becomes evident that this organism is a dangerous pathogen. *S. aureus* is capable of infecting almost every tissue and organ, causing a wide range of acute and chronic diseases (47). These can vary from minor skin infections, such as impetigo, to life-threatening diseases such as pneumonia, endocardatis and meningitis (13, 23, 47, 55). *S. aureus* is one of the five most common causes of nosocomial infections often causing post-surgical wound infections and chronic infections due to the formation of biofilms on indwelling medical devices (14, 28, 32). Interestingly, *S. aureus* is able to gain resistance against

many antibiotics. This first became apparent several years after the introduction of penicillin. In the meantime, *S. aureus* strains have acquired resistance to the most commonly used antibiotics with the oxacillin resistant form (methicillin-resistant *S. aureus*, MRSA) being the most important cause of antibiotic resistant health care-associated infections worldwide (Annual ReportEARSS 2009; 30). In 1980 the first community–acquired MRSA infection was reported in the USA. These strains infected apparently healthy individuals without healthcare-associated risk factors (10, 21, 38, 40, 82, 83). CA-MRSA strains are commonly found on the skin, thereby facilitating skin to skin spread (20, 46, 52, 87). Vancomycin has been the last resort antibiotic against MRSA strains, but unfortunately, in 1996 the first vancomycin intermediate resistant strains (VISA) were reported (39, 63, 71). Since then several cases have been reported describing strains with reduced susceptibility to glycopeptide antibiotics (glycopeptide intermediate-level resistant *S. aureus*-GISA strains) (11, 26, 27). Today not only intermediate but also completely resistant (VRSA) strains to vancomycin and other glycopeptides have been reported (3, 17, 19, 76, 77, 88). Because of the increasing rise of multiple antibiotic resistant *S. aureus* strains there is an urgent need for new strategies to prevent or intervene with staphylococcal infections. This requires a more thorough understanding of the virulence mechanisms employed by *S. aureus* as well as the identification of suitable targets for novel interventions. Pathogenic bacteria such as *S. aureus* utilize many different mechanisms to invade and/or escape the host immune defenses, to establish themselves on the site of infection, and to invade different niches in order to find new sources of nutrients. The research presented in this thesis was specifically focused on two of these mechanisms, namely surface translocation and biofilm formation.

Surface translocation

Bacterial translocation is one of the most remarkable features of bacterial life. It enables bacteria to establish symbiotic and pathogenic associations with plants and animals. Potential benefits of translocation include increased access to nutrients, avoidance of toxins, access to preferred colonization sites within the host and increased efficiency of transmission from one host individual to another. Bacteria are capable of colonizing many different environmental niches. Depending on factors such as surface conditions and nutrient availability, bacterial colonization can remain local or the bacteria can spread to other niches where more nutrients are available (33, 37). In 1972, J. Henrischen identified six different categories of bacterial surface motility: swimming, swarming, gliding, twitching, sliding and darting. Swimming and swarming movements are dependent on flagella, twitching has been shown to require type IV pili, as do some forms of gliding and sliding. Spreading and darting are forms of passive translocation. There is a strong correlation between the production of surfactants such as lipopeptides, lipopolysaccharides (LPS) and glycolipids and the sliding/spreading phenomenon (33).

Chapter 1

Factors affecting bacterial motility

There are several conditions, such as moisture that favor motility. This is evident from the motility behavior of many bacterial species including bacilli, pseudomonads and *Escherichia coli* (34, 35, 62, 67). These bacteria will swarm optimally at agar concentrations ranging from 0.5% to 0.7%. *Bacillus subtillis* mutants defective in surfactin production, the main surfactant molecule of this organism, have swarming defects. External addition of surfactin rescues the swarming of defective mutants (43). *Salmonella typhimurium* mutants defective in LPS (lipopolysaccharide) biosynthesis are also defective in swarming and can be rescued by external addition of surfactin from *B. subtillis* suggesting that LPS acts as a surfactant (53, 78). The phenol-soluble modulins (PSMs) have biosurfactant properties and promote colony spreading in *S. aureus*. Surfactants such as surfactin are exolipids that lower the surface tension and improve surface wettability, allowing liquids to spread on hydrophobic surfaces. These and other exolipids not only promote swarming motility, but also allow bacterial cells to spread in the absence of a mechanism for active motility and are likely to play an important role in microbial colonization of hydrophobic surfaces (25, 44, 50, 53, 81). Temperature can also affect motility. For example, in *S. aureus* colony spreading is inhibited below 30°C or above 42°C. Most likely, this temperature effect also relates to surface wettability. In *Serratia marcescens*, swimming, swarming and sliding are inhibited at temperatures above 32°C. Inhibition of sliding is likely due to the absence of synthesis of the surfactant serrawettin at higher temperatures (1, 42). A final important factor for motility is nutrient availability. For example, swarming of many organisms is not observed in minimal media, and gliding without pili or colony spreading are favored in nutrient-rich conditions (25, 78).

Quorum-sensing and bacterial motility

In *Serratia liquefaciens* swarming is controlled by two quorum-sensing regulators that belong to the class of N-acyl homoserine lactones (HSLs) (25). They do so by binding to a transcriptional activator that regulates the production of the surfactant serrawettin, which is required for colony expansion. In *Pseudomonas aeruginosa*, quorum-sensing systems are responsible for the production of rhamnolipid, which appears to facilitate swarming. The twitching motility encountered in many other microorganisms is influenced by cell density, cell contact-dependent intercellular signals, as well as the nutritional state of the cell (33, 73, 85, 91). Lastly, as documented in this thesis, colony spreading in *S. aureus* is dependent on the staphylococcal 'accessory gene regulator' (*agr*) quorum-sensing system (81).

Biofilm formation

A biofilm is a sessile bacterial community embedded in a self-produced extracellular polymeric substance (EPS) where cells adhere to each other and/or to (host) surfaces (18, 22, 31, 69). In most natural environments, a biofilm consists of a multispecies microbial community. In contrast, foreign–body related infections appear to be mainly caused by biofilm-associated

staphylococci (22, 29, 51, 69). There are two stages involved in the formation of a staphylococcal biofilm. The formation of a biofilm begins with the attachment of free-floating bacteria to a surface. This stage is partly mediated by cell wall- associated adhesins and is often reversible. The second stage involves biofilm development and maturation where multiple layers of biofilm are being formed (Figure 2). Bacteria living in biofilms display complex rearrangements and secondary structures, such as microcolonies, which enables better diffusion of nutrients (7, 22). Cells that are embedded in a biofilm undergo a phenotypic shift in behavior in which many genes are differentially regulated compared to planktonic conditions (2, 5). This altered gene regulation confers to the bacteria resistance to antibiotics, disinfectants and to the host immune defenses (12, 22, 54, 59, 68, 75, 92).

Another important stage in the lifecycle of a biofilm is the dispersal of cells from the biofilm. The dispersal enables cells leaving a biofilm to spread and colonize new surfaces. Many factors have been 'accused' of playing a role in the detachment of clusters of cells from a mature biofilm. There are three different categories of biofilms: proteinaceous biofilms, external (e-)DNA-dependent biofilms and polysaccharide intercellular adhesin (PIA-)dependent biofilms (15, 16, 49, 74, 89). All of these are compounds of the EPS. Depending on the type of biofilm, different factors contribute to the dispersal of the cells. For example, in eDNA-dependent biofilms nucleases are crucial for dispersal (49), and in the case of proteinaceous biofilms extracellular proteases facilitate the dispersal of cells (6).
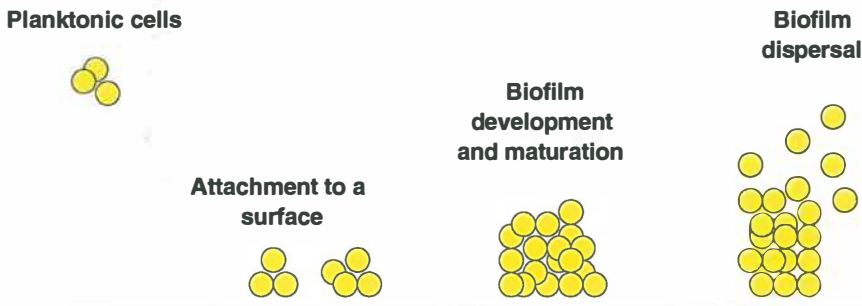
Figure 2. Schematic representation of the different stages in biofilm formation, maturation and dispersal.

Various studies have shown the influence of the staphylococcal *agr* system on biofilm formation (8, 45, 84, 92). The contribution of the *agr* system to biofilm development is dependent on the biofilm growth conditions. Yarwood *et al.* have shown that in most areas of a biofilm and at most times the *agr*-dependent virulence genes are not expressed. In contrast, cells that do express *agr* appear to be released from the biofilm (92). A model proposed by Projan and Novick for *agr* expression by staphylococci in abscess infections states that when bacteria are crowding within a localized infection, the *agr*-dependent quorum response is activated. This results in the synthesis of extracellular factors, enabling the staphylococci to escape the abscess and spread to new sites (61).

Chapter 1

The staphylococcal quorum-sensing system *agr*

The staphylococcal *agr* locus encodes a quorum-sensing system, which is expressed from the divergent promoters designated P2 and P3. The P2 transcript covers a 4-gene operon containing *agrB*, *agrD*, *agrC* and *agrA*. The AgrA and AgrC proteins constitute a classical two-component signaling module and AgrB and AgrD combine to generate the activating autoinducing peptide (AIP). The AIP binds to and activates AgrA and, in turn, the activated AgrA activates its own promoter P2 and the adjacent P3 promoter (56-58). AgrB is a transmembrane protein that appears to be involved in processing of the AgrD product into the AIP octapeptide, secretion of AIP and modification of AIP by the formation of a cyclic thiolactone bond between an internal cysteine and the carboxyl terminus. AgrC is a histidine kinase that binds the extracellular AIP and in turn modulates the activity of the response regulator ArgA. The activation of the AgrA leads to increased transcription from both the P2 and P3 promoters in the late-log phase of growth, when the concentration of the AIP in the medium is high (Figure 3) (92). Increased transcription of P3 results in dramatically increased levels of RNAIII, a regulatory RNA molecule. This RNA is in fact the effector molecule of the *agr* locus. The RNAIII not only acts as a regulatory RNA by itself, but it also encodes the toxin δ-haemolysin in its 5´ end. Overall, the most important function of RNAIII is to act as a regulatory RNA (similar to non-coding regulatory RNAs) that up-regulates the transcription, and in some cases the translation of secreted proteins and down-regulates the transcription of cell wall-associated proteins (24). The mechanism by which RNAIII regulates its targets is currently not completely understood. RNAIII may bind to individual transcription factors, causing allosteric modifications that affect their ability to bind to target sequences, but it is also possible that RNAIII affects the stability of the target gene transcripts (4, 55). Such a coordination of virulence gene expression may play an important role in the colonization and spreading of *S. aureus* in the host. Initially, bacteria will be present in small numbers, expressing their cell surface virulence factors in order to evade the immune system of the host. When the first site of infection becomes depleted of nutrients due to increased bacterial numbers, the bacteria increase the production of secreted factors, allowing them to escape from this site and spread through the host (92).
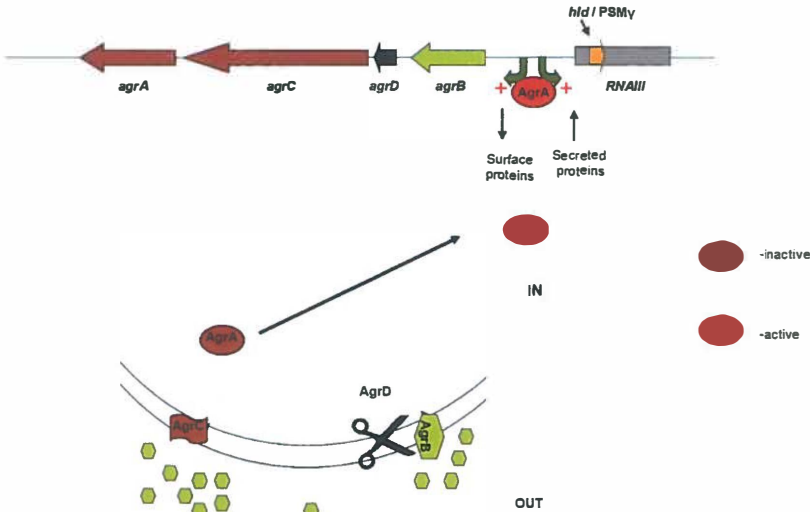
Figure 3.
Schematic representation of the staphylococcal Agr quorum-sensing system.

Heatley *et al.* showed that the δ-toxin encoded by RNAIII, also known as PSMγ, has strong surfactant properties (36). Furthermore, Vuong *et al.* proposed that δ-toxin may also serve as a surfactant *in vivo* thereby preventing adherence of staphylococcal cells to surfaces. Surfactants may also decrease the adherence of staphylococci to biomaterials. In an acute infection, the expression of secreted virulence factors controlled by *agr*, seems to be important for bacterial virulence, whereas in chronic infections an *agr⁻* phenotype contributes to increased persistence. Somerville *et al.* showed that during growth in aerobic conditions, mutations arise in the *agr* locus, creating a mixed bacterial population (e.g., α-hemolytic and non-hemolytic). This population heterogeneity enhances the likelihood of subsequent selection of an *S. aureus agr* variant most capable of surviving in a specific environment. All identified mutations were located in the coding region of *agrC* or within the intergenic region between *agrC* and *agrA*. These mutations increase the growth yield, thereby enhancing the mutant's ability to place progeny into the next generation (72).

Traber *et al.* have shown that spontaneous mutations in the *agr* locus are not simply a laboratory phenomenon, as they found the same mutation in the *agrA* that is present in the laboratory strain RN4220, in serial blood cultures from a hospital patient with staphylococcal endocarditis. This mutation results in a partial defect of *agrA* gene function, causing a considerable delay in the activation of the *agr* locus. In turn, this causes a delayed activation of the RNAIII, which results in δ and α haemolysin negative phenotypes (79). The timing of *agr* activation may thus be an important factor in the regulation of virulence factors *in vitro*. This shows that a single nucleotide change, even outside an open reading frame, can have a dramatic global effect on bacterial gene expression.

Chapter 1

More evidence for the selection for *agr* negative phenotypes in chronic infections is emerging. In mixed strain infection experiments, functions performed by the wild-type strain (agr^+) may assist the non-haemolytic group (agr^-) in establishing infection (65; Tsompanidou *et al.* submitted). Specifically, Traber *et al.* reported that *agr* defective mutants are detectable in clinical samples, that these mutants arise and persist during infections, and that they are not the result of post-isolation handling. Thus these *agr* mutants represent an important subset of the clinical *S. aureus* strains. Other staphylococcal isolates have been obtained in which the population was continuously heterogeneous with respect to *agr* functionality, with the agr^+ and agr^- variants having otherwise indistinguishable chromosomal backgrounds (80).

The relatively high frequency of naturally occurring *agr* mutants supports the hypothesis that *agr* mutants have adapted to an ecological niche in which adhesive properties are important (84). Wright *et al.* monitored in real time the activity of the *agr* system in abscess formation and suggested that an early rapid burst of *agr* activity, and thus excessive production of secreted virulence factors is critical for the prolonged survival of the bacteria (90). All of this data suggests that whereas secreted virulence factors that are up-regulated by RNAIII may be important during the acute phase of infection, loss of *agr* function may enhance the long-term survival of staphylococci in the host and contribute to persistent infections known also as biofilms. On the other hand, the high levels of *agr* activity are thought to be the reason for the increased virulence of CA-MRSA. In this thesis, it is reported that colony spreading motility can occur only when the *agr* locus is active (81). Thus, CA-MRSA strains have more tendencies to spread, which suggests that this type of motility may play a role in the transmission of these strains.

The *S. aureus* phenol-soluble modulin peptides (PSMs)

S. aureus secretes eight PSM peptides. Four of these are encoded by the *psmα* operon, two by the *psmβ* operon, one by the RNAIII gene (i.e. *psmγ* or *hld*; Figure 4), and one by the *psm-mec* gene located on the 'staphylococcal cassette chromosome *mec*' (SCC*mec*) types II and III. These peptides are produced in high amounts by CA-MRSAs and are considered to be key virulence factors of *S. aureus* that contribute to the increased virulence of CA-MRSAs (88). The PSMs have leukocidal, surfactant and antibacterial properties (88, 41). When present in low amounts, PSMs and especially PSMβ peptides can induce biofilm formation. In contrast, when present in high amounts they can inhibit biofilm formation and dissemination of cells from the biofilm (86). In addition, when present in high amounts they can also induce colony spreading, thereby allowing the bacteria to translocate over surfaces (Chapter 3). Due to their antibacterial properties, PSMs enhance the survival rates of *S. aureus* by killing competing organisms. Thus, by using the different properties of the PSM peptides, *S. aureus* can escape the immune defenses, translocate to new niches that can be rich in nutrients and can outcompete other organisms that prefer the same niches. In this way, PSMs contribute to the success of *S. aureus* as an important pathogen.
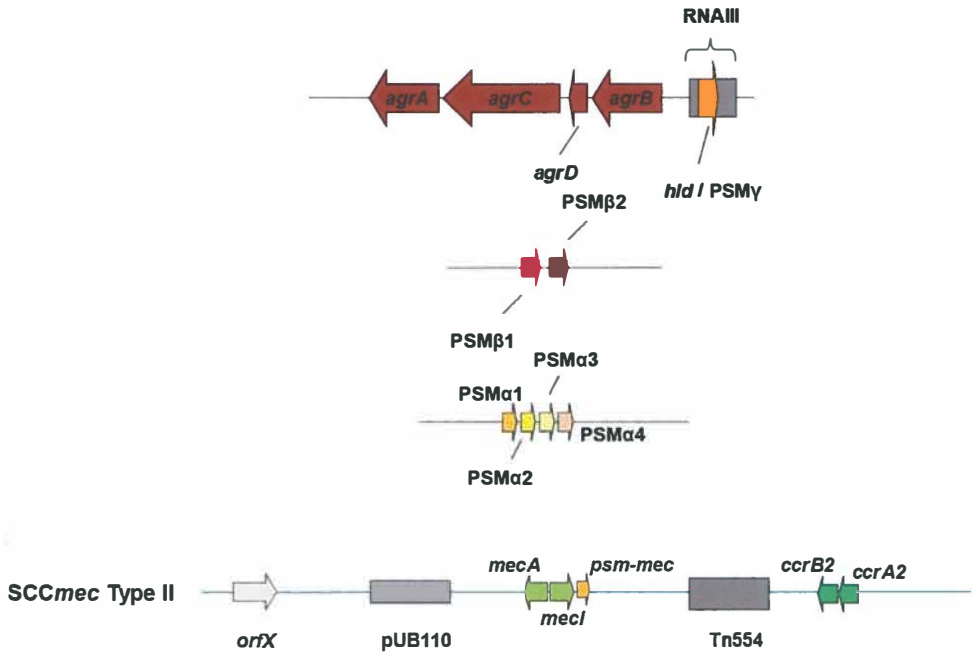
Figure 4. The eight PSM peptides of *S. aureus*

Chapter 1

Scope of this thesis

S. aureus is one of the main causative agents of nosocomial infections. In addition, *S. aureus* has an amazing ability of acquiring resistance to almost all antibiotics used in its treatment. Thus, there is a great need for unraveling more of its virulence mechanisms and the factors involved in its capabilities to cause disease. *S. aureus* possesses a large arsenal of virulence factors some of which are not encoded by the genomes of all known *S. aureus* isolates. Moreover, the expression of the well conserved genes for virulence factors is highly variable. Therefore, in order to prevent and fight *S. aureus* infections, the effort must be focused on "invariant" virulence factors and mechanisms (20, 94). As outlined in **Chapter 1**, the research described in this thesis was primarily focused on determinants for staphylococcal motility that help this organism to translocate over wet surfaces and to escape from biofilms. In addition, to obtain new insights into general Gram-positive bacterial motility phenomena a combined systems and synthetic biology approach was followed using the genetically amenable model organism *B. subtilis*.

Chapter 2 describes a comparison of clinical isolates and laboratory strains for their ability to spread on soft agar plates; a phenomenon called colony spreading. This study revealed that not all strains share the ability to spread. Only strains with an active *agr* locus have the ability to spread, whereas strains where the *agr* locus remains silent are unable to spread. As pointed out in Chapter 1, the staphylococcal quorum-sensing system is a global regulator of virulence genes and one of the best studied two-component regulatory systems.

Chapter 3 describes a dissection of the roles of PSMs in colony spreading and biofilm formation. The PSM peptides are tightly regulated by *agr* and they are only produced by *agr* positive strains. These peptides have surfactant properties and thereby allow *S. aureus* cells to spread over surfaces. There are seven PSM peptides encoded by the staphylococcal core genome. The surfactant properties of these seven peptides are very variable. The PSMα3 and PSMγ/hld have the strongest surfactant properties. In *agr* negative strains the PSM peptides are not produced, and the results in Chapter 3 show that this is why these mutants are unable to spread by themselves. Most importantly, PSM peptides and especially PSMα3 and PSMγ can promote the spreading of *S. aureus* cells that escape from catheter-associated biofilms. Furthermore, the results show that PSMα3 and both PSMβ peptides can strongly inhibit biofilm formation of *S. aureus*.

Chapter 4 reports on the antagonizing effect of several surface-exposed LPxTG proteins on colony spreading. When the *srtA* gene was deleted from *S. aureus agr* positive strains, these strains showed a substantial increase in their spreading capabilities. SrtA is responsible for the correct coupling of LPxTG proteins to the cell surface. In the absence of *srtA*, *S. aureus* fails to display these proteins on the surface. The results described in Chapter 4 imply that the hyper-spreading phenotype observed in the *srtA* mutant strains is indirectly caused by the loss of surface display of LPxTG proteins. Specifically, the fibronectin-binding proteins FnbpA and

General Introduction and Scope of this Thesis

FnbpB, and the clumping factors ClfA and ClfB were implicated in the hyper-spreading phenotype of the *srtA* mutant. Importantly, the spreading-limiting role of cell surface-bound proteins was also observed for *S. epidermidis*. It can therefore be concluded that cell wall-associated factors that promote a sessile lifestyle of *S. aureus* and *S. epidermidis* antagonize the colony spreading motility of these bacteria.

In **Chapter 5** the properties of *S. aureus* and *S. epidermidis* *srtA* mutants are described and compared to their respective parental strains. The localization of many sortase substrates was altered in the respective mutants. These included the *S. aureus* surface protein G (SasG) and the accumulation-associated protein (Aap) from *S. epidermidis*. Moreover, biofilm formation was also affected in the *srtA* mutant strains. Complementation studies revealed that the phenotype could be fully complemented with *srtA* from either *S. aureus* or *S. epidermidis*. Partial complementation was observed with *srtC* from *S. epidermidis*, whereas the *yhcS* gene encoding a sortase from *B. subtilis* did not complement the *S. aureus* and *S. epidermidis* *srtA* mutations.

Chapter 6 reports on the analyses of 286 *B. subtilis* mutants lacking large non-essential chromosomal regions. These mutant strains were analyzed for swimming, swarming and pellicle formation. Deletions with an altered phenotype were transferred to undomesticated *B. subtillis* strains to confirm the phenotype. The results reveal a major role for the two-component regulatory system CssRS, the phosphate transport system Pst and several genes of unknown function in the motility of *B. subtilis*.

Finally, **Chapter 7** gives a general overview and discussion on the findings described in this thesis. Suggestions for future research are presented.

Chapter 1

References

1. **Alberti, L. and R. M. Harshey.** 1990. Differentiation of *Serratia marcescens* 274 into swimmer and swarmer cells. J. Bacteriol. 172:4322-4328.
2. **An, D. and M. R. Parsek.** 2007. The promise and peril of transcriptional profiling in biofilm communities. Curr. Opin. Microbiol. 10:292-296.
3. **Appelbaum, P. C.** 2006. The emergence of vancomycin-intermediate and vancomycin-resistant *Staphylococcus aureus*. Clin. Microbiol. Infect. 12 Suppl 1:16-23.
4. **Arvidson, S. and K. Tegmark.** 2001. Regulation of virulence determinants in *Staphylococcus aureus*. Int. J. Med. Microbiol. 291:159-170.
5. **Beenken, K. E., P. M. Dunman, F. McAleese, D. Macapagal, E. Murphy, S. J. Projan, J. S. Blevins, and M. S. Smeltzer.** 2004. Global gene expression in *Staphylococcus aureus* biofilms. J. Bacteriol. 186:4665-4684.
6. **Boles, B. R. and A. R. Horswill.** 2008. Agr-mediated dispersal of *Staphylococcus aureus* biofilms. PLoS Pathog. 4:e1000052.
7. **Branda, S. S., S. Vik, L. Friedman, and R. Kolter.** 2005. Biofilms: the matrix revisited. Trends Microbiol. 13:20-26.
8. **Cafiso, V., T. Bertuccio, M. Santagati, V. Demelio, D. Spina, G. Nicoletti, and S. Stefani.** 2007. *agr*-Genotyping and transcriptional analysis of biofilm-producing *Staphylococcus aureus*. FEMS Immunol. Med. Microbiol. 51:220-227.
9. **Cavalier-Smith, T.** 2006. Cell evolution and Earth history: stasis and revolution. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 361:969-1006.
10. **Centers for Disease Control (CDC).** 1981. Community-acquired methicillin-resistant *Staphylococcus aureus* infections--Michigan. MMWR Morb. Mortal. Wkly. Rep. 30:185-187.
11. **Centers for Disease Control and Prevention (CDC).** 2000. *Staphylococcus aureus* with reduced susceptibility to vancomycin--Illinois, 1999. MMWR Morb. Mortal. Wkly. Rep. 48:1165-1167.
12. **Ceri, H., M. E. Olson, C. Stremick, R. R. Read, D. Morck, and A. Buret.** 1999. The Calgary Biofilm Device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. J. Clin. Microbiol. 37:1771-1776.
13. **Cheng, A. G., H. K. Kim, M. L. Burts, T. Krausz, O. Schneewind, and D. M. Missiakas.** 2009. Genetic requirements for *Staphylococcus aureus* abscess formation and persistence in host tissues. FASEB J. 23:3393-3404.
14. **Costerton, J. W., L. Montanaro, and C. R. Arciola.** 2005. Biofilm in implant infections: its production and regulation. Int. J. Artif. Organs 28:1062-1068.
15. **Cramton, S. E., C. Gerke, N. F. Schnell, W. W. Nichols, and F. Gotz.** 1999. The intercellular adhesion (*ica*) locus is present in *Staphylococcus aureus* and is required for biofilm formation. Infect. Immun. 67:5427-5433.
16. **Cucarella, C., C. Solano, J. Valle, B. Amorena, I. Lasa, and J. R. Penades.** 2001. Bap, a *Staphylococcus aureus* surface protein involved in biofilm formation. J. Bacteriol. 183:2888-2896.
17. **Cui, L., X. Ma, K. Sato, K. Okuma, F. C. Tenover, E. M. Mamizuka, C. G. Gemmell, M. N. Kim, M. C. Ploy, N. El-Solh, V. Ferraz, and K. Hiramatsu.** 2003. Cell wall thickening is a common feature of vancomycin resistance in *Staphylococcus aureus*. J. Clin. Microbiol. 41:5-14.
18. **Davey, M. E. and G. A. O'toole.** 2000. Microbial biofilms: from ecology to molecular genetics. Microbiol. Mol. Biol. Rev. 64:847-867.
19. **de Niederhausern, S., M. Bondi, P. Messi, R. Iseppi, C. Sabia, G. Manicardi, and I. Anacarso.** 2011. Vancomycin-resistance transferability from VanA enterococci to *Staphylococcus aureus*. Curr. Microbiol. 62:1363-1367.
20. **Diep, B. A. and M. Otto.** 2008. The role of virulence determinants in community-associated MRSA pathogenesis. Trends Microbiol. 16:361-369.

General Introduction and Scope of this Thesis

21. **Diep, B. A., G. F. Sensabaugh, N. Somboonna, H. A. Carleton, and F. Perdreau-Remington.** 2004. Widespread skin and soft-tissue infections due to two methicillin-resistant *Staphylococcus aureus* strains harboring the genes for Panton-Valentine leucocidin. J. Clin. Microbiol. 42:2080-2084.

22. **Donlan, R. M. and J. W. Costerton.** 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. Clin. Microbiol. Rev. 15:167-193.

23. **Dubrac, S., P. Bisicchia, K. M. Devine, and T. Msadek.** 2008. A matter of life and death: cell wall homeostasis and the WalKR (YycGF) essential signal transduction pathway. Mol. Microbiol. 70:1307-1322.

24. **Dunman, P. M., E. Murphy, S. Haney, D. Palacios, G. Tucker-Kellogg, S. Wu, E. L. Brown, R. J. Zagursky, D. Shlaes, and S. J. Projan.** 2001. Transcription profiling-based identification of *Staphylococcus aureus* genes regulated by the *agr* and/or *sarA* loci. J. Bacteriol. 183:7341-7353.

25. **Eberl, L., S. Molin, and M. Givskov.** 1999. Surface motility of *Serratia liquefaciens* MG1. J. Bacteriol. 181:1703-1712.

26. **Fridkin, S. K.** 2001. Vancomycin-intermediate and -resistant *Staphylococcus aureus*: what the infectious disease specialist needs to know. Clin. Infect. Dis. 32:108-115.

27. **Fridkin, S. K., J. R. Edwards, J. M. Courval, H. Hill, F. C. Tenover, R. Lawton, R. P. Gaynes, J. E. McGowan Jr, and Intensive Care Antimicrobial Resistance Epidemiology (ICARE) Project and the National Nosocomial Infections Surveillance (NNIS) System Hospitals.** 2001. The effect of vancomycin and third-generation cephalosporins on prevalence of vancomycin-resistant enterococci in 126 U.S. adult intensive care units. Ann. Intern. Med. 135:175-183.

28. **Furukawa, S., S. L. Kuchma, and G. A. O'Toole.** 2006. Keeping their options open: acute versus persistent infections. J. Bacteriol. 188:1211-1217.

29. **Gotz, F.** 2002. Staphylococcus and biofilms. Mol. Microbiol. 43:1367-1378.

30. **Grundmann, H., M. Aires-de-Sousa, J. Boyce, and E. Tiemersma.** 2006. Emergence and resurgence of methicillin-resistant *Staphylococcus aureus* as a public-health threat. Lancet 368:874-885.

31. **Hall-Stoodley, L., J. W. Costerton, and P. Stoodley.** 2004. Bacterial biofilms: from the natural environment to infectious diseases. Nat. Rev. Microbiol. 2:95-108.

32. **Harris, L. G. and R. G. Richards.** 2006. Staphylococci and implant surfaces: a review. Injury 37 Suppl 2:S3-14.

33. **Harshey, R. M.** 2003. Bacterial motility on a surface: many ways to a common goal. Annu. Rev. Microbiol. 57:249-273.

34. **Harshey, R. M.** 1994. Bees aren't the only ones: swarming in Gram-negative bacteria. Mol. Microbiol. 13:389-394.

35. **Harshey, R. M. and T. Matsuyama.** 1994. Dimorphic transition in *Escherichia coli* and *Salmonella typhimurium*: surface-induced differentiation into hyperflagellate swarmer cells. Proc. Natl. Acad. Sci. U. S. A. 91:8631-8635.

36. **Heatley, N. G.** 1971. A new method for the preparation and some properties of staphylococcal delta-haemolysin. J. Gen. Microbiol. 69:269-278.

37. **Henrichsen, J.** 1972. Bacterial surface translocation: a survey and a classification. Bacteriol. Rev. 36:478-503.

38. **Herold, B. C., L. C. Immergluck, M. C. Maranan, D. S. Lauderdale, R. E. Gaskin, S. Boyle-Vavra, C. D. Leitch, and R. S. Daum.** 1998. Community-acquired methicillin-resistant *Staphylococcus aureus* in children with no identified predisposing risk. JAMA 279:593-598.

39. **Hiramatsu, K., N. Aritaka, H. Hanaki, S. Kawasaki, Y. Hosoda, S. Hori, Y. Fukuchi, and I. Kobayashi.** 1997. Dissemination in Japanese hospitals of strains of *Staphylococcus aureus* heterogeneously resistant to vancomycin. Lancet 350:1670-1673.

40. **Hota, B., C. Ellenbogen, M. K. Hayden, A. Aroutcheva, T. W. Rice, and R. A. Weinstein.** 2007. Community-associated methicillin-resistant *Staphylococcus aureus* skin and soft tissue infections at a public hospital: do public housing and incarceration amplify transmission? Arch. Intern. Med. 167:1026-1033.

Chapter 1

41. **Joo, H. S., G. Y. Cheung, and M. Otto.** 2011. Antimicrobial activity of community-associated methicillin-resistant *Staphylococcus aureus* is caused by phenol-soluble modulin derivatives. J. Biol. Chem. 286:8933-8940.

42. **Kaito, C. and K. Sekimizu.** 2007. Colony spreading in *Staphylococcus aureus*. J. Bacteriol. 189:2553-2557.

43. **Kearns, D. B. and R. Losick.** 2003. Swarming motility in undomesticated *Bacillus subtilis*. Mol. Microbiol. 49:581-590.

44. **Kohler, T., L. K. Curty, F. Barja, C. van Delden, and J. C. Pechere.** 2000. Swarming of *Pseudomonas aeruginosa* is dependent on cell-to-cell signaling and requires flagella and pili. J. Bacteriol. 182:5990-5996.

45. **Kong, K. F., C. Vuong, and M. Otto.** 2006. Staphylococcus quorum-sensing in biofilm formation and infection. Int. J. Med. Microbiol. 296:133-139.

46. **Lee, N. E., M. M. Taylor, E. Bancroft, P. J. Ruane, M. Morgan, L. McCoy, and P. A. Simon.** 2005. Risk factors for community-associated methicillin-resistant *Staphylococcus aureus* skin infections among HIV-positive men who have sex with men. Clin. Infect. Dis. 40:1529-1534.

47. **Lowy, F. D.** 1998. *Staphylococcus aureus* infections. N. Engl. J. Med. 339:520-532.

48. **Lu, J., Y. Nogi, and H. Takami.** 2001. *Oceanobacillus iheyensis* gen. nov., sp. nov., a deep-sea extremely halotolerant and alkaliphilic species isolated from a depth of 1050 m on the Iheya Ridge. FEMS Microbiol. Lett. 205:291-297.

49. **Mann, E. E., K. C. Rice, B. R. Boles, J. L. Endres, D. Ranjit, L. Chandramohan, L. H. Tsang, M. S. Smeltzer, A. R. Horswill, and K. W. Bayles.** 2009. Modulation of eDNA release and degradation affects *Staphylococcus aureus* biofilm maturation. PLoS One 4:e5822.

50. **Matsuyama, T., K. Kaneda, Y. Nakagawa, K. Isa, H. Hara-Hotta, and I. Yano.** 1992. A novel extracellular cyclic lipopeptide which promotes flagellum-dependent and -independent spreading growth of *Serratia marcescens*. J. Bacteriol. 174:1769-1776.

51. **Mayberry-Carson, K. J., B. Tober-Meyer, J. K. Smith, D. W. Lambe Jr, and J. W. Costerton.** 1984. Bacterial adherence and glycocalyx formation in osteomyelitis experimentally induced with *Staphylococcus aureus*. Infect. Immun. 43:825-833.

52. **Miller, L. G. and B. A. Diep.** 2008. Clinical practice: colonization, fomites, and virulence: rethinking the pathogenesis of community-associated methicillin-resistant *Staphylococcus aureus* infection. Clin. Infect. Dis. 46:752-760.

53. **Mireles, J. R., 2nd, A. Toguchi, and R. M. Harshey.** 2001. *Salmonella enterica serovar typhimurium* swarming mutants with altered biofilm-forming abilities: surfactin inhibits biofilm formation. J. Bacteriol. 183:5848-5854.

54. **Molin, S. and T. Tolker-Nielsen.** 2003. Gene transfer occurs with enhanced efficiency in biofilms and induces enhanced stabilisation of the biofilm structure. Curr. Opin. Biotechnol. 14:255-261.

55. **Novick, R. P.** 2003. Autoinduction and signal transduction in the regulation of staphylococcal virulence. Mol. Microbiol. 48:1429-1449.

56. **Novick, R. P. and E. Geisinger.** 2008. Quorum-sensing in staphylococci. Annu. Rev. Genet. 42:541-564.

57. **Novick, R. P., H. F. Ross, S. J. Projan, J. Kornblum, B. Kreiswirth, and S. Moghazeh.** 1993. Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. EMBO J. 12:3967-3975.

58. **Novick, R. P., S. J. Projan, J. Kornblum, H. F. Ross, G. Ji, B. Kreiswirth, F. Vandenesch, and S. Moghazeh.** 1995. The *agr* P2 operon: an autocatalytic sensory transduction system in *Staphylococcus aureus*. Mol. Gen. Genet. 248:446-458.

59. **Oie, S., Y. Huang, A. Kamiya, H. Konishi, and T. Nakazawa.** 1996. Efficacy of disinfectants against biofilm cells of methicillin-resistant *Staphylococcus aureus*. Microbios 85:223-230.

60. **Peacock, S. J., I. de Silva, and F. D. Lowy.** 2001. What determines nasal carriage of *Staphylococcus aureus*? Trends Microbiol. 9:605-610.

61. **Projan, S. J., and R. P Novick.** 1997. The molecular basis of pathogenicity, p. 55-81. In K. B. Crossley and G. L. Archer (ed.), The staphylococci in human disease. Churchill Livingstone Inc., New York, N.Y.

62. **Rashid, M. H. and A. Kornberg.** 2000. Inorganic polyphosphate is needed for swimming, swarming, and twitching motilities of *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. U. S. A. 97:4885-4890.

63. **Sancak, B., S. Ercis, D. Menemenlioglu, S. Colakoglu, and G. Hascelik.** 2005. Methicillin-resistant *Staphylococcus aureus* heterogeneously resistant to vancomycin in a Turkish university hospital. J. Antimicrob. Chemother. 56:519-523.

64. **Schopf, J. W.** 2006. Fossil evidence of Archaean life. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 361:869-885.

65. **Schwan, W. R., M. H. Langhorne, H. D. Ritchie, and C. K. Stover.** 2003. Loss of hemolysin expression in *Staphylococcus aureus agr* mutants correlates with selective survival during mixed infections in murine abscesses and wounds. FEMS Immunol. Med. Microbiol. 38:23-28.

66. **Sears, C. L.** 2005. A dynamic partnership: celebrating our gut flora. Anaerobe 11:247-251.

67. **Senesi, S., F. Celandroni, S. Salvetti, D. J. Beecher, A. C. Wong, and E. Ghelardi.** 2002. Swarming motility in *Bacillus cereus* and characterization of a *fliY* mutant impaired in swarm cell differentiation. Microbiology 148:1785-1794.

68. **Shiau, A. L. and C. L. Wu.** 1998. The inhibitory effect of *Staphylococcus epidermidis* slime on the phagocytosis of murine peritoneal macrophages is interferon-independent. Microbiol. Immunol. 42:33-40.

69. **Shirtliff, M. E., J. T. Mader, and A. K. Camper.** 2002. Molecular interactions in biofilms. Chem. Biol. 9:859-871.

70. **Shivaji, S., P. Chaturvedi, G. S. Reddy, and K. Suresh.** 2005. *Pedobacter himalayensis* sp. nov., from the Hamta glacier located in the Himalayan mountain ranges of India. Int. J. Syst. Evol. Microbiol. 55:1083-1088.

71. **Smith, T. L. and W. R. Jarvis.** 1999. Antimicrobial resistance in *Staphylococcus aureus*. Microbes Infect. 1:795-805.

72. **Somerville, G. A., S. B. Beres, J. R. Fitzgerald, F. R. DeLeo, R. L. Cole, J. S. Hoff, and J. M. Musser.** 2002. In vitro serial passage of *Staphylococcus aureus*: changes in physiology, virulence factor production, and *agr* nucleotide sequence. J. Bacteriol. 184:1430-1437.

73. **Spormann, A. M.** 1999. Gliding motility in bacteria: insights from studies of *Myxococcus xanthus*. Microbiol. Mol. Biol. Rev. 63:621-641.

74. **Steinberger, R. E. and P. A. Holden.** 2005. Extracellular DNA in single- and multiple-species unsaturated biofilms. Appl. Environ. Microbiol. 71:5404-5410.

75. **Stewart, P. S. and J. W. Costerton.** 2001. Antibiotic resistance of bacteria in biofilms. Lancet 358:135-138.

76. **Taj, Y., F. E. Abdullah, and S. U. Kazmi.** 2010. Current pattern of antibiotic resistance in *Staphylococcus aureus* clinical isolates and the emergence of vancomycin resistance. J. Coll. Physicians Surg. Pak. 20:728-732.

77. **Tenover, F. C., L. M. Weigel, P. C. Appelbaum, L. K. McDougal, J. Chaitram, S. McAllister, N. Clark, G. Killgore, C. M. O'Hara, L. Jevitt, J. B. Patel, and B. Bozdogan.** 2004. Vancomycin-resistant *Staphylococcus aureus* isolate from a patient in Pennsylvania. Antimicrob. Agents Chemother. 48:275-280.

78. **Toguchi, A., M. Siano, M. Burkart, and R. M. Harshey.** 2000. Genetics of swarming motility in *Salmonella enterica serovar typhimurium*: critical role for lipopolysaccharide. J. Bacteriol. 182:6308-6321.

79. **Traber, K. and R. Novick.** 2006. A slipped-mispairing mutation in AgrA of laboratory strains and clinical isolates results in delayed activation of *agr* and failure to translate delta- and alpha-haemolysins. Mol. Microbiol. 59:1519-1530.

80. **Traber, K. E., E. Lee, S. Benson, R. Corrigan, M. Cantera, B. Shopsin, and R. P. Novick.** 2008. *agr* function in clinical *Staphylococcus aureus* isolates. Microbiology 154:2265-2274.

81. **Tsompanidou, E., M. J. Sibbald, M. A. Chlebowicz, A. Dreisbach, J. W. Back, J. M. van Dijl, G. Buist, and E. L. Denham.** 2011. Requirement of the *agr* locus for colony spreading of *Staphylococcus aureus*. J. Bacteriol. 193:1267-1272.

82. **Udo, E. E. and W. B. Grubb.** 1993. Genetic analysis of methicillin-resistant *Staphylococcus aureus* from a Nigerian hospital. J. Med. Microbiol. 38:203-208.

Chapter 1

83. **Vandenesch, F., T. Naimi, M. C. Enright, G. Lina, G. R. Nimmo, H. Heffernan, N. Liassine, M. Bes, T. Greenland, M. E. Reverdy, and J. Etienne.** 2003. Community-acquired methicillin-resistant *Staphylococcus aureus* carrying Panton-Valentine leukocidin genes: worldwide emergence. Emerg. Infect. Dis. 9:978-984.

84. **Vuong, C., H. L. Saenz, F. Gotz, and M. Otto.** 2000. Impact of the *agr* quorum-sensing system on adherence to polystyrene in *Staphylococcus aureus*. J. Infect. Dis. 182:1688-1693.

85. **Wall, D., P. E. Kolenbrander, and D. Kaiser.** 1999. The *Myxococcus xanthus pilQ* (*sglA*) gene encodes a secretin homolog required for type IV pilus biogenesis, social motility, and development. J. Bacteriol. 181:24-33.

86. **Wang, R., B. A. Khan, G. Y. Cheung, T. H. Bach, M. Jameson-Lee, K. F. Kong, S. Y. Queck, and M. Otto.** 2011. *Staphylococcus epidermidis* surfactant peptides promote biofilm maturation and dissemination of biofilm-associated infection in mice. J. Clin. Invest. 121:238-248.

87. **Wang, R., K. R. Braughton, D. Kretschmer, T. H. Bach, S. Y. Queck, M. Li, A. D. Kennedy, D. W. Dorward, S. J. Klebanoff, A. Peschel, F. R. DeLeo, and M. Otto.** 2007. Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. Nat. Med. 13:1510-1514.

88. **Weigel, L. M., D. B. Clewell, S. R. Gill, N. C. Clark, L. K. McDougal, S. E. Flannagan, J. F. Kolonay, J. Shetty, G. E. Killgore, and F. C. Tenover.** 2003. Genetic analysis of a high-level vancomycin-resistant isolate of *Staphylococcus aureus*. Science 302:1569-1571.

89. **Whitchurch, C. B., T. Tolker-Nielsen, P. C. Ragas, and J. S. Mattick.** 2002. Extracellular DNA required for bacterial biofilm formation. Science 295:1487.

90. **Wright, J. S., 3rd, K. E. Traber, R. Corrigan, S. A. Benson, J. M. Musser, and R. P. Novick.** 2005. The *agr* radiation: an early event in the evolution of staphylococci. J. Bacteriol. 187:5585-5594.

91. **Yang, Z., X. Ma, L. Tong, H. B. Kaplan, L. J. Shimkets, and W. Shi.** 2000. *Myxococcus xanthus dif* genes are required for biogenesis of cell surface fibrils essential for social gliding motility. J. Bacteriol. 182:5793-5798.

92. **Yarwood, J. M. and P. M. Schlievert.** 2003. Quorum-sensing in Staphylococcus infections. J. Clin. Invest. 112:1620-1625.

93. **Zeigler, D. R., Z. Pragai, S. Rodriguez, B. Chevreux, A. Muffler, T. Albert, R. Bai, M. Wyss, and J. B. Perkins.** 2008. The origins of 168, W23, and other *Bacillus subtilis* legacy strains. J. Bacteriol. 190:6983-6995.

94. **Ziebandt, A. K., H. Kusch, M. Degner, S. Jaglitz, M. J. Sibbald, J. P. Arends, M. A. Chlebowicz, D. Albrecht, R. Pantucek, J. Doskar, W. Ziebuhr, B. M. Broker, M. Hecker, J. M. van Dijl, and S. Engelmann.** 2010. Proteomics uncovers extreme heterogeneity in the *Staphylococcus aureus* exoproteome due to genomic plasticity and variant gene regulation. Proteomics 10:1634-1644.

"The mind is a strange land with many walls but no distance."

Tom Harper - Secrets of the Dead

Chapter 2

Requirement of the *agr* locus for colony spreading of *Staphylococcus aureus*

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Chapter 2

Abstract

The important human pathogen *Staphylococcus aureus* is known to spread on soft agar plates. Here we show that colony spreading of *S. aureus* involves the *agr* quorum-sensing system. This can be related to the *agr*-dependent expression of biosurfactants, such as phenol-soluble modulins, suggesting a connection between spreading motility and virulence.

Requirement of the *agr* locus for colony spreading of *Staphylococcus aureus*

The Gram-positive bacterium *Staphylococcus aureus* is frequently encountered among the human microbiota (41). For most individuals, *S. aureus* is an apparently harmless commensal. However, once *S. aureus* passes the primary barriers imposed by the human skin or mucosa, it becomes evident that this organism is in fact a dangerous pathogen. *S. aureus* is then capable of infecting almost every tissue and organ, causing a wide range of acute and chronic diseases (16,31). This ability to cause infections depends on a diverse array of cell wall-associated and extracellular virulence factors (44,49,64). The expression of many *S. aureus* virulence genes is coordinated by the *accessory gene regulator* (*agr*) quorum sensing system (33,42,46), which responds to cell density-dependent stimuli. At high cell densities or in confined compartments, this system up-regulates the expression of secreted virulence factors and down-regulates the expression of cell wall-associated virulence factors (38-40,42). Consequently, cell wall proteins and surface adhesins are expressed during the early colonising stages of infection, whereas secreted proteins, such as haemolysins, lipases and proteases are expressed at later tissue-damaging stages (14). Notably, when *S. aureus* is cultured *in vitro*, all haemolysins are up-regulated at the transition from the late exponential to stationary phase (38-40,42,64).

Activity of the *agr* system involves two major transcripts named RNAII and RNAIII. RNAII covers the *agrABCD* operon. The membrane protein AgrB is involved in (i) processing of the AgrD product into the *a*ctivating auto-*i*nducing octa-*p*eptide AIP, (ii) secretion of AIP, and (iii) modification of AIP. AgrC is a histidine kinase that binds the extracellular AIP and, in turn, modulates the activity of the response regulator AgrA which determines the synthesis of RNAII and RNAIII (14,38-40,63). RNAIII is the effector molecule of the *agr* locus that up-regulates the transcription and, in some cases, the translation of secreted proteins (2,37). Conversely, RNAIII down-regulates the transcription of cell wall-associated proteins (11). Furthermore, the 5´ end of RNAIII encodes the toxin δ-haemolysin (also known as phenol-soluble modulin γ [PSMγ]).

Bacterial pathogens often employ motility mechanisms for host colonisation. Almost 40 years ago, Henrischen made a distinction between six different categories of bacterial surface motility, which he referred to as swimming, swarming, gliding, twitching, sliding and darting (18). Swimming and swarming are dependent on flagella, whereas twitching has been shown to require type IV pili, as do some forms of gliding. Sliding and darting are forms of passive bacterial movement (16). Sliding is correlated with the production of surfactants, such as lipopeptides, lipopolysaccharides (LPS) or glycolipids (16). It was proposed that expansion forces of dividing *S. aureus* cells cause the motility phenomenon that was named darting motility (18). More recently, a different form of *S. aureus* motility was defined as colony spreading (18,26), which resembles sliding as it is independent of flagella or pili. In 2008 the so-called *fudoh* gene was implicated in the inhibition of colony spreading (25). This gene is present on the type II and III staphylococcal chromosomal cassettes *mec* (SCC*mec*) of certain methicillin-resistant *S. aureus* (MRSA) strains (25), but not on the six other presently known types of SCC*mec* (*i.e.* types I and IV-VIII) (7-9,23). The underlying mechanisms of colony spreading as well as the precise role of the *fudoh* gene in this process have thus far remained elusive.

Therefore, the aim of the present studies was to define key determinants for colony spreading of *S. aureus*.

Correlation between staphylococcal colony spreading and the quorum sensing system agr

To determine which genetic features are important for colony spreading, a collection of MRSA and MSSA strains with different characteristics (Table 1) were tested for their ability to spread on tryptic soy soft agar plates (0.24% agar). The spreading assay was performed as described by Kaito *et al.* (25,26) with minor modifications. Each plate (10 ml) was dried for approximately 10 min in a laminar flow cabinet. From an overnight culture in tryptic soy broth an aliquot of 2 μl was spotted in the centre of a plate, which was subsequently dried for 5 min under laminar flow. The plates were then incubated overnight at 37°C. Images were recorded with a G:box (Syngene, Leusden, the Netherlands). The results are summarized in Table 2 and some representative images are presented in Figure 1. When the ability to spread was compared to other features of the tested strains, it became apparent that there was a clear correlation between spreading and the presence of an intact *agr* system for quorum sensing (Table 2). For example, it was shown previously that *S. aureus* NCTC8325, MRSA252, MSSA476, MW2, RF122, Newman and USA300 express RNAIII (19,50). These strains were clearly able to spread. *S. aureus* COL expresses low levels of RNAIII due to an *agr* defect (19,50), and our results show that this strain has a minimal spreading ability (Table 2). The heavily mutagenized *S. aureus* strain RN4220 has a mutation in *agrA*, which results in an *agr⁻* phenotype. Consistent with the notion that *agr* might have a role in colony spreading, our results show that *S. aureus* RN4220 does not spread (Fig. 1). Furthermore, we tested clinical *S. aureus* isolates from patients with different staphylococcal infections (65). Previous analyses had shown that not all of these strains express RNAIII (65). Our present results show that all *agr⁺* clinical isolates do spread, whereas the *agr⁻* isolates are unable to spread (Table 2).

The *agr* system controls the expression of staphylococcal haemolysins via RNAIII. Lack of haemolytic activity is typical for *agr* defective stains and mutants with delayed *agr* activation (51). To confirm the correlation between colony spreading and *agr* activity, all strains used in this study were tested for haemolytic activity on 5% sheep blood agar plates ((13); Table 2). The α- and β-haemolysin activities were directly detectable on blood agar plates. δ-Haemolysin production was detected by measuring its synergistic activity with the β-haemolysin produced by strain RN4220, which at the same time inhibits the α-haemolysin.

Requirement of the *agr* locus for colony spreading of *Staphylococcus aureus*

Table 1. Bacterial strains and plasmids

Strains	Description[a]	Reference
NCTC8325	HA-MSSA strain, *agr⁺*, *rsbU⁻*	(35)
NCTC8325⁻	HA-MSSA, *agr⁻*, *rsbU⁻*	This work
NCTC8325-4	Derivative of NCTC8325, cured of all prophages; 11 bp deletion in *rsbU*	(36)
NCTC8325-4 *agr⁻*	NCTC8325-4 derivative, *agr::tet*	(6)
RN4220	Restriction-deficient derivative of NCTC 8325, cured of all known prophages	(28)
SH1000	NCTC8325-4 derivative, *rsbU⁺*, *agr⁺*	(22)
SH1000⁻	NCTC8325-4 derivative, *rsbU⁺*, *agr⁻*	This work
HG001	NCTC8325 derivative, *rsbU⁺*, *agr⁺*	(43)
HG001⁻	NCTC8325 derivative, *rsbU⁺*, *agr⁻*	This work
RN6390	NCTC8325 derivative, prophage-cured	(42)
RN6911	RN6390 derivative, *agr::tetM*, *sarA⁺*; Tc^R	(34)
SA113	ATCC35556 derivative; PIA-dependent biofilm producer	(24)
15981	Clinical isolate; biofilm positive in TSBg	(53)
Newman	ATCC25904; high-level clumping factor production; σ^B+	(12)
Newman Δ*agr*	Newman derivative, Δ*agr::tetM*	(59)
N315	MRSA	(29)
COL	MRSA	(15,47)
Isolate C	HA-MRSA	(65)
Isolate D	HA-MRSA	(65)
Isolate E	HA-MSSA	(65)
Isolate F	HA-MSSA	(65)
Isolate R	CA-MSSA	(65)
Isolate V	CA-MSSA	(65)
Isolate X	HA-MSSA	(65)
Isolate Y	HA MRSA	(65)
UMCG-M2	CA-MSSA; resulted from *in vivo* MRSA to MSSA conversion of strain UMCG-M4 (formerly isolate A)	(7,65)
UMCG-M4	CA-MRSA (also referred to as isolate B)	(7,65)
UMCG-M4 I	MRSA; derivative of the UMCG-M4 strain that has lost haemolysin activity upon *in vitro* cultivation at 41°C	(7)
USA300	CA-MRSA	(10)
LAC USA300	CA-MRSA	(58)
LAC USA300 ΔPSMα	LAC USA300 with a PSMα gene locus deletion	(58)
Mu50	HA-VISA	(29)
MRSA252	HA-MRSA	(21)
MSSA476	CA-MSSA	(21)
MW2	CA-MRSA	(3)
RF122	Bovine mastitis isolate	(20)
Plasmids		
pRN6662	pSK267 with *S. aureus agrA*	(1)
pALC2073RNAIII	pALC2073 with RNAIII coding region	(54)

[a] HA, hospital-acquired; CA, community-acquired; MRSA, methicillin-resistant *S. aureus*, MSSA, methicillin-sensitive *S. aureus*; VISA, vancomycin intermediate resistant *S. aureus*

Table 2. Properties of investigated *S. aureus* strains in relation to colony spreading and haemolytic activity

Strain	MRSA/ MSSA	Spreading	*agr*	Haemolysis	*rsbU*	SCC*mec*	*fudoh*
NCTC8325	HA-MSSA	+	+	+	-	-	-
RN4220	MSSA	-	-	-	-	-	-
SH1000⁺	MSSA	+	+	+	+	-	-
NCTC8325-4	MSSA	+	+	+	-	-	-
HG001	MSSA	+	+	+	+	-	-
RN6390	MSSA	+	+	+	-	-	-
RN6911 Δ*agr::tetM*	MSSA	-	-	-	-	-	-
SA113	MSSA	-	-	-	-	-	-
15981	MSSA	-	-	-	+	-	-
Newman	MSSA	+	+	+	+	-	-
N315	HA-MRSA	-	-	-	unknown	II	+
COL	HA-MRSA	+/-	+/-	-	+	I	-
UMCG-M2 (Isolate A)	CA-MSSA	+	+	+	unknown	-	-
UMCG-M4 (Isolate B)	CA-MRSA	+	+	+	unknown	V(5C2&5)	-
Isolate C	HA-MRSA	-	-	-	unknown	unknown	-
Isolate D	HA-MRSA	-	-	-	unknown	unknown	-
Isolate E	HA-MSSA	+	+	+	unknown	-	-
Isolate F	HA-MSSA	+	+	+	unknown	-	-
Isolate R	CA-MSSA	+	+	+	unknown	-	-
Isolate V	CA-MSSA	+	+	+	unknown	-	-
Isolate X	HA-MSSA	-	-	-	unknown	-	-
Isolate Y	HA-MRSA	-	-	-	unknown	unknown	-
USA300	CA-MRSA	+	+	+	+	IV	-
LAC USA300	CA-MRSA	+	+	+	unknown	IV	-
Mu50	HA-VISA	-	-	-	+	II	+
MRSA252	HA-MRSA	+	+	-	+	II	+
MSSA476	CA-MSSA	+	+	+	+	SCC*hsd*	-
MW2	CA-MRSA	+	+	+	+	IV	-
RF122	Bovine mastitis	+	+	+	+	-	-

Thus, the synergistic activity of β- and δ-haemolysin is reflected by bright arrow-like zones on blood agar plates when the respective strains are striped in close proximity to each other (1). As expected, all strains with a functional *agr* locus showed clear δ-haemolysin activity, which directly confirmed the production of RNAIII.

Requirement of the *agr* locus for colony spreading of *Staphylococcus aureus*
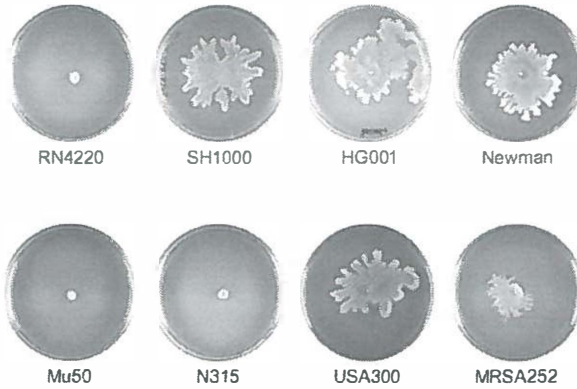
Figure 1. **Colony spreading of different** *S. aureus* **strains on soft agar plates.** From an overnight culture, an aliquot of 2 μl was spotted in the middle of a TSA plate, which was then incubated overnight at 37°C. The analyses include standard laboratory strains of *S. aureus* (*i.e.* RN4220, SH1000, HG001), as well as community-acquired (*i.e.* USA300) and hospital-acquired (*i.e.* N315, Mu50, MRSA252, Newman) strains.

An intact agr *system is required for colony spreading*

Somerville *et al.* reported that during growth in aerobic conditions, spontaneous mutations in *agr* occur, thereby creating mixed bacterial populations with cells that are either α-haemolytic or non-haemolytic (50). Notably, this population heterogeneity occurs not only in laboratory cultures, but also *in vivo* where it may enhance the ability of *S. aureus* to withstand the stress and insults imposed by the human immune defences (4,32,50,51). By plating *agr*+ strains on blood agar plates, we also observed this population heterogeneity, which we exploited to test whether the non-haemolytic cells had also lost the ability to spread. This is exemplified for non-haemolytic variants of the laboratory strain SH1000 (denoted as SH1000⁻) and the clinical isolate UMCG-M4 (denoted as UMCG-M4 I; Table 3).

Table 3. *S. aureus* strains used to confirm the role of *agr* in colony spreading and haemolytic activity

Strain	MRSA/ MSSA	Spreading	*agr*	Haemo-lysis	*rsbU*	SCC*mec*	*fudoh*
NCTC8325⁻	MSSA	-	-	-	-	-	-
RN4220⁻ pRN6662	MSSA	+	restored	+	-	-	-
RN4220⁻ pALC2073RNAIII	MSSA	+	restored	+	-	-	-
SH1000⁻	MSSA	-	-	-	+	-	-
SH1000⁻ pRN6662	MSSA	+	restored	+	+	-	-
NCTC8325-4 *agr*⁻	MSSA	-	-	-	-	-	-
HG001-	MSSA	-	-	-	+	-	-
Newman Δ*agr*	MSSA	-	-	-	+	-	-
UMCG-M4 I	MRSA	-	-	-	unknown	V(5C2&5)	-

Chapter 2

Spontaneous *agr* mutations are known to arise preferentially in the coding region of *agrC*, or the intergenic region between *agrC* and *agrA*, thereby preventing the translation of *agrA* (1,51). This implies that *agr* defects can be complemented at least partially by ectopic expression of RNAIII, and in some cases also by ectopic *agrA* expression (1,71). Accordingly, we performed a complementation analysis to verify the requirement of an intact *agr* system for colony spreading using the *agrA* mutant *S. aureus* strain RN4220 and the spontaneous *agr* mutant SH1000⁻. Both strains were complemented with the pALC2073RNAIII plasmid for RNAIII expression, or pRN6662 for expression of an intact *agrA* gene (39). As shown in Figure 2A, both complemented strains carrying pALC2073RNAIII or pRN6662 were able to spread. It should be noticed however that the spreading ability of the *agr*-complemented strains RN4220 and SH1000⁻ is less pronounced than that of known *agr*⁺ strains (Fig. 1). Likewise, the haemolytic activity of the complemented strains was also partially restored (Fig. 2B). Taken together, these results show unambiguously that an intact *agr* system is required for colony spreading of *S. aureus* (Table 3).

In contrast to *agr*, we observed no obvious correlations between colony spreading and *rsbU* mutations or the presence of SCC*mec* elements (Fig. 1). The lack of detectable effects of *rsbU* mutations means that reduced levels of the accessory sigma factor B (σ^B) do not have a strong impact on spreading (22,60). More noticeable was the observation that the *S. aureus* MRSA252 strain, which carries the SCC*mec* type II with a *fudoh* gene, did spread on soft agar plates (Fig. 1). This was an unexpected finding as Kaito *et al.* (26) have reported that the presence of the *fudoh* gene in SCC*mec* type II would suppress the colony spreading phenotype in MRSA strains (25). In line with this idea, the tested strains N315 and Mu50 that carry the *fudoh* gene within their type II SCC*mec* were indeed unable to spread (Fig. 1). However, the strains N315 and Mu50 are *agr*⁻, whereas MRSA252 is *agr*⁺, which suggests that *agr* is more generally important for spreading than the *fudoh* gene. The latter view is consistent with our finding that *agr* is a key determinant for spreading in MSSA strains (Table 2).

Requirement of the *agr* locus for colony spreading of *Staphylococcus aureus*
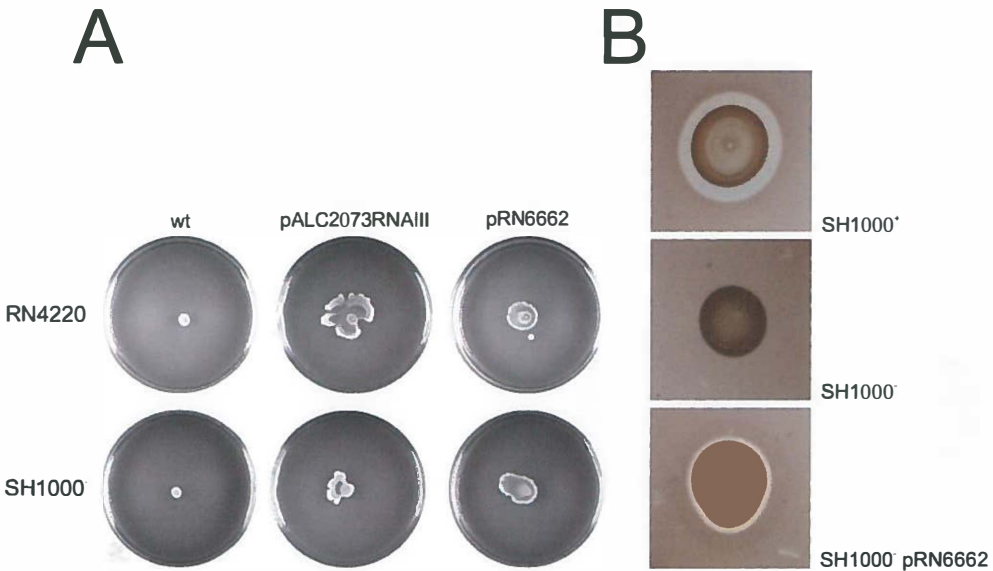
Figure 2. Involvement of the *S. aureus agr* system and phenol-soluble modulins in colony spreading. To complement the *agr* mutations in *S. aureus* strains RN4220 or SH1000⁻, the respective cells were transformed either with plasmid pALC2073RNAIII for RNAIII production or with plasmid pRN6662 for AgrA production. (**A**) Colony spreading by complemented *agr* mutant strains of *S. aureus*. (**B**) Haemolysis by *agr*⁺ (SH1000⁺), *agr*⁻ (SH1000⁻) and *agr*-complemented (SH1000⁻ pRN6662) variants of *S. aureus* SH1000. Note that haemolysis was partially restored in the *S. aureus agr*⁻ strain upon introduction of pRN6662 encoding *agrA*.

The *agr*⁻ phenotype of *S. aureus* has been connected with the enhancement of biofilm formation *in vitro* and *in vivo*, where *agr* mutants form a thicker biofilm than *agr*⁺ strains (27,55-57,61,62). Indeed, all tested *agr*⁻ strains were stronger biofilm formers than their *agr*⁺ counterparts, and this was even true for the *agrA*-complemented RN4220 strain (data not shown). Furthermore, it was reported that upregulation of RNAIII was associated with escape of *S. aureus* from biofilms grown in flow cells (61). These findings suggest that the *agr* system plays a decisive role in the choice of *S. aureus* cells between a sessile and a non-sessile lifestyle. Specifically, Boles *et al*. showed that *agr*⁻ cells have the ability to attach to surfaces and form biofilm due to the low expression levels of detachment factors such as hydrolases, proteases and surfactants (5). Surfactants are especially interesting in this respect, because surfactant molecules lower the surface tension of surface-air interfaces thereby improving 'surface wettability', which allows liquids to spread on hydrophobic surfaces. This property of surfactants could be directly connected to colony spreading since surface motility of bacteria is dependent on moist conditions (16). Notably, the δ-haemolysin (PSMγ) encoded by RNAIII has strong surfactant properties (17,56). This would directly link RNAIII production to colony spreading. Additionally, all other PSMs of *S aureus* have surfactant properties and they are tightly controlled by the *agr* system (11,45). To test the possible involvement of such compounds, we tested colony spreading for a

mutant of *S. aureus* strain LAC USA300 lacking the *psm*-α operon (kindly provided by M. Otto). Indeed, the spreading ability of this mutant was strongly reduced compared to the parental LAC USA300 strain, and spreading of the mutant strain was restored by addition of the four chemically synthesized PSMα peptides (Fig. 3). This directly shows the involvement of the PSMα peptides in colony spreading. Importantly, these PSMs have been implicated in leukocyte killing, which suggests a potential connection between spreading motility and staphylococcal virulence (58).
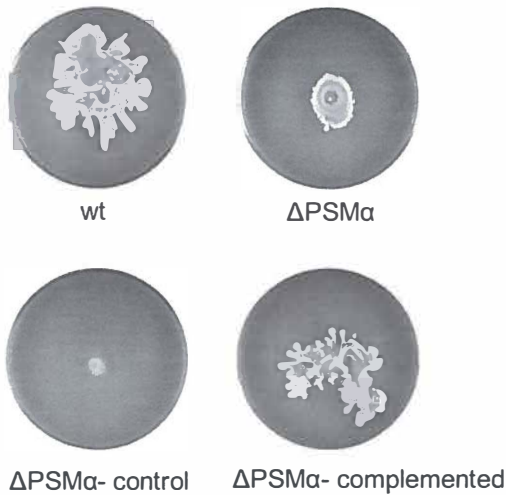
Figure 3. Phenol-soluble modulins α promote colony spreading. The role of PSMs in colony spreading was tested with the *S. aureus* LAC USA300 ΔPSMα strain, which lacks all four PSMα peptides. As a control, the parental strain *S. aureus* LAC USA300 (marked wt) was used. To verify that the spreading defect of *S. aureus* LAC USA300 ΔPSMα was due to the absence of the PSMα peptides, spreading was tested upon addition of a mixture of the four chemically synthesized PSMα peptides to the cells. PSMα1-4 with a C-terminal four-residue glycine spacer and an ε-amino biotinyl lysine were synthesized by standard Fmoc solid-phase peptide synthesis. The crude peptides were purified by reversed-phase high-performance liquid chromatography and the molecular masses of the peptides were confirmed by electro-spray ionization mass spectrometry. The purified PSMα1-4 peptides were then dissolved to a final concentration of 15 mM in dimethyl sulfoxide (DMSO) and 10 mM dithiothreitol (DTT). For the present experiment, the α1, α2, α3 and α4 peptides were mixed in a 4.4:1:1.5:2.5 ratio, which is in accordance with the ratios of these peptides encountered in the medium of *S. aureus* USA300 (30). Next, the mixture was 10-fold diluted in PBS. 20 μl of an overnight culture of the ΔPSMα mutant strain were centrifuged and subsequently resuspended in 20 μl of the diluted peptide mixture (ΔPSMα-complemented). As a control, ΔPSMα cells were resuspended in 20 μl of PBS with a 10-fold dilution of 10 mM DTT in DMSO without the PSMα1-4 peptides (ΔPSMα-control). 2 μl of the resuspended cells were added on soft agar plates to test colony spreading. All experiments were performed in duplicate and repeated three times.

Requirement of the *agr* locus for colony spreading of *Staphylococcus aureus*

General conclusion and outlook

Taken together, our present findings show that an intact *agr* system is required for the movement of *S. aureus* over surface-air interfaces by colony spreading. A key question that remains to be answered in future studies is whether *agr*-dependent 'colony spreading' plays a role during *S. aureus* infections. Clearly, many examples of the co-existence of *agr*+ and *agr*- variants of *S. aureus in vivo* have been reported (48,52,57). This might suggest a division of tasks within an infecting *S. aureus* population, where non-sessile *agr*+ cells would have a relatively high potential for spreading over surfaces and invasive growth, while sessile *agr*- cells would have a relatively high potential for colonisation possibly through the formation of biofilms.

Chapter 2

Reference

1. **Adhikari, R. P., S. Arvidson, and R. P. Novick**. 2007. A nonsense mutation in *agrA* accounts for the defect in *agr* expression and the avirulence of *Staphylococcus aureus* 8325-4 *traP::kan*. Infect.Immun. **75**:4534-4540.

2. **Arvidson, S. and K. Tegmark**. 2001. Regulation of virulence determinants in *Staphylococcus aureus*. Int.J.Med.Microbiol. **291**:159-170.

3. **Baba, T., F. Takeuchi, M. Kuroda, H. Yuzawa, K. Aoki, A. Oguchi, Y. Nagai, N. Iwama, K. Asano, T. Naimi, H. Kuroda, L. Cui, K. Yamamoto, and K. Hiramatsu**. 2002. Genome and virulence determinants of high virulence community-acquired MRSA. Lancet **359**:1819-1827.

4. **Björklind, A. and S. Arvidson**. 1980. Mutants of *Staphylococcus aureus* affected in the regulation of exoprotein synthesis. FEMS Microbiol.Lett. **7**:202-206.

5. **Boles, B. R. and A. R. Horswill**. 2008. *agr*-mediated dispersal of *Staphylococcus aureus* biofilms. PLoS.Pathog. **4**:e1000052.

6. **Chan, P. F. and S. J. Foster**. 1998. The role of environmental factors in the regulation of virulence-determinant expression in *Staphylococcus aureus* 8325-4. Microbiology **144 (Pt 9)**:2469-2479.

7. **Chlebowicz, M. A., K. Nganou, S. Kozytska, J. P. Arends, S. Engelmann, H. Grundmann, K. Ohlsen, J. M. van Dijl, and G. Buist**. 2010. Recombination between *ccrC* genes in a type V (5C2&5) staphylococcal cassette chromosome *mec* (SCC*mec*) of *Staphylococcus aureus* ST398 leads to conversion from methicillin resistance to methicillin susceptibility *in vivo*. Antimicrob.Agents Chemother. **54**:783-791.

8. **Chongtrakool, P., T. Ito, X. X. Ma, Y. Kondo, S. Trakulsomboon, C. Tiensasitorn, M. Jamklang, T. Chavalit, J. H. Song, and K. Hiramatsu**. 2006. Staphylococcal cassette chromosome *mec* (SCC*mec*) typing of methicillin-resistant *Staphylococcus aureus* strains isolated in 11 Asian countries: a proposal for a new nomenclature for SCC*mec* elements. Antimicrob.Agents Chemother. **50**:1001-1012.

9. **Deurenberg, R. H. and E. E. Stobberingh**. 2008. The evolution of *Staphylococcus aureus*. Infect.Genet.Evol. **8**:747-763.

10. **Diep, B. A., S. R. Gill, R. F. Chang, T. H. Phan, J. H. Chen, M. G. Davidson, F. Lin, J. Lin, H. A. Carleton, E. F. Mongodin, G. F. Sensabaugh, and F. Perdreau-Remington**. 2006. Complete genome sequence of USA300, an epidemic clone of community-acquired meticillin-resistant *Staphylococcus aureus*. Lancet **367**:731-739.

11. **Dunman, P. M., E. Murphy, S. Haney, D. Palacios, G. Tucker-Kellogg, S. Wu, E. L. Brown, R. J. Zagursky, D. Shlaes, and S. J. Projan**. 2001. Transcription profiling-based identification of *Staphylococcus aureus* genes regulated by the *agr* and/or *sarA* loci. J.Bacteriol. **183**:7341-7353.

12. **Duthie, E. S. and L. L. Lorenz**. 1952. Staphylococcal coagulase; mode of action and antigenicity. J.Gen.Microbiol. **6**:95-107.

13. **ELEK, S. D. and E. LEVY**. 1950. Distribution of haemolysins in pathogenic and non-pathogenic staphylococci. J.Pathol.Bacteriol. **62**:541-554.

14. **George, E. A. and T. W. Muir**. 2007. Molecular mechanisms of *agr* quorum sensing in virulent staphylococci. Chembiochem. **8**:847-855.

15. **Gill, S. R., D. E. Fouts, G. L. Archer, E. F. Mongodin, R. T. Deboy, J. Ravel, I. T. Paulsen, J. F. Kolonay, L. Brinkac, M. Beanan, R. J. Dodson, S. C. Daugherty, R. Madupu, S. V. Angiuoli, A. S. Durkin, D. H. Haft, J. Vamathevan, H. Khouri, T. Utterback, C. Lee, G. Dimitrov, L. Jiang, H. Qin, J. Weidman, K. Tran, K. Kang, I. R. Hance, K. E. Nelson, and C. M. Fraser**. 2005. Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant *Staphylococcus aureus* strain and a biofilm-producing methicillin-resistant *Staphylococcus epidermidis* strain. J.Bacteriol. **187**:2426-2438.

16. **Harshey, R. M**. 2003. Bacterial motility on a surface: many ways to a common goal. Annu.Rev.Microbiol. **57**:249-273.

17. **Heatley, N. G**. 1971. A new method for the preparation and some properties of staphylococcal d-haemolysin. J.Gen.Microbiol. **69**:269-278.

18. **Henrichsen, J**. 1972. Bacterial surface translocation: a survey and a classification. Bacteriol.Rev. **36**:478-503.

Requirement of the *agr* locus for colony spreading of *Staphylococcus aureus*

19. **Herbert, S., A. K. Ziebandt, K. Ohlsen, T. Schäfer, M. Hecker, D. Albrecht, R. Novick, and F. Götz.** 2010. Repair of global regulators in *Staphylococcus aureus* 8325 and comparative analysis with other clinical isolates. Infect.Immun. **78**:2877-2889.

20. **Herron, L. L., R. Chakravarty, C. Dwan, J. R. Fitzgerald, J. M. Musser, E. Retzel, and V. Kapur.** 2002. Genome sequence survey identifies unique sequences and key virulence genes with unusual rates of amino Acid substitution in bovine *Staphylococcus aureus*. Infect.Immun. **70**:3978-3981.

21. **Holden, M. T., E. J. Feil, J. A. Lindsay, S. J. Peacock, N. P. Day, M. C. Enright, T. J. Foster, C. E. Moore, L. Hurst, R. Atkin, A. Barron, N. Bason, S. D. Bentley, C. Chillingworth, T. Chillingworth, C. Churcher, L. Clark, C. Corton, A. Cronin, J. Doggett, L. Dowd, T. Feltwell, Z. Hance, B. Harris, H. Hauser, S. Holroyd, K. Jagels, K. D. James, N. Lennard, A. Line, R. Mayes, S. Moule, K. Mungall, D. Ormond, M. A. Quail, E. Rabbinowitsch, K. Rutherford, M. Sanders, S. Sharp, M. Simmonds, K. Stevens, S. Whitehead, B. G. Barrell, B. G. Spratt, and J. Parkhill.** 2004. Complete genomes of two clinical *Staphylococcus aureus* strains: evidence for the rapid evolution of virulence and drug resistance. Proc.Natl.Acad.Sci U.S.A **101**:9786-9791.

22. **Horsburgh, M. J., J. L. Aish, I. J. White, L. Shaw, J. K. Lithgow, and S. J. Foster.** 2002. s^B modulates virulence determinant expression and stress resistance: characterization of a functional *rsbU* strain derived from *Staphylococcus aureus* 8325-4. J.Bacteriol. **184**:5457-5467.

23. **International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC).** 2009. Classification of staphylococcal cassette chromosome *mec* (SCC*mec*): guidelines for reporting novel SCC*mec* elements. Antimicrob.Agents Chemother. **53**:4961-4967.

24. **Iordanescu, S. and M. Surdeanu.** 1976. Two restriction and modification systems in *Staphylococcus aureus* NCTC8325. J.Gen.Microbiol. **96**:277-281.

25. **Kaito, C., Y. Omae, Y. Matsumoto, M. Nagata, H. Yamaguchi, T. Aoto, T. Ito, K. Hiramatsu, and K. Sekimizu.** 2008. A novel gene, *fudoh*, in the SCCmec region suppresses the colony spreading ability and virulence of *Staphylococcus aureus*. PLoS.ONE. **3**:e3921.

26. **Kaito, C. and K. Sekimizu.** 2007. Colony spreading in *Staphylococcus aureus*. J.Bacteriol. **189**:2553-2557.

27. **Kong, K. F., C. Vuong, and M. Otto.** 2006. *Staphylococcus* quorum sensing in biofilm formation and infection. Int.J.Med.Microbiol. **296**:133-139.

28. **Kreiswirth, B. N., S. Löfdahl, M. J. Betley, M. O'Reilly, P. M. Schlievert, M. S. Bergdoll, and R. P. Novick.** 1983. The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. Nature **305**:709-712.

29. **Kuroda, M., T. Ohta, I. Uchiyama, T. Baba, H. Yuzawa, I. Kobayashi, L. Cui, A. Oguchi, K. Aoki, Y. Nagai, J. Lian, T. Ito, M. Kanamori, H. Matsumaru, A. Maruyama, H. Murakami, A. Hosoyama, Y. Mizutani-Ui, N. K. Takahashi, T. Sawano, R. Inoue, C. Kaito, K. Sekimizu, H. Hirakawa, S. Kuhara, S. Goto, J. Yabuzaki, M. Kanehisa, A. Yamashita, K. Oshima, K. Furuya, C. Yoshino, T. Shiba, M. Hattori, N. Ogasawara, H. Hayashi, and K. Hiramatsu.** 2001. Whole genome sequencing of meticillin-resistant *Staphylococcus aureus*. Lancet **357**:1225-1240.

30. **Li, M., B. A. Diep, A. E. Villaruz, K. R. Braughton, X. Jiang, F. R. DeLeo, H. F. Chambers, Y. Lu, and M. Otto.** 2009. Evolution of virulence in epidemic community-associated methicillin-resistant *Staphylococcus aureus*. Proc.Natl.Acad.Sci.U.S.A **106**:5883-5888.

31. **Lowy, F. D.** 1998. *Staphylococcus aureus* infections. N.Engl.J.Med. **339**:520-532.

32. **McNamara, P. J. and J. J. Iandolo.** 1998. Genetic instability of the global regulator *agr* explains the phenotype of the *xpr* mutation in *Staphylococcus aureus* KSI9051. J.Bacteriol. **180**:2609-2615.

33. **Morfeldt, E., L. Janzon, S. Arvidson, and S. Löfdahl.** 1988. Cloning of a chromosomal locus (*exp*) which regulates the expression of several exoprotein genes in *Staphylococcus aureus*. Mol.Gen.Genet. **211**:435-440.

34. **Nesin, M., P. Svec, J. R. Lupski, G. N. Godson, B. Kreiswirth, J. Kornblum, and S. J. Projan.** 1990. Cloning and nucleotide sequence of a chromosomally encoded tetracycline resistance determinant, *tetA*(M), from a pathogenic, methicillin-resistant strain of *Staphylococcus aureus*. Antimicrob.Agents Chemother. **34**:2273-2276.

Chapter 2

35. **Novick, R.** 1967. Properties of a cryptic high-frequency transducing phage in *Staphylococcus aureus*. Virology **33**:155-166.

36. **Novick, R. P.** 1990. The staphylococcus as a molecular genetic system, p. 1-37. *In*: R. P. Novick (ed.), Molecular Biology of the Staphylococci. VCH Publishers, New York.

37. **Novick, R. P.** 2003. Autoinduction and signal transduction in the regulation of staphylococcal virulence. Mol.Microbiol. **48**:1429-1449.

38. **Novick, R. P. and E. Geisinger**. 2008. Quorum sensing in staphylococci. Annu.Rev.Genet. **42**:541-564.

39. **Novick, R. P., S. J. Projan, J. Kornblum, H. F. Ross, G. Ji, B. Kreiswirth, F. Vandenesch, and S. Moghazeh**. 1995. The *agr* P2 operon: an autocatalytic sensory transduction system in *Staphylococcus aureus*. Mol.Gen.Genet. **248**:446-458.

40. **Novick, R. P., H. F. Ross, S. J. Projan, J. Kornblum, B. Kreiswirth, and S. Moghazeh**. 1993. Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. EMBO J. **12**:3967-3975.

41. **Peacock, S. J., I. de Silva, and F. D. Lowy**. 2001. What determines nasal carriage of *Staphylococcus aureus*? Trends Microbiol. **9**:605-610.

42. **Peng, H. L., R. P. Novick, B. Kreiswirth, J. Kornblum, and P. Schlievert**. 1988. Cloning, characterization, and sequencing of an accessory gene regulator (*agr*) in *Staphylococcus aureus*. J.Bacteriol. **170**:4365-4372.

43. **Pohl, K., P. Francois, L. Stenz, F. Schlink, T. Geiger, S. Herbert, C. Goerke, J. Schrenzel, and C. Wolz**. 2009. CodY in *Staphylococcus aureus*: a regulatory link between metabolism and virulence gene expression. J.Bacteriol. **191**:2953-2963.

44. **Projan, S. J., S. Brown-Skrobot, P. M. Schlievert, F. Vandenesch, and R. P. Novick**. 1994. Glycerol monolaurate inhibits the production of b-lactamase, toxic shock toxin-1, and other staphylococcal exoproteins by interfering with signal transduction. J.Bacteriol. **176**:4204-4209.

45. **Queck, S. Y., B. A. Khan, R. Wang, T. H. Bach, D. Kretschmer, L. Chen, B. N. Kreiswirth, A. Peschel, F. R. DeLeo, and M. Otto**. 2009. Mobile genetic element-encoded cytolysin connects virulence to methicillin resistance in MRSA. PLoS.Pathog. **5**:e1000533.

46. **Recsei, P., B. Kreiswirth, M. O'Reilly, P. Schlievert, A. Gruss, and R. P. Novick**. 1986. Regulation of exoprotein gene expression in *Staphylococcus aureus* by agar. Mol.Gen.Genet. **202**:58-61.

47. **Shafer, W. M. and J. J. Iandolo**. 1979. Genetics of staphylococcal enterotoxin B in methicillin-resistant isolates of *Staphylococcus aureus*. Infect.Immun. **25**:902-911.

48. **Shopsin, B., A. Drlica-Wagner, B. Mathema, R. P. Adhikari, B. N. Kreiswirth, and R. P. Novick**. 2008. Prevalence of *agr* dysfunction among colonizing *Staphylococcus aureus* strains. J.Infect.Dis. **198**:1171-1174.

49. **Sibbald, M. J., A. K. Ziebandt, S. Engelmann, M. Hecker, A. de Jong, H. J. Harmsen, G. C. Raangs, I. Stokroos, J. P. Arends, J. Y. Dubois, and J. M. van Dijl**. 2006. Mapping the pathways to staphylococcal pathogenesis by comparative secretomics. Microbiol.Mol.Biol.Rev. **70**:755-788.

50. **Somerville, G. A., S. B. Beres, J. R. Fitzgerald, F. R. DeLeo, R. L. Cole, J. S. Hoff, and J. M. Musser**. 2002. *In vitro* serial passage of *Staphylococcus aureus*: changes in physiology, virulence factor production, and *agr* nucleotide sequence. J.Bacteriol. **184**:1430-1437.

51. **Traber, K. and R. Novick**. 2006. A slipped-mispairing mutation in AgrA of laboratory strains and clinical isolates results in delayed activation of *agr* and failure to translate d- and a-haemolysins. Mol.Microbiol. **59**:1519-1530.

52. **Traber, K. E., E. Lee, S. Benson, R. Corrigan, M. Cantera, B. Shopsin, and R. P. Novick**. 2008. *agr* function in clinical *Staphylococcus aureus* isolates. Microbiology **154**:2265-2274.

53. **Valle, J., A. Toledo-Arana, C. Berasain, J. M. Ghigo, B. Amorena, J. R. Penadés, and I. Lasa**. 2003. SarA and not s^B is essential for biofilm development by *Staphylococcus aureus*. Mol.Microbiol. **48**:1075-1087.

54. **Vergara-Irigaray, M., J. Valle, N. Merino, C. Latasa, B. García, L. M. Ruiz de, I, C. Solano, A. Toledo-Arana, J. R. Penadés, and I. Lasa**. 2009. Relevant role of fibronectin-binding proteins in *Staphylococcus aureus* biofilm-associated foreign-body infections. Infect.Immun. **77**:3978-3991.

Requirement of the *agr* locus for colony spreading of *Staphylococcus aureus*

55. **Vuong, C., M. Durr, A. B. Carmody, A. Peschel, S. J. Klebanoff, and M. Otto**. 2004. Regulated expression of pathogen-associated molecular pattern molecules in *Staphylococcus epidermidis*: quorum-sensing determines pro-inflammatory capacity and production of phenol-soluble modulins. Cell Microbiol. **6**:753-759.

56. **Vuong, C., S. Kocianova, Y. Yao, A. B. Carmody, and M. Otto**. 2004. Increased colonization of indwelling medical devices by quorum-sensing mutants of *Staphylococcus epidermidis in vivo*. J.Infect.Dis. **190**:1498-1505.

57. **Vuong, C., H. L. Saenz, F. Gotz, and M. Otto**. 2000. Impact of the *agr* quorum-sensing system on adherence to polystyrene in *Staphylococcus aureus*. J.Infect.Dis. **182**:1688-1693.

58. **Wang, R., K. R. Braughton, D. Kretschmer, T. H. Bach, S. Y. Queck, M. Li, A. D. Kennedy, D. W. Dorward, S. J. Klebanoff, A. Peschel, F. R. DeLeo, and M. Otto**. 2007. Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. Nat.Med. **13**:1510-1514.

59. **Wolz, C., D. McDevitt, T. J. Foster, and A. L. Cheung**. 1996. Influence of agr on fibrinogen binding in *Staphylococcus aureus* Newman. Infect.Immun. **64**:3142-3147.

60. **Wu, S., L. H. de, and A. Tomasz**. 1996. Sigma-B, a putative operon encoding alternate sigma factor of *Staphylococcus aureus* RNA polymerase: molecular cloning and DNA sequencing. J.Bacteriol. **178**:6036-6042.

61. **Yao, Y., D. E. Sturdevant, and M. Otto**. 2005. Genomewide analysis of gene expression in *Staphylococcus epidermidis* biofilms: insights into the pathophysiology of *S. epidermidis* biofilms and the role of phenol-soluble modulins in formation of biofilms. J.Infect.Dis. **191**:289-298.

62. **Yarwood, J. M., D. J. Bartels, E. M. Volper, and E. P. Greenberg**. 2004. Quorum sensing in *Staphylococcus aureus* biofilms. J.Bacteriol. **186**:1838-1850.

63. **Yarwood, J. M. and P. M. Schlievert**. 2003. Quorum sensing in *Staphylococcus* infections. J.Clin.Invest **112**:1620-1625.

64. **Ziebandt, A. K., D. Becher, K. Ohlsen, J. Hacker, M. Hecker, and S. Engelmann**. 2004. The influence of *agr* and s^B in growth phase dependent regulation of virulence factors in *Staphylococcus aureus*. Proteomics. **4**:3034-3047.

65. **Ziebandt, A. K., H. Kusch, M. Degner, S. Jaglitz, M. J. Sibbald, J. P. Arends, M. A. Chlebowicz, D. Albrecht, R. Pantucek, J. Doskar, W. Ziebuhr, B. M. Bröker, M. Hecker, J. M. van Dijl, and S. Engelmann**. 2010. Proteomics uncovers extreme heterogeneity in the *Staphylococcus aureus* exoproteome due to genomic plasticity and variant gene regulation. Proteomics. **10**:1634-1644.

"Ο κόσμος σκηνή, ο βίος πάροδος. Ήλθες, είδες, απήλθες"

"The world is a theater, life is a tragedy. You come, you see, you leave"

Dimokritos (ca. 470-361 BC, Abdera, Xanthi, Greece)

Chapter 3

Distinct roles of phenol-soluble modulins in spreading of *Staphylococcus aureus* on wet surfaces

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Chapter 3

Abstract

The human pathogen *Staphylococcus aureus* is renowned for the rapid colonization of wounds, the formation of biofilms on medical implants, and for causing food poisoning. Here we have investigated which secreted factors are used by *S. aureus* cells to facilitate their spreading over wet surfaces, starting either from planktonic or biofilm-associated states. Our present proteomics analyses pinpoint phenol-soluble modulins (PSMs) as prime candidate facilitators of the spreading process. By use of synthetic PSM peptides and different *psm* mutant strains, we have subsequently dissected the roles of particular PSMs in staphylococcal spreading and the inhibition of biofilm formation. Importantly, we show that PSM-mediated motility of *S. aureus* facilitates the rapid colonization of wet surfaces next to catheters and the colonization of fresh meat. These findings implicate spreading motility in catheter-associated infections and food spoilage.

Distinct roles of phenol-soluble modulins in spreading of *Staphylococcus aureus* on wet surfaces

Introduction

Staphylococcus aureus is an opportunistic human pathogen that can cause a wide range of acute and chronic diseases, which range from superficial skin infections to life-threatening endocarditis and sepsis (28, 42). The ability of this Gram-positive bacterium to cause these infections depends on the production of secreted and cell wall-associated virulence factors. Of increasing concern is the ability of *S. aureus* to acquire resistance against antibiotics, as underscored by the global spread of methicillin-resistant *S. aureus* (MRSA) lineages.

Intriguingly, recent proteomics studies have revealed an enormous diversity in the production of virulence factors by different isolates of *S. aureus*, and only a few of these seem to be invariantly produced (8, 9, 62). Amongst the most commonly identified staphylococcal virulence factors, especially in the community-associated (CA-)MRSA lineages, are the so-called phenol-soluble modulins (PSMs) (39). These PSMs are short, amphipathic, α-helical peptides that have leukocidal activity and biosurfactant properties (47, 48, 58). The growth media of *S. aureus* cultures contain both N-terminally formylated and deformylated PSMs, suggesting that these virulence factors are substrates for the bacterial N-formyl-methionine deformylase (53)(58).

To date, eight PSMs have been identified in *S. aureus*. These include the four PSMα1-4 peptides (22 residues each), the PSMβ1 and PSMβ2 peptides (44 residues each), PSMγ (25 residues) and the recently reported PSM-mec (22 residues). The PSMα peptides are encoded by the *psmα* operon, the PSMβ peptides by the *psmβ* operon, and PSMγ by the *hld* gene. Notably, the *hld* gene is embedded within the regulatory RNAIII molecule that is encoded by the *agr* locus. The gene for PSM-mec was identified in MRSA strains carrying the staphylococcal cassette chromosome *mec* (SCC*mec*) types II or III. The expression of all *psm* genes is controlled by the Agr system for quorum sensing (3, 46, 47, 48, 51). This system modulates gene expression such that cell wall-associated virulence factors (e.g. the immunoglobulin G-binding protein A) are most highly expressed at low cell densities, and that secreted virulence factors (e.g. the PSMs) are most highly expressed at high cell densities (11, 31, 35-37, 43, 49, 61).

Notably, PSMs have been implicated in the high virulence of CA-MRSA lineages, which are readily transmitted by direct contact with a carrier (32, 58). The investigated CA-MRSA isolates produce higher amounts of the PSM peptides than the generally less virulent nosocomial MRSA isolates (19, 58). The PSMα peptides have the strongest leukolytic, pro-inflammatory and chemotactic activities (58). Consistently, a strain lacking *psmα* had a decreased ability to cause skin lesions in mice and rabbits (26, 58). Further to this, Wang *et al.,* have shown that the mortality rates and the levels of the inflammatory cytokine TNF-α in the blood of mice infected with *psmα* or *psmγ* mutant strains were substantially reduced. The PSMβ peptides appear less important for cytolysis and inflammation but, in low concentrations, they seem to promote biofilm formation by *Staphylococcus epidermidis*. High amounts of the same PSMβ peptides do, however, promote the detachment of staphylococcal cells from biofilms both *in vitro* and *in vivo* (57).

45

Chapter 3

Although *S. aureus* was originally believed to be non-motile, recent studies have shown that this organism is capable of spreading over wet surfaces (14, 15, 55). We have previously shown that a mix of the four PSMα peptides can promote this so-called colony spreading phenotype (55), and we hypothesized that this relates to their strong surfactant properties (58). The Agr system is an important determinant for colony spreading due to its control over the synthesis of PSMs (55). However, Agr is also needed for biofilm formation, which gives it a decisive role in the choice between motile and sessile lifestyles of *S. aureus*. To date, very little was known about the roles of individual PSM peptides in colony spreading, and how this relates to their impact on biofilm formation. Therefore, the present studies were aimed at dissecting the roles of the different PSM peptides in these processes. Furthermore, we wanted to test whether N-terminally formylated and deformylated PSMs are equally potent in colony spreading and biofilm dispersal. To achieve these objectives we constructed multiple *psm* mutant strains, which were then incubated in the presence or absence of synthetic PSMs. Importantly, our results show that PSMα3 and PSMγ have key roles in spreading, and that PSMα3 and PSMβ1 are the most potent in biofilm dispersal. Furthermore, our observations link PSMα3 and PSMγ to the spreading of staphylococcal cells from catheter-related biofilms and they suggest that PSM-mediated spreading plays a major role in the movement of *S. aureus* over biotic surfaces.

Experimental Procedures

Bacterial strains and growth conditions

The bacterial strains and plasmids that were used in this study are listed in Table 1. *Escherichia coli* strains were grown in Luria-Bertani broth (LB) at 37°C under vigorous shaking. *S. aureus* strains were grown in Tryptic Soy Broth (TSB) at 37°C under vigorous shaking, or on tryptic soy agar (TSA) plates. Where necessary, antibiotics were added in the following concentrations: ampicillin (Ap), 100 μg/ml (for *E. coli*); erythromycin (Em), 5 μg/ml (for *S. aureus*); chloramphenicol (Cm), 10 μg/ml (for *S. aureus*).

Colony spreading assay

The colony spreading assay was performed essentially as described by Kaito *et al.*, (14), but with minor previously described modifications (55). To detect colony spreading of the *S. aureus* strains SH1000 and Newman along the growth curve, these strains were grown in TSB for 24 hours. Samples were collected at hourly intervals for the first 7 hours and after 24 hours of growth. All samples were immediately tested for colony spreading. Equal amounts of cells from each time point were spotted on the 0.24% TSA plates. All spreading assays were repeated at least five times. For the complementation of spreading with the surfactin from *B. subtilis*, 2 μl of a 10 μg/ml stock solution was spotted in the center of the TSA plates prior to the inoculation of *S. aureus* mutant strains. Pure *B. subtilis* surfactin was purchased from Sigma-Aldrich.

Distinct roles of phenol-soluble modulins in spreading of *Staphylococcus aureus* on wet surfaces

Table 1. Bacterial strains and plasmids

Strains	Description[a]	Reference
E. coli DH5α	λ φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA1 endA hsdR17*(ʳk ᵐk⁻)*supE44 thi-1 gyrA relA1*	(12)
E. coli TOP10	Cloning host for TOPO vector; F⁻ *mcr*A Δ(*mrr-hsdRMS-mcrBC*) λ⁻ φ80d*lacZ*ΔM15 Δ*lacX74 recA1 araD139* Δ(*ara-leu*)7697 *galU galK rpsL* (Strʳ) *endA1 nupG*	Invitrogen Life Technologies
RN4220	Restriction-deficient derivative of NCTC 8325, cured of all known prophages	(22)
NCTC8325	HA-MSSA strain, *agr⁺*, *rsbU⁻*	(34)
NCTC8325	HA-MSSA strain, *agr⁻*, *rsbU⁻*	(55)
NCTC8325 *Δpsmα*	NCTC8325 lacking the *psmα* genes	This work
NCTC8325 *Δpsmβ*	NCTC8325 lacking the *psmβ* genes	This work
NCTC8325 *Δpsmα Δpsmβ*	NCTC8325 lacking the *psmα* and *psmβ* genes	This work
SH1000	NCTC8325-4 derivative, *rsbU⁺*, *agr⁺*	(13)
SH1000	NCTC8325-4 derivative, *rsbU⁺*, *agr⁻*	(55)
SH1000 *Δpsmα*	SH1000 lacking the *psmα* genes	This work
SH1000 *Δpsmβ*	SH1000 lacking the *psmβ* genes	This work
SH1000 *Δpsmα Δpsmβ*	SH1000 lacking the *psmα* and *psmβ* genes	This work
HG001	NCTC8325 derivative, *rsbU⁺*, *agr⁺*	(45)
HG001	NCTC8325 derivative, *rsbU⁺*, *agr⁻*	(55)
HG001 *Δpsmα*	HG001 lacking the *psmα* genes	This work
HG001 *Δpsmβ*	HG001 lacking the *psmβ* genes	This work
HG001 *Δpsmα Δpsmβ*	HG001 lacking the *psmα* and *psmβ* genes	This work
Newman	ATCC25904; high-level clumping factor production; σ^{B+}	(10)
Newman Δ*agr*	Newman derivative, Δ*agr::tetM*	(59)
Newman *Δpsmα*	Newman lacking the *psmα* genes	This work
Newman *Δpsmβ*	Newman lacking the *psmβ* genes	This work
Newman *Δpsmα Δpsmβ*	Newman lacking the *psmα* and *psmβ* genes	This work
LAC USA300	CA-MRSA	(58)
LAC USA300 *Δpsmα*	LAC USA300 lacking the *psmα* genes	(58)
Plasmids		
pUC18	Apʳ, ColE1, φ80d*lacZ*; *lac* promoter	(33)
TOPO	pCR-Blint II-TOPO vector; Kmʳ	Invitrogen Life Technologies
pMAD	*E. coli*/*S. aureus* shuttle vector that is temperature-sensitive in *S. aureus* and contains the *bgaB* gene; Emʳ Apʳ	(1)
pRIT5H	*E. coli*–*S. aureus* shuttle vector, Cmʳ, *spa* promoter	(23)
pSW4-GFPopt	pSW4 plasmid containing 729-bp *AseI/Bam*HI-cloned *gfpopt*	(50)
GFPopt-pRIT5H	pRIT5H with *gfpopt* gene; Apʳ, Kmʳ	This work
pAH9	*sarA* promoter P₁-RFP	(2)

[a] CA, community-acquired; HA, hospital-acquired; MRSA, methicillin-resistant *S. aureus*

Chapter 3

Mass spectrometric analyses of culture supernatants

Strains Newman (*agr$^+$*, *agr$^-$* or Δ*psmα*), LAC USA300 (*agr$^+$* or Δ*psmα*), NCTC8325 (*agr$^+$* or *agr$^-$*) and HG001 (*agr$^+$* or *agr$^-$*) were grown in TSB. At OD$_{600}$ of 2 and 6, 3 ml culture samples were collected, and cells were separated from the growth medium by centrifugation (8000 × g, 4°C, 10 min). To precipitate the secreted proteins, the medium fractions were incubated at 4°C with 10% tricholoroacetic acid overnight. The proteins were pelleted (20000 × g, 4°C, 20 min), washed with acetone and subsequently dissolved in 8 M urea. Protein concentrations were determined using the Bio-Rad *DC*^{TM} protein assay according to the protocol of the supplier. The samples (2 μg) were reduced with 10 mM DTT (Duchefa Biochemie) for 30 min and alkylated with 10 mM iodoacetamide (Sigma-Aldrich) for another 30 min in the dark. Finally, the protein samples were incubated overnight at 37°C with 40 ng of trypsin (Promega). Peptides were purified using ZipTips (9), separated and analyzed by LC-MS/MS using an Easy-nLCII HPLC system (Thermo Fisher Scientific, Waltham, MA) coupled directly to an LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA). The Easy-nLCII was equipped with a self-packed analytical column (C18-material (Luna 3u C18(2)100A, Phenomenex®), 100μm i.D. x 200 mm column). Peptide elution was performed by application of a binary gradient of buffer A (0.1% (v/v) acetic acid) and B (99.9% (v/v) acetonitrile, 0.1% (v/v) acetic acid) over a period of 80 min with a flow rate of 300 nl/min. The mass spectrometric analyses were performed as described by Miller *et al.* (30). A non-redundant database was constructed, that contained all available *S. aureus* protein sequences (uniprot) that differ in at least one amino acid residue (E. Tsompanidou, unpublished data available on request). This database was subsequently used for the data base search (including a concatenated reversed database, 40098 entries). The database search was performed with Sequest using a fragment ion mass tolerance of 1.00 Da and a parent ion tolerance of 10 ppm. Oxidation of methionine and carbamidomethylation of cysteine were specified in Sequest as variable modifications. Validation of MS/MS-based peptide and protein identifications was performed with Scaffold (version Scaffold 3.3.2, Proteome Software Inc., Portland, OR). Peptide identifications were accepted if they exceeded specific database search engine thresholds. Sequest identifications required at least deltaCn scores of greater than 0.10 and XCorr scores of greater than 1.9, 2.2, 3.8 and 3.8 for singly, doubly, triply and quadruply charged peptides. All experiments have been conducted in independent duplicates. Proteins were only accepted as being identified if they were detected in both biological replicates per sample set. With these filter parameters the false positive rate was below 1%.

Construction of a GFP expressing vector

For constitutive expression of GFP in *S. aureus* the *gfpopt* gene was amplified from plasmid pSW4-GFPopt using primers GFPoptFR and GFPoptRV (Table 2). The amplified PCR product was then cloned in plasmid pRIT5H using the *Eco*RI and *Sal*I restriction sites. Expression of GFP was detected using the IVIS Spectrum from Caliper Life Sciences using the specific filter for GFP (excitation: 465 nm; emission: 520nm).

Distinct roles of phenol-soluble modulins in spreading of *Staphylococcus aureus* on wet surfaces

Table 2. Primers used in these studies

Primer	Sequence (5'→3')[a]
psmα-Fl	ctgcataacctccttatttctaatc
psmα-Rl	**AATCGTCAGTCAGTCACCATGGCA**taagattacctcctttgcttatga
psmα-F2	**TGCCATGGTGACTGACTGACGTAA**taatttaagcgaattgaatacttaaa
psmα-R2	ggctaggacatgtatgtgtctta
psmβ-Fl	GGATCCgtaatcacggaactcttttgttt
psmβ-Rl	**AATCGTCAGTCAGTCACCATGGCA**tgaaaacactccttaaaatttaaatt
psmβ-F2	**TGCCATGGTGACTGACTGACGTAA**tataataactaatattctttaaaataaactggg
psmβ-R2	GGATCCtgatatcctgtttcttcagatataaaatatc
GFPoptFR	GAATTC*AAGGAGGA*AAAAACATATGTCAAAAGGAGAAGAATTAT
GFPoptRV	GTCGACttacttatataattcatccattcc

[a]The overlap in Rl and F2 primers used to merge flanking regions by PCR is shown in **boldface**. Restriction sites used for cloning are underlined. A ribosome-binding site is indicated in *italics*.

Construction of PSM mutant strains of S. aureus

Mutants of *S. aureus* were constructed using the temperature-sensitive plasmid pMAD (Arnoud et al., 2004) and previously described procedures (21). Primers were designed using the genome sequence of *S. aureus* NCTC8325 (http://www.ncbi.nlm.nih.gov/nuccore/NC007795). To delete the *psmα* and/or *psmβ* operons, the primer pairs with the designations F1/R1 and F2/R2 were used for PCR amplification of the respective upstream and downstream regions (each ~500 bp) (Table 2). The R1 and F2 primers contain a 24-bp linker sequence to fuse the flanking regions by PCR prior to cloning in pMAD. The resulting plasmids were used to transform *S. aureus* strain RN4220 via electroporation. Next, the plasmids were isolated from the RN4220 strain and used to transform the *S. aureus* SH1000, HG001, NCTC8325 or Newman strains via electroporation in order to delete their *psmα* and/or *psmβ* operons through subsequent plasmid integration and excision steps (52). At the end of the procedure, white colonies were screened for the absence of the *psmα* and/or *psmβ* genes by colony PCR using primers F1 and R2.

Complementation of psm mutations by synthetic PSM peptides

The PSMα1-4, PSMβ1-2, PSMγ and PSM-mec peptides were synthesized as described previously with a C-terminal 4-residue glycyl spacer and an ε-amino biotinyl lysine (55). All peptides were dissolved in DMSO plus 10 mM DTT to a concentration of 12 mM. The peptides were then diluted 10-fold in Phosphate-buffered saline (PBS), and 2 µl of each peptide solution was spotted in the center of a soft TSA plate prior to the inoculation of the *S. aureus* mutant strain to be tested for spreading.

Chapter 3

Colony spreading of S. aureus on catheters or pork

Catheters were positioned on the soft agar plates and 2 µl of bacteria grown overnight were spotted next to the catheter before the plates were incubated overnight at 37°C. Subsequently, the catheters were transferred to fresh soft TSA plates and incubated again overnight at 37°C. Catheters that were colonized with *agr⁻* strains were transferred to fresh soft TSA plates, where 2 µl of PSM peptide solution was spotted prior to the transfer of the catheters. The plates were then incubated overnight at 37°C. Images were recorded with a G:box (Syngene, Leusden, the Netherlands).

Pieces of pork meat were placed in sterile petri dishes and approximately in the center, 2 µl of bacteria grown overnight in TSB were spotted. The meat was incubated at 37°C for 24 h, after which images were recorded with a Sony cyber-shot camera.

Inhibition of biofilm formation by synthetic PSM peptides

Biofilm assays were performed based on the methods described by Christensen *et al.* (4). Bacteria were grown overnight at 37°C in TSB supplemented with 0.25% glucose (TSBg). The culture was diluted 1:200 in TSBg and 200 µl of this cell suspension was used to inoculate sterile 96-well polystyrene microtiter plates. Prior to the inoculation of bacteria, 2 µl of 1.2 mM PSM peptide solutions were used to pretreat the bottom of a well. After 24 h of growth at 37°C in standing conditions the plates were washed twice with 200 µl sterile PBS, air-dried in an inverted position and stained with 0.1% safranin for 30 s. Wells were rinsed again, and the absorbance was determined at 490 nm. Each strain was inoculated in triplicate and the assay was performed four times.

Results

Secreted factors regulated by agr are responsible for colony spreading

We have previously shown that the *agr* locus, which regulates the synthesis of secreted virulence factors, is required for colony spreading of *S. aureus*. This suggested that secreted virulence factors are of prime importance for colony spreading. Another indication for the role of secreted factors in spreading came from the observation that colonies of *S. aureus* cells that are capable of spreading were surrounded by a transparent halo (Fig. 1A). This halo was absent from non-spreading *agr* mutant colonies. Furthermore, when overnight grown *agr*-proficient bacteria were spotted on soft agar plates, spreading of the cultured cells was visible after a few seconds, suggesting that any secreted factors needed for spreading were already present in the culture. In fact, this area of rapid spreading remained clearly visible on the plates (marked by a box in Fig. 1B), which was due to the emergence of subsequent 'waves' of spreading cells from the initial spreading zone. This suggested that the factors needed for spreading were not synthesized continuously.

Distinct roles of phenol-soluble modulins in spreading of *Staphylococcus aureus* on wet surfaces
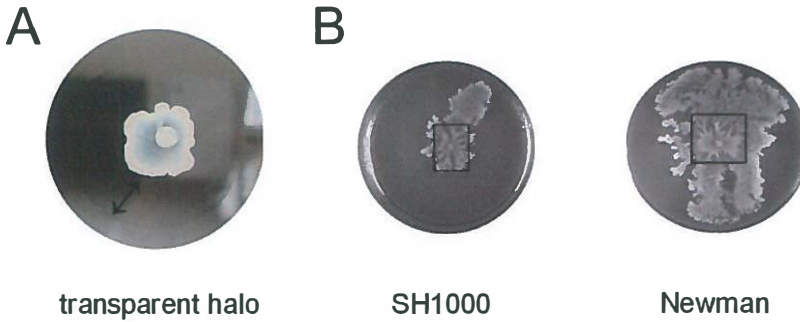

Figure 1. Characteristic features of *S. aureus* spreading motility. **A,** A Transparent 'halo' is produced by cells of spreading colonies. A similar halo is also generated by surfactants that are spotted on soft agar plates (not shown). **B,** Spreading motility involves a rapid phase of spreading by the cells spotted on a plate. Subsequently, waves of cells emerge from the cells in the initial spreading zone. The boxes mark the rapid spreading zones that emerged from the sites of inoculation of strains SH1000 and Newman on soft agar plates.

To investigate whether the spreading factors were synthesized growth phase-dependently, *S. aureus* cells were grown to different growth stages and tested for the rapid spreading phenotype. Indeed, the rapid spreading was not observed for cells in the early exponential growth phase (Fig. 2A, time point t3), but it started when the cells reached the late exponential phase (t5) and continued in the stationary phase (t7). Consistent with our previous findings, these time points correspond with the activation of the Agr system (36). To verify that secreted factors promote colony spreading, *agr*⁻ cells from different strains were resuspended in filtered supernatants of overnight grown *agr*⁺ strains and the cell suspension was spotted on soft agar plates. As shown in Figure 2B, the filtered supernatants were able to promote colony spreading of the *agr*⁻ cells. Together, these results demonstrate that secreted factors regulated via the Agr system are both needed and sufficient for colony spreading of *S. aureus*.

As the spent growth medium of *agr*⁺ cells can promote the spreading of *agr*⁻ cells, we wondered whether the *agr*⁻ cells would also spread if they were grown in the presence of *agr*⁺ cells. To test this, an overnight culture of an *agr*⁻ derivative of *S. aureus* HG001 expressing the green fluorescent protein (GFP) was mixed with an overnight culture of the authentic *S. aureus* HG001 strain (*agr*⁺) expressing the fluorescent mCherry protein. Next aliquots of the mixed culture were transferred to soft agar plates and incubated overnight. Images were taken using the IVIS Spectrum with the specific filters for GFP (excitation: 465 nm; emission: 520 nm) and mCherry (excitation: 570 nm; emission: 620 nm) to monitor the spreading of the two different strains. GFP was only detected at the edges of the colony spreading area, indicating that the *agr*⁻ strain was very well able to spread over the soft agar together with the *agr*⁺ strain (Fig. 2C). This implies that non-spreading *S. aureus* cells can spread on the soft agar plates with the help of secreted factors from the spreading *S. aureus* cells.

Chapter 3

Mass spectrometric identification of secreted proteins potentially involved in colony spreading

To investigate which proteinaceous factors are involved in colony spreading, culture supernatants of *agr*$^+$, *agr*$^-$ and *Δpsma* variants of the *S. aureus* strains Newman, LAC USA300, NCTC8325 and HG001 were analyzed using mass spectrometry (MS). As expected, the MS analyses revealed many strain-dependent differences between the investigated *S. aureus* strains (E. Tsompanidou, unpublished data available on request). Importantly, the only proteins that were common in the media of all tested *agr*$^+$ strains and absent from the media of all tested *agr*$^-$ strains were PSMα2, PSMα3, PSMα4, PSMβ1, PSMβ2 and the staphylococcal lipase 1. Only the identified PSM peptides have potential surfactant properties that might promote colony spreading, and therefore we focused all our following studies on these peptides.
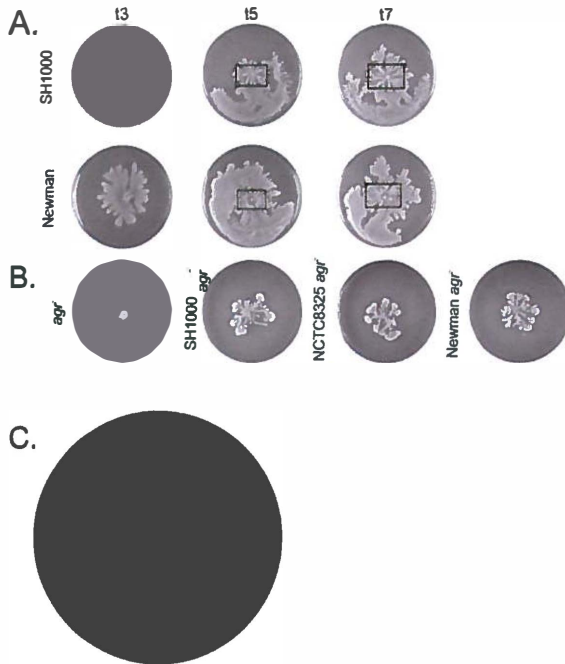
Figure 2. Colony spreading depends on the growth phase of inoculated *S. aureus* cells and on secreted factors produced by *agr*$^+$ strains. A, Colony spreading by planktonic cells of *S. aureus* strains SH1000 or Newman collected from cultures in different growth stages; t3 corresponds to the early exponential growth phase (OD$_{600}$ 1.5), t5 to the late exponential phase (OD$_{600}$ 6.8), and t7 to early stationary phase (OD$_{600}$ 9.0). **B,** Filter-sterilized culture medium of *agr*$^+$ cells of *S. aureus* strain Newman promotes rapid spreading of *agr*$^-$ cells of strains SH1000, NCTC8325 and Newman. As shown for *agr*$^-$ cells of strain SH1000, no spreading is observed when fresh medium is used (left plate labelled *agr*$^-$). **C,** *agr*$^-$ cells carrying plasmid GFPopt-pRIT5H for expression of GFP were co-inoculated with *agr*$^+$ cells carrying plasmid pAH9 for expression of mCherry. GFP fluorescence of the *agr*$^-$ cells is detectable at the edges of the spreading zone (green color), whereas mCherry fluorescence of the *agr*$^+$ cells (red color) is detectable in the entire area covered by spreading.

Distinct roles of phenol-soluble modulins in spreading of *Staphylococcus aureus* on wet surfaces

Dissection of PSM function with synthetic peptides reveals major roles for PSMα3 and PSMγ in colony spreading

Surfactants lower the surface tension of liquid-to-air interfaces, and due to this activity they facilitate the motility of many different bacterial species (17, 20, 27). To test whether a molecule with surfactant properties would indeed be sufficient for colony spreading of *S. aureus*, we made use of the lipopeptide surfactin that is produced by another Gram-positive bacterium named *Bacillus subtilis*. It should be noted that surfactin was previously shown to be essential for the swarming motility of *B. subtilis* (17). Indeed, the addition of surfactin to soft agar plates unambiguously restored the colony spreading by all the *agr⁻* strains tested, as exemplified in Figure 3 for an *agr* mutant of *S. aureus* SH1000 (plates in top row). Therefore, we also tested synthetic PSMα1-4, PSMβ1-2, PSMγ and PSM-mec peptides for their ability to promote colony spreading. To this end, all PSMs were individually spotted at the same concentrations on soft agar plates. This analyses showed that the PSMα3 and PSMγ peptides strongly promoted the colony spreading by otherwise non-spreading *agr⁻* strains. Other PSMs, such as PSMα1, PSMα2 and PSMβ1 promoted colony spreading to lesser extents, but PSMα4, PSM β2 and PSM-mec did not promote spreading at all (Fig. 3). Based on genetic studies, it was previously proposed that PSM-mec might inhibit colony spreading (16). We therefore tested the synthetic PSM-mec peptide for possible inhibitory effects, but unfortunately, no such effects were detectable in our experimental setting (Fig. 3, plates in bottom row). Furthermore, our studies show that N-terminally formylated PSMs promote spreading equally well as the non-formylated peptides (Fig. 3). It thus seems that both the formylated and non-formylated forms of PSMα3 and PSMγ contribute to the movement of *S. aureus* cells over wet surfaces.

Genetic dissection of PSM function reveals additive effects in colony spreading

As underscored by our proteomics analyses, *agr*-deficient strains are completely defective in the synthesis of PSMs. To identify the contributions of the *psmα* and *psmβ* operons to spreading, the respective single and double mutant strains were constructed. Next, the spreading ability of these mutants was compared to the parental strain and to the equivalent *agr* mutant, which is fully PSM-deficient.

To quantify spreading activity, all images of spreading assays were analyzed with ImageJ and the area covered by the cells was determined. Figure 4 shows that deletion of the *psmβ* operon had a moderate but significant effect on spreading. Deletion of the *psmα* operon had a more severe effect on spreading and the spreading activity of the *psmα psmβ* double mutant was even lower (Fig. 4). Notably, the spreading activity of the double mutant was still higher than that of the *agr* mutant, which suggests that the remaining activity of the double mutant is due to the production of PSMγ. This view is consistent with the above finding that the synthetic PSMγ is sufficient to restore spreading of *agr* mutant strains (Fig. 3). A comparison of the results obtained for the single and double *psmα psmβ* mutant variants of strains Newman, SH1000 and HG001 shows that the deletion of the *psmα* operon has the strongest negative impact on

spreading, which is fully consistent with the findings obtained with the synthetic peptides added at equimolar concentrations. Furthermore, our genetic dissection of the function of PSM-encoding loci shows that they contribute additively to colony spreading.
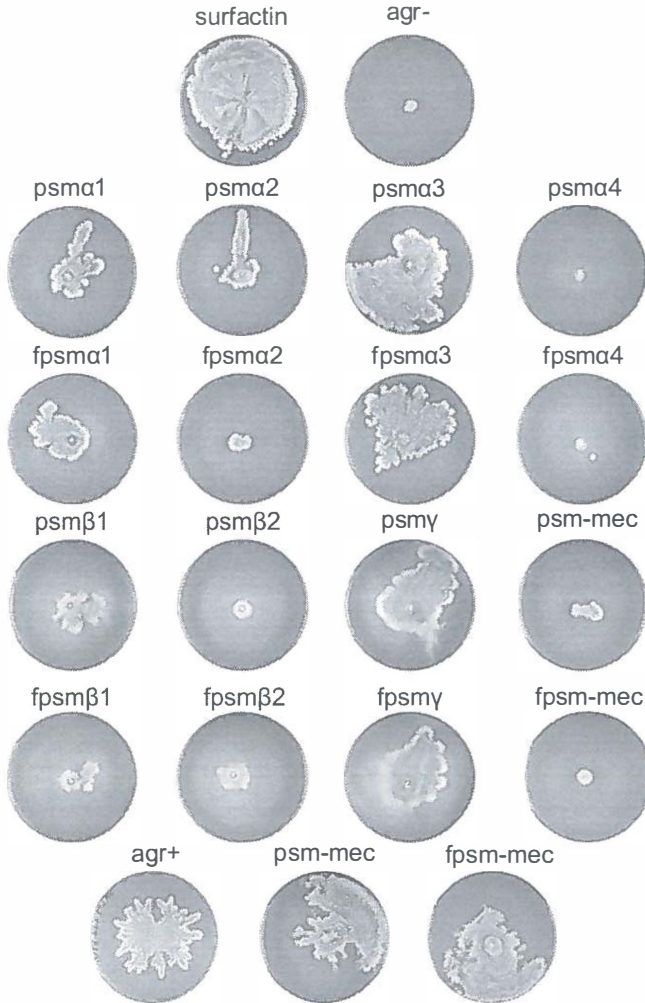
Figure 3. Particular synthetic PSM peptides facilitate spreading of *agr⁻* cells. To identify factors that facilitate spreading of *agr⁻* cells, the pure surfactin from *B. subtilis* (top row, left plate) or chemically synthesized PSMs from *S. aureus* (plate rows 2-5) were spotted in the centre of soft agar plates prior to the inoculation with cells of *S. aureus* SH1000 *agr⁻*. Both N-terminally formylated PSMs (marked with an 'f') and non-formylated peptides were used in the assay. Additionally, the PSM-mec peptides were tested for a potentially inhibitory role in spreading by cells of the *S. aureus* SH1000 *agr⁺* strain (bottom row).

Distinct roles of phenol-soluble modulins in spreading of *Staphylococcus aureus* on wet surfaces

Inhibition of biofilm formation by synthetic PSMs

The Agr system of *S. aureus* is known to contribute to biofilm dispersal (2) and in *S. epidermidis* PSMs were recently shown to contribute to this process (44). For these reasons, we verified the possible biofilm-antagonizing activity of our complete set of formylated and deformylated synthetic PSM peptides. When the wells of a 96-well polystyrene plate were pre-treated with synthetic PSM peptides, all PSMs except PSMα4 and PSM-mec had statistically significant biofilm-antagonizing properties irrespective of their formylation state as was shown for agr^+ cells of *S. aureus* SH1000 (Fig. 5). What stood out in these experiments was the very strong effect of PSMβ1 and PSMβ2, which are relatively poor promoters of spreading.

A similar but less pronounced observation was made for PSMα1 and PSMα2. Conversely PMSγ had a relatively moderate negative impact on biofilm formation (Fig. S1), whereas it is a strong promoter of spreading (Fig. 3). The most moderate effects on biofilm formation, if any, were observed for PSMα4 and PSM-mec, which were also incapable of promoting colony spreading in our assays. On average, the PSMα3 turned out to be most potent in preventing biofilm formation and promoting colony spreading. These findings show that individual PSMs differ substantially in their ability to inhibit biofilm formation and to promote spreading. While some are very effective in both processes, others appear to have more specialized functions in one or the other process.

Spreading activity of S. aureus cells from catheter-associated biofilms or on pork meat

PSMs allow *S. aureus* to move across wet surfaces. In the above assays this was demonstrated starting with planktonic cells growing in a broth. However, we wanted to know whether spreading would be detectable also in assays that mimic clinically relevant surfaces and conditions. In one approach, we therefore tested whether *S. aureus* cells grown in a catheter-associated biofilm have the ability to spread.
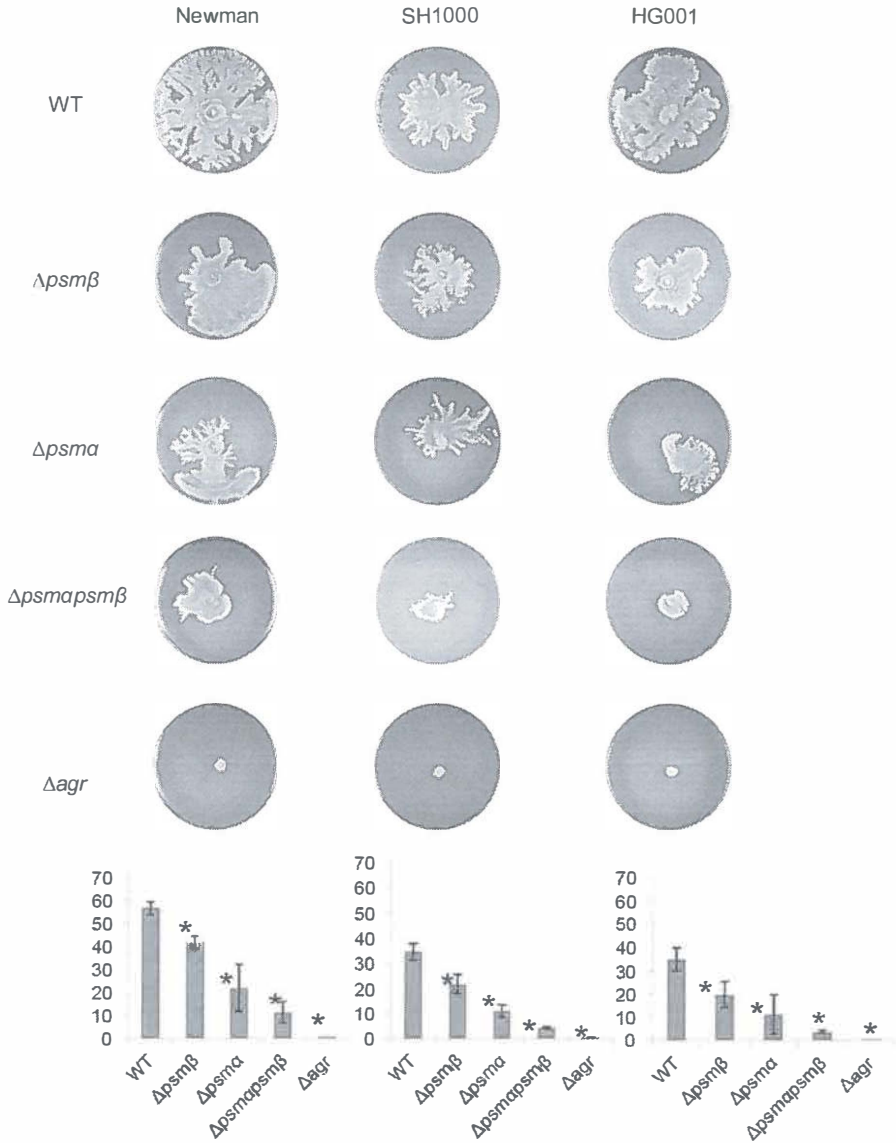
Figure 4. Additive effects of *psma* and *psmβ* gene deletions on the spreading of *S. aureus* cells. The *psma* and/or *psmβ* loci of the *S. aureus* strains Newman, SH1000 or HG001 were deleted and the effects on colony spreading were compared with the effects of an *agr* mutation. Subsequently, the spreading areas of the investigated mutant and parental strains were determined by ImageJ and statistical analyses were performed based on triplicate measurements for each individual strain. The graphs show the areas covered in arbitrary units.

Distinct roles of phenol-soluble modulins in spreading of *Staphylococcus aureus* on wet surfaces
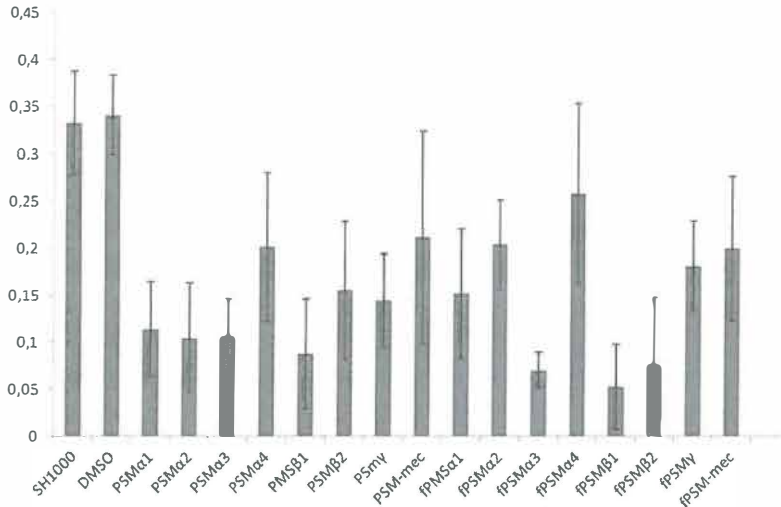
Figure 5. Particular synthetic PSM peptides inhibit biofilm formation. The wells of 96-well polystyrene dishes were pre-treated with synthetic PSM peptides prior the inoculation with *agr*⁺ cells of *S. aureus* strain SH1000. After 24 h of growth at 37°C in standing conditions, biofilm formation was assayed by staining with 0.1% safranin.

As shown in Figure 6, both *agr*⁺ and *agr* strains were able to form biofilms on catheter material (plates 8 and 2, respectively). Interestingly, when the catheters were transferred to fresh TSA plates, the *agr*⁺ strains were well able to detach and spread away from the catheter material (plate 9). This phenomenon was not observed for the tested *agr*⁻ strains (plate 3). Importantly however, when these catheters were transferred to fresh plates on which 2 µl of PSMα3 or PSMγ, were spotted beforehand, the *agr*⁻ strains were also able to detach and spread away from the catheter (plates 4 and 5, respectively). In contrast, the PSMβ1 did not facilitate the spreading of *agr*⁻ cells from the catheter material (plate 6). These findings suggest that the ability of PSMs to promote spreading is important for *S. aureus* to move away from a biofilm and to colonize the surrounding wet surface.

This idea was further tested by studying colony spreading on fresh pork meat. As predicted, *agr*⁺ cells were substantially more efficient than *agr*⁻ cells in colonizing pieces of pork meat upon overnight incubation at 37°C. In the experiment shown in Figure 7, the *agr*⁺ cells colonized an area that was on average ~2.5-fold larger than the area colonized by *agr*⁻ cells. It thus seems that colony spreading has a general role in the colonization of wet surfaces by *S. aureus*.

A.

B.
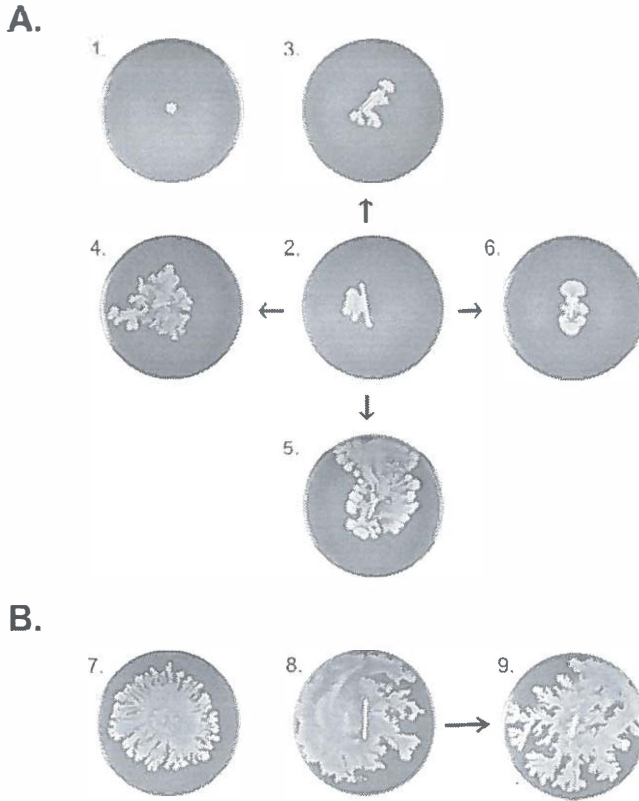
Figure 6. The spreading of *S. aureus* cells from catheter-associated biofilms is facilitated by particular PSM peptides. A, To investigate whether the spreading of cells from a catheter-associated biofilm is facilitated by PSMs, biofilms of *agr⁻* cells of *S. aureus* Newman were grown on ~1 cm long strips of catheter material. These strips were then incubated on soft agar plates under differing conditions. **1.** Negative control plate, showing that the used *S. aureus* Newman *agr⁻* strain is unable to spread; **2.** Biofilms of *agr⁻* cells were grown on catheter strips placed on a soft agar plate as shown with plate 2; **3.** Catheter strip with a biofilm (as on plate 2) transferred to a fresh soft TSA plate without further additions. Some 'outgrowth' of the cells is observed but no spreading. **4.** Catheter strip with a biofilm (as on plate 2) transferred to a soft TSA plate to which the PSMα3 peptide was added prior to the positioning of the catheter strip. **5.** Catheter strip with a biofilm transferred to a plate with the PSMγ peptide (as in 4). **6.** Catheter strip with a biofilm transferred to a plate with the PSMβ1 peptide (as in 4). **B.** Contol experiments with *agr⁺* cells of *S. aureus* Newman. **7.** Positive control plate showing colony spreading of *agr⁺* cells. **8.** Colony spreading of Newman *agr⁺* along a catheter strip. **9.** Catheter strip from plate 8 transferred to a fresh soft TSA plate without added PSMs.

Distinct roles of phenol-soluble modulins in spreading of *Staphylococcus aureus* on wet surfaces

SH1000 *agr⁺*   SH1000 *agr⁻*
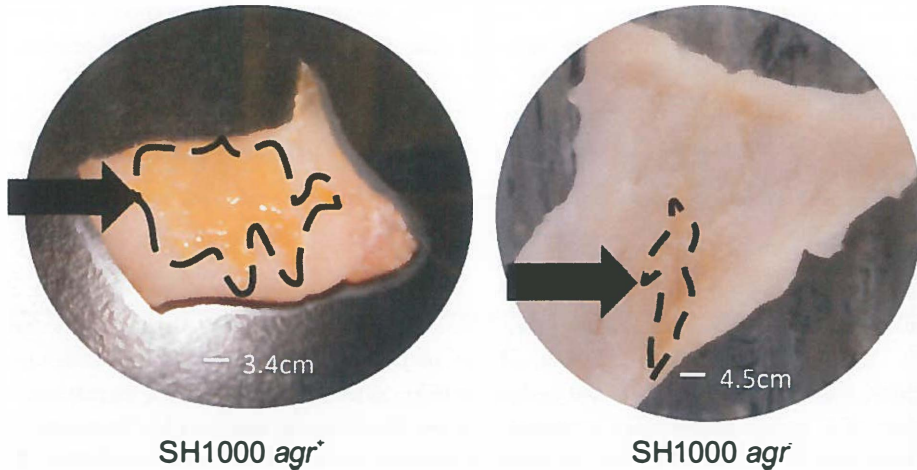
Figure 7. Spreading of *S. aureus* on meat. Overnight grown *S. aureus* SH1000 *agr⁺* or *agr⁻* cells were spotted on pork meat, which was subsequently incubated overnight at 37°C. SH1000 *agr⁺* cells covered a 2.5-fold larger area than SH1000 *agr⁻* cells. The spreading areas are marked with dashed lines.

Discussion

Colony spreading and PSMs

The present studies have focused attention on the role of secreted factors that are needed for the rapid colony spreading phenotype of *S. aureus*. The first indication for an important role of secreted factors in colony spreading was the presence of a transparent halo around the expanding colonies. Subsequent analyses showed that culture supernatants of spreaders were sufficient to make non-spreaders move on a wet soft agar surface, and that the main common components in the media of spreaders were PSMs. With synthetic PSM peptides we subsequently demonstrated conclusively that several PSMs of *S. aureus* are sufficient to promote colony spreading of otherwise non-spreading strains. This view was confirmed by mutagenesis experiments in which the *psmα* and/or *psmβ* operons were deleted. Taken together our present findings show that PSMα3 and PSMγ are the key players in colony spreading, and that the other PSMα's and PSMβ's have minor roles in spreading. We observed no role for the PSM-mec neither in the promotion nor the inhibition of spreading. The latter observations are intriguing, because it has been reported that the PSM-mec and/or the PSM-mec mRNA can inhibit colony spreading (16). Since the synthetic PSM-mec peptides gave no phenotype, whereas other synthetic PSM peptides were active, it seems most likely that the previously reported effects do not relate to a translated product but rather to a regulatory effect of the *psm-mec* gene. We were unable to assess this possibility with the strains used in our studies, because they lack type II or type III SCC*mec* elements that encode PSM-mec. In any case, it is safe to conclude from our studies that addition of the PSM-mec peptide did not interfere with the function of the main spreading-promoting PSMα3 and PSMγ peptides. Indeed, an independent study confirms that deletion of the *psm-mec*

gene does not affect the production of the other PSMs as compared to the respective parental strains (3). Lastly, it was previously reported that *S. aureus* secretes both N-terminally formylated and deformylated PSM peptides. Our present data show that the removal of the N-terminal formyl group has no consequences with respect to the activity of PSMs in colony spreading. This indicates that the surfactant properties of PSM peptides are not substantially influenced by N-terminal formylation or deformylation.

Biofilm formation and PSMs

S. aureus can cause biofilm-associated infections, and this bacterium is notorious for the formation of biofilms on catheters and other indwelling medical devices. Already in the early 1990's, *S. aureus* was regarded as one of the major causative agents of catheter-related infections, leading to catheter loss and peritonitis (63). Since then a significant increase in the incidence of *S. aureus* bacteremias attributed to indwelling vascular catheters has been observed (29). Importantly, the staphylococci embedded in biofilms are protected against antibiotics, host immune defenses and desiccation. This leads to persistent infections and often the catheter or other indwelling medical devices must be removed (5-7, 40, 41). Here we show that PSM peptides have certain biofilm-antagonizing effects. In the first place, PSMα3, PSMβ1 and PSMβ2 are very effective in preventing the formation of biofilms on polystyrene plates. Secondly and perhaps more importantly, PSMα3 and PSMγ allow *S. aureus* to leave catheter-associated biofilms and to rapidly colonize a surrounding wet surface. This suggests that PSMs might play a similar role in catheter-associated infections, although we have to emphasize that the assay used here, only very remotely reflects the *in vivo* situation. Notably, the CA-MRSA strain USA300 was shown to disperse much faster and more completely from biofilms than the laboratory strain SH1000 (24), which was also used in our present biofilm-related experiments. This is consistent with the view that PSMs are involved in biofilm dispersal, especially since CA-MRSA strains like USA300 are known to produce substantially higher amounts of PSMs than other *S. aureus* strains. Taken together, our results demonstrate that certain PSM peptides (i.e. PSMβ1 and PSMβ2) effectively block initiation of biofilm formation, that other PSM peptides (i.e. PSMγ) effectively facilitate the colonization of wet surfaces by cells that have detached from a biofilm, and that yet other PSM peptides can have both effects (i.e. PSMα3). Interestingly, Periasamy *et al.* have also shown that PSMs interfere with biofilm formation and contribute to the detachment of *S. aureus* from biofilms (44). In the latter study, the strongest negative effects on biofilm formation were observed for PSMα1 and PSMα2. In decreasing order of efficiency, PSMα4, PSMα3, PSMβ1 and PSMβ2/γ were less effective in *S. aureus* biofilm inhibition. Thus, although the general effect is similar, we find that individual PSMs inhibit biofilm formation at different efficiencies. This most likely relates to differences in the assay conditions and strains that were used in the different studies.

Distinct roles of phenol-soluble modulins in spreading of *Staphylococcus aureus* on wet surfaces

Is colony spreading by S. aureus clinically relevant?

A key question in the analysis of colony spreading is whether this property is clinically relevant. As a first approach to answer this question, we recently tested the spreading ability of 500 different clinical isolates that are representative for invasive *S. aureus* infections in Europe. More than 85% of these strains were able to spread (our unpublished observations). While this does not tell us that spreading was important for the actual infections, this finding does show that spreading is a very common feature of strains that have caused invasive infections in humans. Accordingly, it is conceivable that invasive strains make use of their spreading ability to move away from catheters and other implanted devices so that they can efficiently colonize wet surfaces of the human body. Clearly, our experiments with catheter material show that *S. aureus* cells originating form a biofilm have a similar spreading ability as planktonic cells. In this light it is not surprising that we find the same PSMs (i.e. PSMα3 and PSMγ) to be most effective in both types of assays. These findings therefore suggest that spreading may be a clinically relevant staphylococcal trait.

It has been observed that most clinical *S. aureus* isolates are agr^+, but agr^- isolates are also isolated from patients. This population heterogeneity is likely to be advantageous for *S. aureus* since agr^+ strains are more potent in initiating infections (54), while the agr^- strains are more potent in the establishment of chronic infections through biofilm formation (60). Our finding that non-spreading cells can 'hitch-hike' along with the spreaders on soft agar plates would suggest that a similar phenomenon might 'help' agr^- cells also in the colonization of wet surfaces, either in the human host or in other habitats. One of these other habitats might be animal meat intended for human consumption. There are many reports on the contamination of meat products with *S. aureus*, including MRSA (18, 38, 56). This is highly unwanted, firstly, because *S. aureus* is renowned as a causative agent of food poisoning (25). In addition, the contamination of food products with *S. aureus*, MRSA in particular, is a high risk factor for frail and immune-compromised individuals who are more susceptible for staphylococcal infections. As shown by our experiments with pork, agr^+ cells can colonize larger surfaces of the investigated meat in shorter periods of time than agr^- cells, reflecting the differences in their spreading ability on soft agar plates. Thus, we hypothesize that spreading is an important parameter at least in food spoilage and, consequently, in food poisoning by *S. aureus*. It will remain a challenge for future studies to verify this hypothesis and to pinpoint any other clinically relevant roles of staphylococcal spreading.

Chapter 3

Acknowledgements

Distinct roles of phenol-soluble modulins in spreading of *Staphylococcus aureus* on wet surfaces

References

1. **Arnaud, M., A. Chastanet, and M. Debarbouille.** 2004. New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, gram-positive bacteria. Appl. Environ. Microbiol. **70:**6887-6891.

2. **Boles, B. R. and A. R. Horswill.** 2008. Agr-mediated dispersal of *Staphylococcus aureus* biofilms. PLoS Pathog. **4:**e1000052.

3. **Chatterjee, S. S., L. Chen, H. S. Joo, G. Y. Cheung, B. N. Kreiswirth, and M. Otto.** 2011. Distribution and Regulation of the Mobile Genetic Element-Encoded Phenol-Soluble Modulin PSM-mec in Methicillin-Resistant *Staphylococcus aureus*. PLoS One **6:**e28781.

4. **Christensen, G. D., W. A. Simpson, J. J. Younger, L. M. Baddour, F. F. Barrett, D. M. Melton, and E. H. Beachey.** 1985. Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. J. Clin. Microbiol. **22:**996-1006.

5. **Costerton, J. W., P. S. Stewart, and E. P. Greenberg.** 1999. Bacterial biofilms: a common cause of persistent infections. Science **284:**1318-1322.

6. **del Pozo, J. L. and R. Patel.** 2007. The challenge of treating biofilm-associated bacterial infections. Clin. Pharmacol. Ther. **82:**204-209.

7. **Donlan, R. M. and J. W. Costerton.** 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. Clin. Microbiol. Rev. **15:**167-193.

8. **Dreisbach, A., J. M. van Dijl, and G. Buist.** 2011. The cell surface proteome of *Staphylococcus aureus*. Proteomics **11:**3154-3168.

9. **Dreisbach, A., K. Hempel, G. Buist, M. Hecker, D. Becher, and J. M. van Dijl.** 2010. Profiling the surfacome of *Staphylococcus aureus*. Proteomics **10:**3082-3096.

10. **Duthie, E. S. and L. L. Lorenz.** 1952. Staphylococcal coagulase; mode of action and antigenicity. J. Gen. Microbiol. **6:**95-107.

11. **George, E. A. and T. W. Muir.** 2007. Molecular mechanisms of agr quorum sensing in virulent staphylococci. Chembiochem **8:**847-855.

12. **Hanahan, D.** 1983. Studies on transformation of Escherichia coli with plasmids. J. Mol. Biol. **166:**557-580.

13. **Horsburgh, M. J., J. L. Aish, I. J. White, L. Shaw, J. K. Lithgow, and S. J. Foster.** 2002. sigmaB modulates virulence determinant expression and stress resistance: characterization of a functional rsbU strain derived from *Staphylococcus aureus* 8325-4. J. Bacteriol. **184:**5457-5467.

14. **Kaito, C. and K. Sekimizu.** 2007. Colony spreading in *Staphylococcus aureus*. J. Bacteriol. **189:**2553-2557.

15. **Kaito, C., Y. Omae, Y. Matsumoto, M. Nagata, H. Yamaguchi, T. Aoto, T. Ito, K. Hiramatsu, and K. Sekimizu.** 2008. A novel gene, fudoh, in the SCCmec region suppresses the colony spreading ability and virulence of *Staphylococcus aureus*. PLoS One **3:**e3921.

16. **Kaito, C., Y. Saito, G. Nagano, M. Ikuo, Y. Omae, Y. Hanada, X. Han, K. Kuwahara-Arai, T. Hishinuma, T. Baba, T. Ito, K. Hiramatsu, and K. Sekimizu.** 2011. Transcription and translation products of the cytolysin gene psm-mec on the mobile genetic element SCCmec regulate *Staphylococcus aureus* virulence. PLoS Pathog. **7:**e1001267.

17. **Kearns, D. B. and R. Losick.** 2003. Swarming motility in undomesticated *Bacillus subtilis*. Mol. Microbiol. **49:**581-590.

18. **Kluytmans, J. A.** 2010. Methicillin-resistant *Staphylococcus aureus* in food products: cause for concern or case for complacency? Clin. Microbiol. Infect. **16:**11-15.

19. **Kobayashi, S. D. and F. R. DeLeo.** 2009. An update on community-associated MRSA virulence. Curr. Opin. Pharmacol. **9:**545-551.

20. **Kohler, T., L. K. Curty, F. Barja, C. van Delden, and J. C. Pechere.** 2000. Swarming of *Pseudomonas aeruginosa* is dependent on cell-to-cell signaling and requires flagella and pili. J. Bacteriol. **182:**5990-5996.

21. **Kouwen, T. R., E. N. Trip, E. L. Denham, M. J. Sibbald, J. Y. Dubois, and J. M. van Dijl.** 2009. The large mechanosensitive channel MscL determines bacterial susceptibility to the bacteriocin sublancin 168. Antimicrob. Agents Chemother. **53:**4702-4711.

Chapter 3

22. **Kreiswirth, B. N., S. Lofdahl, M. J. Betley, M. O'Reilly, P. M. Schlievert, M. S. Bergdoll, and R. P. Novick.** 1983. The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. Nature **305:**709-712.

23. **Kuroda, M., R. Ito, Y. Tanaka, M. Yao, K. Matoba, S. Saito, I. Tanaka, and T. Ohta.** 2008. *Staphylococcus aureus* surface protein SasG contributes to intercellular autoaggregation of *Staphylococcus aureus*. Biochem. Biophys. Res. Commun. **377:**1102-1106.

24. **Lauderdale, K. J., C. L. Malone, B. R. Boles, J. Morcuende, and A. R. Horswill.** 2010. Biofilm dispersal of community-associated methicillin-resistant *Staphylococcus aureus* on orthopedic implant material. J. Orthop. Res. **28:**55-61.

25. **Le Loir, Y., F. Baron, and M. Gautier.** 2003. *Staphylococcus aureus* and food poisoning. Genet. Mol. Res. **2:**63-76.

26. **Li, M., G. Y. Cheung, J. Hu, D. Wang, H. S. Joo, F. R. Deleo, and M. Otto.** 2010. Comparative analysis of virulence and toxin expression of global community-associated methicillin-resistant *Staphylococcus aureus* strains. J. Infect. Dis. **202:**1866-1876.

27. **Lindum, P. W., U. Anthoni, C. Christophersen, L. Eberl, S. Molin, and M. Givskov.** 1998. N-Acyl-L-homoserine lactone autoinducers control production of an extracellular lipopeptide biosurfactant required for swarming motility of Serratia liquefaciens MG1. J. Bacteriol. **180:**6384-6388.

28. **Lowy, F. D.** 1998. *Staphylococcus aureus* infections. N. Engl. J. Med. **339:**520-532.

29. **Malanoski, G. J., M. H. Samore, A. Pefanis, and A. W. Karchmer.** 1995. *Staphylococcus aureus* catheter-associated bacteremia. Minimal effective therapy and unusual infectious complications associated with arterial sheath catheters. Arch. Intern. Med. **155:**1161-1166.

30. **Miller, M., A. Dreisbach, A. Otto, D. Becher, J. Bernhardt, M. Hecker, M. P. Peppelenbosch, and J. M. van Dijl.** 2011. Mapping of interactions between human macrophages and *Staphylococcus aureus* reveals an involvement of MAP kinase signaling in the host defense. J. Proteome Res. **10:**4018-4032.

31. **Morfeldt, E., L. Janzon, S. Arvidson, and S. Lofdahl.** 1988. Cloning of a chromosomal locus (exp) which regulates the expression of several exoprotein genes in *Staphylococcus aureus*. Mol. Gen. Genet. **211:**435-440.

32. **Nguyen, D. M., L. Mascola, and E. Brancoft.** 2005. Recurring methicillin-resistant *Staphylococcus aureus* infections in a football team. Emerg. Infect. Dis. **11:**526-532.

33. **Norrander, J., T. Kempe, and J. Messing.** 1983. Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. Gene **26:**101-106.

34. **Novick, R.** 1967. Properties of a cryptic high-frequency transducing phage in *Staphylococcus aureus*. Virology **33:**155-166.

35. **Novick, R. P. and E. Geisinger.** 2008. Quorum sensing in staphylococci. Annu. Rev. Genet. **42:**541-564.

36. **Novick, R. P., H. F. Ross, S. J. Projan, J. Kornblum, B. Kreiswirth, and S. Moghazeh.** 1993. Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. EMBO J. **12:**3967-3975.

37. **Novick, R. P., S. J. Projan, J. Kornblum, H. F. Ross, G. Ji, B. Kreiswirth, F. Vandenesch, and S. Moghazeh.** 1995. The agr P2 operon: an autocatalytic sensory transduction system in *Staphylococcus aureus*. Mol. Gen. Genet. **248:**446-458.

38. **O'Brien, A. M., B. M. Hanson, S. A. Farina, J. Y. Wu, J. E. Simmering, S. E. Wardyn, B. M. Forshey, M. E. Kulick, D. B. Wallinga, and T. C. Smith.** 2012. MRSA in conventional and alternative retail pork products. PLoS One **7:**e30092.

39. **Otto, M.** 2010. Basis of virulence in community-associated methicillin-resistant *Staphylococcus aureus*. Annu. Rev. Microbiol. **64:**143-162.

40. **Otto, M.** 2008. Staphylococcal biofilms. Curr. Top. Microbiol. Immunol. **322:**207-228.

41. **Otto, M.** 2006. Bacterial evasion of antimicrobial peptides by biofilm formation. Curr. Top. Microbiol. Immunol. **306:**251-258.

42. **Peacock, S. J., I. de Silva, and F. D. Lowy.** 2001. What determines nasal carriage of *Staphylococcus aureus*? Trends Microbiol. **9:**605-610.

Distinct roles of phenol-soluble modulins in spreading of *Staphylococcus aureus* on wet surfaces

43. **Peng, H. L., R. P. Novick, B. Kreiswirth, J. Kornblum, and P. Schlievert.** 1988. Cloning, characterization, and sequencing of an accessory gene regulator (agr) in *Staphylococcus aureus*. J. Bacteriol. **170:**4365-4372.

44. **Periasamy, S., H. S. Joo, A. C. Duong, T. H. Bach, V. Y. Tan, S. S. Chatterjee, G. Y. Cheung, and M. Otto.** 2012. How *Staphylococcus aureus* biofilms develop their characteristic structure. Proc. Natl. Acad. Sci. U. S. A.

45. **Pohl, K., P. Francois, L. Stenz, F. Schlink, T. Geiger, S. Herbert, C. Goerke, J. Schrenzel, and C. Wolz.** 2009. CodY in *Staphylococcus aureus*: a regulatory link between metabolism and virulence gene expression. J. Bacteriol. **191:**2953-2963.

46. **Projan, S. J., S. Brown-Skrobot, P. M. Schlievert, F. Vandenesch, and R. P. Novick.** 1994. Glycerol monolaurate inhibits the production of beta-lactamase, toxic shock toxin-1, and other staphylococcal exoproteins by interfering with signal transduction. J. Bacteriol. **176:**4204-4209.

47. **Queck, S. Y., M. Jameson-Lee, A. E. Villaruz, T. H. Bach, B. A. Khan, D. E. Sturdevant, S. M. Ricklefs, M. Li, and M. Otto.** 2008. RNAIII-independent target gene control by the agr quorum-sensing system: insight into the evolution of virulence regulation in *Staphylococcus aureus*. Mol. Cell **32:**150-158.

48. **Queck, S. Y., B. A. Khan, R. Wang, T. H. Bach, D. Kretschmer, L. Chen, B. N. Kreiswirth, A. Peschel, F. R. Deleo, and M. Otto.** 2009. Mobile genetic element-encoded cytolysin connects virulence to methicillin resistance in MRSA. PLoS Pathog. **5:**e1000533.

49. **Recsei, P., B. Kreiswirth, M. O'Reilly, P. Schlievert, A. Gruss, and R. P. Novick.** 1986. Regulation of exoprotein gene expression in *Staphylococcus aureus* by agar. Mol. Gen. Genet. **202:**58-61.

50. **Sastalla, I., K. Chim, G. Y. Cheung, A. P. Pomerantsev, and S. H. Leppla.** 2009. Codon-optimized fluorescent proteins designed for expression in low-GC gram-positive bacteria. Appl. Environ. Microbiol. **75:**2099-2110.

51. **Sibbald, M. J., A. K. Ziebandt, S. Engelmann, M. Hecker, A. de Jong, H. J. Harmsen, G. C. Raangs, I. Stokroos, J. P. Arends, J. Y. Dubois, and J. M. van Dijl.** 2006. Mapping the pathways to staphylococcal pathogenesis by comparative secretomics. Microbiol. Mol. Biol. Rev. **70:**755-788.

52. **Sibbald, M. J., T. Winter, M. M. van der Kooi-Pol, G. Buist, E. Tsompanidou, T. Bosma, T. Schafer, K. Ohlsen, M. Hecker, H. Antelmann, S. Engelmann, and J. M. van Dijl.** 2010. Synthetic effects of secG and secY2 mutations on exoproteome biogenesis in *Staphylococcus aureus*. J. Bacteriol. **192:**3788-3800.

53. **Somerville, G. A., A. Cockayne, M. Durr, A. Peschel, M. Otto, and J. M. Musser.** 2003. Synthesis and deformylation of *Staphylococcus aureus* delta-toxin are linked to tricarboxylic acid cycle activity. J. Bacteriol. **185:**6686-6694.

54. **Traber, K. E., E. Lee, S. Benson, R. Corrigan, M. Cantera, B. Shopsin, and R. P. Novick.** 2008. agr function in clinical *Staphylococcus aureus* isolates. Microbiology **154:**2265-2274.

55. **Tsompanidou, E., M. J. Sibbald, M. A. Chlebowicz, A. Dreisbach, J. W. Back, J. M. van Dijl, G. Buist, and E. L. Denham.** 2011. Requirement of the agr locus for colony spreading of *Staphylococcus aureus*. J. Bacteriol. **193:**1267-1272.

56. **van Loo, I. H., B. M. Diederen, P. H. Savelkoul, J. H. Woudenberg, R. Roosendaal, A. van Belkum, N. Lemmens-den Toom, C. Verhulst, P. H. van Keulen, and J. A. Kluytmans.** 2007. Methicillin-resistant *Staphylococcus aureus* in meat products, the Netherlands. Emerg. Infect. Dis. **13:**1753-1755.

57. **Wang, R., B. A. Khan, G. Y. Cheung, T. H. Bach, M. Jameson-Lee, K. F. Kong, S. Y. Queck, and M. Otto.** 2011. *Staphylococcus epidermidis* surfactant peptides promote biofilm maturation and dissemination of biofilm-associated infection in mice. J. Clin. Invest. **121:**238-248.

58. **Wang, R., K. R. Braughton, D. Kretschmer, T. H. Bach, S. Y. Queck, M. Li, A. D. Kennedy, D. W. Dorward, S. J. Klebanoff, A. Peschel, F. R. DeLeo, and M. Otto.** 2007. Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. Nat. Med. **13:**1510-1514.

59. **Wolz, C., D. McDevitt, T. J. Foster, and A. L. Cheung.** 1996. Influence of agr on fibrinogen binding in *Staphylococcus aureus* Newman. Infect. Immun. **64:**3142-3147.

60. **Yarwood, J. M. and P. M. Schlievert.** 2003. Quorum sensing in Staphylococcus infections. J. Clin. Invest. **112:**1620-1625.

Chapter 3

61. **Ziebandt, A. K., D. Becher, K. Ohlsen, J. Hacker, M. Hecker, and S. Engelmann.** 2004. The influence of agr and sigmaB in growth phase dependent regulation of virulence factors in *Staphylococcus aureus*. Proteomics **4:**3034-3047.

62. **Ziebandt, A. K., H. Kusch, M. Degner, S. Jaglitz, M. J. Sibbald, J. P. Arends, M. A. Chlebowicz, D. Albrecht, R. Pantucek, J. Doskar, W. Ziebuhr, B. M. Broker, M. Hecker, J. M. van Dijl, and S. Engelmann.** 2010. Proteomics uncovers extreme heterogeneity in the *Staphylococcus aureus* exoproteome due to genomic plasticity and variant gene regulation. Proteomics **10:**1634-1644.

63. **Zimmerman S.W., O'Brien M., Wiedenhoeft F.A., and Johnson C.A.** 1988. *Staphylococcus aureus* Peritoneal Catheter-Related Infections: A Cause of Catheter Loss and Peritonitis. Peritoneal Dialysis International **8:**191-194.

"Υγιεία τίμιον αλλ' ευμετάστατον."

"Health is precious but volatile."

Plutarxos, 47-120 (Chaeronea, Boeotia, Greece)

Chapter 4

The sortase A substrates FnbpA, FnbpB, ClfA and ClfB antagonize colony spreading of *Staphylococcus aureus*

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Chapter 4

Abstract

Staphylococcus aureus is an important human pathogen that is renowned both for its rapid transmission within hospitals and the community, and for the formation of antibiotic resistant biofilms on medical implants. Recently, it was shown that *S. aureus* is able to spread over wet surfaces. This motility phenomenon is promoted by the surfactant properties of secreted phenol-soluble modulins (PSMs), which are also known to inhibit biofilm formation. The aim of the present studies was to determine whether any cell surface-associated *S. aureus* proteins have an impact on colony spreading. To this end, we analyzed the spreading capabilities of strains lacking non-essential components of the protein export and sorting machinery. Interestingly, our analyses reveal that the absence of sortase A (SrtA) causes a hyper-spreading phenotype. SrtA is responsible for covalent anchoring of various proteins to the staphylococcal cell wall. Accordingly, we show that the hyper-spreading phenotype of *srtA* mutant cells is an indirect effect that relates to the sortase substrates FnbpA, FnbpB, ClfA and ClfB. These surface-exposed staphylococcal proteins are known to promote biofilm formation, cell-cell interactions and adherence to host cells. The hyper-spreading phenotype of *srtA* mutant staphylococcal cells was subsequently validated in *Staphylococcus epidermidis*. We therefore conclude that cell wall-associated factors that promote a sessile lifestyle of *S. aureus* and *S. epidermidis* antagonize the colony spreading motility of these bacteria.

The sortase A substrates FnbpA, FnbpB, ClfA and ClfB antagonize colony spreading of *Staphylococcus aureus*

Introduction

Staphylococcus aureus is an opportunistic human pathogen that is currently a leading cause of infections throughout the world. This Gram-positive bacterium can cause a wide variety of both acute and chronic diseases ranging from superficial skin infections to life-threatening endocarditis and sepsis (28, 48). The ability of *S. aureus* to cause these infections is due to the production of secreted and cell wall-associated virulence factors that are coordinately expressed. These factors include proteins that are necessary for host colonization, invasion, biofilm formation, toxicogenesis, immune evasion or spreading throughout the host.

To sort proteins to their correct extracytoplasmic locations, Gram-positive bacteria have several pathways for protein targeting and transport. *S. aureus* contains at least six of these pathways (55). Most proteins, including virulence factors are translocated across the cytoplasmic membrane via the Sec pathway. These proteins are synthesized in the cytoplasm with an N-terminal Sec-type signal peptide that directs them to the Sec translocase, which is embedded in the membrane (18, 51, 52, 55, 65). The Sec translocase can only facilitate the membrane passage of proteins in an unfolded state (8, 47). Upon translocation, type I signal peptidases cleave the signal peptide to liberate the proteins from the membrane. Various folding catalysts can then assist the folding of the translocated proteins into their active and protease-resistant conformation (46, 55, 62, 63). Some proteins that are translocated via the Sec pathway are retained in the membrane or cell wall. When a translocated protein lacks a specific signal for retention in these subcellular compartments, it is usually secreted into the extracellular milieu (30, 55). Proteins can be bound to the cell wall either in a non-covalent manner via specific binding domains, or covalently through the enzymatic activity of so-called sortases.

Gram-positive bacteria employ sortases to covalently link exported proteins with a special C-terminal LPxTG motif to the cell wall. These sortases are membrane-bound transpeptidases that cleave the peptide bond between the Thr and Gly residues of the LPxTG motif, and catalyze the formation of an amide bond between the carboxyl group of the Thr residue and the free amino end of a pentaglycine cross bridge in peptidoglycan precursors (11, 14, 27, 37, 54, 57). The sortase A (SrtA) enzyme from *S. aureus* is a prototypical member of the sortase family (31, 32, 59, 60). *S. aureus* strains lacking the *srtA* gene are unable to retain and display LPxTG proteins at the cell surface. As a consequence, *srtA* mutant strains are defective in the establishment of acute infections (32).

There are 19 staphylococcal proteins that carry a C-terminal LPxTG motif and 2 that carry a C-terminal LPxAG motif (13, 30, 38, 50, 55). These include protein A (Spa), two fibronectin-binding proteins (FnbpA and FnbpB) (21), two clumping factors (ClfA and ClfB), three cell wall-anchored proteins with large serine-aspartate repeat domains (SdrC, SdrD and SdrE) (22), a collagen–binding protein (Can), a plasmin-sensitive protein (Pls) (53), FmtB (24), and eleven staphylococcal surface (Sas) proteins. For some of these proteins a direct role in biofilm formation has been reported. This applies to Spa (35, 58), FnbpA and FnbpB (12, 44, 45, 66).

Chapter 4

We have previously shown that *S. aureus* cells can employ secreted phenol–soluble modulins (PSMs) for their translocation over wet surfaces. At the same time, certain PSMs are very effective in preventing biofilm formation (49) Tsompanidou *et al.* submitted]. The PSMs thus seem to have a decisive role in the transitions between sessile and motile lifestyles of *S. aureus*. While the role of secreted PSMs in spreading motility has been established, it was so far not known whether any cell-associated factors are also involved in this process. Therefore, the primary aim of the present studies was to identify cell-associated factors that impact on spreading motility. As a first approach to find out whether any cell surface-associated proteins may be involved in spreading, we investigated spreading motility of mutant strains lacking non-essential components of the protein export and sorting machinery. Interestingly, this revealed that *srtA* mutant cells are more efficient spreaders than the corresponding parental strains. Further analyses showed that this relates to the spreading-limiting roles of the sortase substrates FnbpA, FnbpB, ClfA and ClfB.

Materials and Methods

Bacterial strains and growth conditions

The bacterial strains and plasmids that were used in the present studies are listed in Table 1. All *Escherichia coli* strains were grown in Luria-Bertani broth (LB) at 37°C under shaking conditions. *S. aureus* and *S. epidermidis* strains were grown in tryptic soy broth (TSB) at 37°C under vigorous shaking. Where necessary, ampicillin 100 µg/ml (for *E. coli*) or erythromycin 5 µg/ml (for *S. aureus* and *S. epidermidis*) were added to the growth medium.

Construction of S. aureus and S. epidermidis mutant strains

The *S. aureus* and *S. epidermidis* mutants lacking secretion machinery genes (Table 1) were constructed using the temperature-sensitive plasmid pMAD (1) and previously described procedures (25). All primers used are listed in Table 2. To delete particular genes, primer pairs with the designations F1/R1 and F2/R2 were used for PCR amplification of the respective upstream and downstream regions (each ~500 bp). Primers R1 and F2 contain an overlap of 24 nucleotides, which served to fuse the amplified 'front' and 'back' flanking regions by PCR. The fused flanking regions were cloned in pMAD, and the resulting plasmids were used to delete the genes between these flanking regions from the *S. aureus* or *S. epidermidis* genome. To this end, the pMAD plasmids carrying the flanking regions were used to transform *S. aureus* strain RN4220 via electroporation. Next, these plasmids were isolated from the RN4220 strain and used to transform electrocompetent cells of *S. aureus* SH1000, NCTC8325 and Newman, or *S. epidermidis* 1457. Upon chromosomal plasmid insertion and excision, white colonies on plates with 80 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside were screened for the correct gene deletion by colony PCR using primers F1 and R2. To delete the *clfA* or *clfB* genes from the *S. aureus* SH1000 genome, the respective allelic replacements with antibiotic resistance markers

The sortase A substrates FnbpA, FnbpB, ClfA and ClfB antagonize colony spreading of *Staphylococcus aureus*

were transferred from the original strains provided by T.J. Foster to the SH1000 strain by transduction with phage φ85 (42, 56, 56).

Table 1 Bacterial strains and plasmids used in the present studies

Strains	Genotype	Reference
E. coli		
E. coli DH5α	λ⁻ φ80d*lac*ZΔM15 Δ(*lacZYA-argF*)U169 *recA1 endA hsdR17*(ʳk⁻ ᵐk⁻)*.sup*E44 *thi-1 gyrA relA1*	(19)
E. coli TOP10	Cloning host for TOPO vector; F⁻ *mc*rA Δ(*mrr-hsdRMS-mcrBC*) λ⁻ φ80d*lac*ZΔM15 Δ*lacX74 recA1 araD*139 Δ(*ara-leu*)7697 *galU galK rps*L (Strʳ) *endA*1 *nupG*	Invitrogen Life Technologies
S. aureus		
RN4220	Restriction-deficient derivative of NCTC8325, cured of all known prophages	(26)
SH1000	NCTC8325-4 derivative, *rsbU⁺, agr⁺*	(20)
SH1000Δ*cidA::km*	*cidA*, Kmʳ	This work
SH1000 Δ*dsbA*	*dsbA*	This work
SH1000 Δ*lgt*	*lgt*	This work
SH1000 Δ*lrgA::km*	*lrgA*, Kmʳ	This work
SH1000 Δ*lspA*	*lspA*	This work
SH1000 Δ*prsA*	*prsA*	This work
SH1000 Δ*secG*	*secG*	(56)
SH1000 Δ*secY2*	*secY2*	(56)
SH1000 Δ*spsA*	*spsA*	This work
SH1000 Δ*srtA*	*srtA*	This work
SH1000 Δ*srtB*	*srtB*	This work
SH1000 Δ*tatA*	*tatA*	This work
SH1000 Δ*tatC::km*	*tatC*, Kmʳ	This work
SH1000 Δ*tatAC*	*tatA tatC*	This work
SH1000 Δ*mscL*	*mscL*	This work
SH1000 Δ*fnbpA*	*fnbpA*	This work
SH1000 Δ*fnbpB*	*fnbpB*	This work
SH1000 Δ*fnbpA* Δ*fnbpB*	*fnbpA, fnbpB*	This work
SH1000 Δ*clfA*	*clfA*	(16)
SH1000 Δ*fnbpA* Δ*fnbpB* Δ*clfA* Δ*clfB*	*fnbpA, fnbpB, clfA, clfB*	(16)
NCTC8325	HA-MSSA strain, *agr⁺, rsbU⁻*	(41)
NCTC8325 Δ*srtA*	*srtA*	This work
Newman	NCTC 8178 clinical isolate	(10)
Newman Δ*srtA*	*srtA*	This work
S. epidermidis		
1457	Biofilm positive strain	(29)
1457 Δ*srtA*	*srtA*	This work

Chapter 4

Plasmids	Relevant properties	Reference
pUC18	Ap^r, ColE1, φ80d*lac*Z; lac promoter	(40)
TOPO	pCR-Blint II-TOPO vector; Km^r	Invitrogen Life Technologies
pCN51	*E. coli/S. aureus* shuttle vector that contains a cadmium-inducible promoter; Ap^r Em^r	(2)
srtA-pCN51	pCN51 with *S. aureus srtA* gene; Ap^r Em^r	This work
srtA^{Se}-pCN51	pCN51 with *S. epidermidis srtA* gene; Ap^r Em^r	This work

Complementation of the srtA mutation

For complementation studies, the *srtA* genes of *S. aureus* and *S. epidermidis* were cloned in plasmid pCN51. Expression of a cloned gene in this plasmid is controlled by a cadmium-inducible promoter. The primers used for the amplification of the *srtA* genes are listed in Table 22 and the restriction sites used for cloning in pCN51 are shown in italics. The resulting plasmids *srtA*-pCN51 and *srtA^{Se}*-pCN51 were used to transform electrocompetent *S. aureus* RN4220 cells, and the transformed cells were plated on TSA plates containing erythromycin. The restriction-modified plasmids were isolated from *S. aureus* RN4220 and then used to transform electrocompetent *S. aureus* SH1000 Δ*srtA*, *S. aureus* NCTC8325 Δ*srtA*, or *S. epidermidis* 1457 Δ*srtA*.

Table 2 Primers used in the present studies

Primer	Sequence (5'→3')
cidA-F1	AATAAAGACTTTTACTTGAAT
cidA-R1[a]	TTAGAACTCCAATTCACCCATGGCCCCCGCGCCATCCCTTTCTAAATA
cidA-F2[a]	CCGCAACTGTCCATACCATGGCCCCCTGATTACGTGCAAGCCTTATTAAT
cidA-R2	GATAGAAGATTCAAATCTTCC
dsbA-F1	ATTTCTTTGGATATTTATATT
dsbA-R1	CTACGTCAGTCAGTCACCATGGCAAATAACTCCTATTCATAT
dsbA-F2	TGCCATGGTGACTGACTGACGTAGTCTTAATTGTTGAGATCA
dsbA-R2	CTTTCGTTATAGTTTTCCCAC
lgt-F1	GGTGTTGGTGTACTAATTACC
lgt-R1	CTACGTCAGTCAGTCACCATGGCATCAACCTACTCCTCACTCTTA
lgt-F2	TGCCATGGTGACTGACTGACGTAGTGATAGTTTGAGGAAATTTTT
lgt-R2	ACATTATTATTCTTTTGCGCC
lrgA-F1	TAAAGCCAAAGATGATAATAA
lrgA-R1[a]	TTAGAACTCCAATTCACCCATGGCCCCCGCCTCCTACGTTTGATTTAA
lrgA-F2[a]	CCGCAACTGTCCATACCATGGCCCCCTAACCACTTAGCACTAAACACACC
lrgA-R2	GTAATTCGGAAAAGCTTTAAG
lspA-F1	CCAATTAAGTGTAGACGATTC
lspA-R1	TTACGTCAGTCAGTCACCATGGCATTTCGTTCCTCCAATCAATCG
lspA-F2	TGCCATGGTGACTGACTGACGTAATGGAGACTTATGAATTTAACA
lspA-R2	CGATATATTTTCTTTTAACAG
prsA-F1	GAAAATGGCTTATATTCTATA
prsA-R1	TTACGTCAGTCAGTCACCATGGCAAGTTGAAACTCCTTTGTAAGT
prsA-F2	TGCCATGGTGACTGACTGACGTAACACAAAACCGAGCGACCGTGG
prsA-R2	TTTGTTATATAGTGGTATTAT

74

The sortase A substrates FnbpA, FnbpB, ClfA and ClfB antagonize colony spreading of *Staphylococcus aureus*

secA2-F1	GTATAAAAGCATGCGGGTGAC
secA2-R1[a]	TTAGAACTCCAATTCACCCATGGCCCCCTTACTTCCCCACCATTCAGTT
secA2-F2[a]	CGCAACTGTCCATACCATGGCCCCCTAAATGAAAAGGGGTAGCGCATGA
secA2-R2	GTCGCATATATAATTTCGCTT
secG-F1	TTAAAACAGGACGCTTTATTG
secG-R1	TTACGTCAGTCAGTCACCATGGCAAAATTGTCCTCCGTTCCTTAT
secG-F2	TGCCATGGTGACTGACTGACGTAAGGTCCGGCGATGTAAATGTCG-
secG-R2	GCGTGCATATTCTAAAAAGCC
secY2-F1	TGTCTGGTTCACAAAGCATTT
secY2-R1	TTACGTCAGTCAGTCACCATGGCAGTTGCACCTCTTTTATATCAA
secY2-F2	TGCCATGGTGACTGACTGACGTAAGGAGGTAATTATGAAATACTT
secY2-R2	GCCTCTCCCTGATCATCAAAA
spsA-F1	TAGAGCTATAATTCCAGTATT
spsA-R1	TTACGTCAGTCAGTCACCATGGCAGATGTCACTCCTTTTTCGATC
spsA-F2	TGCCATGGTGACTGACTGACGTAAAAAGAGGTGTCAAAATTGAAA
spsA-R2	CCAACAATTTGGTCTTCATCA
srtA-F1	AATGGTGTAGTAATTGACTAG
srtA-R1	TTACGTCAGTCAGTCACCATGGCAACGTTAAGGCTCCTTTTATAC
srtA-F2	TGCCATGGTGACTGACTGACGTAATCTATTACGCTAATGGATGAA-
srtA-R2	CTCACATTACTTACTATTAAT
srtB-F1	TGAAAATATGGAGCGACGTAT
srtB-R1	TTACGTCAGTCAGTCACCATGGCAAAAAATCCTCTTTTATTAACG
srtB-F2	TGCCATGGTGACTGACTGACGTAAACAGAAAAGAGGATAATTATG
srtB-R2	ATCAAAATGATATAATTGATG
tatA-F1	TTATGGCATTTACATTATCTG
tatA-R1	CTACGTCAGTCAGTCACCATGGCAGATAATCAACCTCACTCATAA
tatA-F2	TGCCATGGTGACTGACTGACGTAGCACTGACCACACCTTACTGGT
tatA-R2	GACCCATAAATAATATTGGTA
tatC-F1	TGATGAAATGGCTGAAGCTGG
tatC-R1[a]	TTAGAACTCCAATTCACCCATGGCCCCCAAAATTTTTACTAACCGATG
tatC-F2[a]	CCGCAACTGTCCATACCATGGCCCCCTAACCTTATACGAATCAATGCTGT
tatC-R2	CGATTAGTAATGGTAATTTGG
kan-F1	GGGGGCCATGGGTGAATTGGAGTTCGTCTTG
kan-R1	GGGGGCCATGGTATGGACAGTTGCGGATGTA
secA2-F3	CGGAATTCGAATCCAGTACGATTTTTAG
secA2-R3	CGGGATCCTCCCGGTAACATACGACCTG
srtA[Se]*-F1*	AACTTTGTTCTTTAGCGTAACGAAT
srtA[Se]*-R1*	**TGCCATGGTGACTGACTGACGTAA**TTATGTTACTCCTTTATATTTATT
srtA[Se]*-F2*	**TTACGTCAGTCAGTCACCATGGC**ATATTCTTATAAGTGAAAGATACGTA
srtA[Se]*-R2*	CTTTATAGATGACTGCTCCAT
srtA-CN 5'	CAGCC<u>GGATCC</u>AATGTATAAAAGGAGCCTTAACGT
srtA-CN 3'	CGGAATTCTTATTTGACTTCTGTAGCTACAAA
srtA[Se]*-CN 5'*	CAGCC<u>GGATCC</u>AATGTATAAAAGGAGCCTTAACGTATGAAGCAGTGGATGAATAGA
srtA[Se]*-CN 3'*	CG GAATTCTTAGTTAATTTGTGTAGCTATGAA

Overlapping nucleotides are shown in bold; restriction sites in primers are underlined
[a] *These primers have an overlap with the kanamycin resistance cassette from pDG783*

Colony spreading assay

The colony spreading assay was performed as described by Kaito *et al* (23), with minor modifications. Briefly, TSB broth supplemented with 0.24% agar was used to prepare TSA soft agar plates. Each plate (10 ml) was dried for approximately 10 min in a laminar flow cabinet for

optimal colony spreading conditions. From a TSB overnight culture of the strain to be tested for spreading, an aliquot of 2 µl was spotted in the centre of a TSA plate and the plates were then dried for an additional 5 min. Finally, upon overnight incubation of the plates at 37°C, the spreading zones were examined and pictures were taken. To induce *srtA* expression from pCN51, soft agar plates were supplemented with 0.25 µM CdSO$_4$.

Results and Discussion

The requirement for non-essential protein secretion machinery components in colony spreading by *S. aureus* was assessed by testing the secretion mutants listed in Table 1 for their ability to spread on TSA soft agar plates. Of all tested strains, only the *srtA* mutant showed a significant change in spreading. Intriguingly, this strain displayed an enhanced colony spreading phenotype as is shown in Figure 1. This spreading phenotype of the *srtA* mutant was completely reversed to the wild-type phenotype by ectopic expression of *srtA* from the plasmid *srtA*-pCN51 (Fig. 1). The *srtA* mutant strains are unable to link LPxTG proteins covalently to the cell wall and, because of this, they are attenuated in virulence. This suggested that the effect of the *srtA* mutation on spreading would also be an indirect consequence of the absence of cell wall coupling of one or more LPxTG proteins.
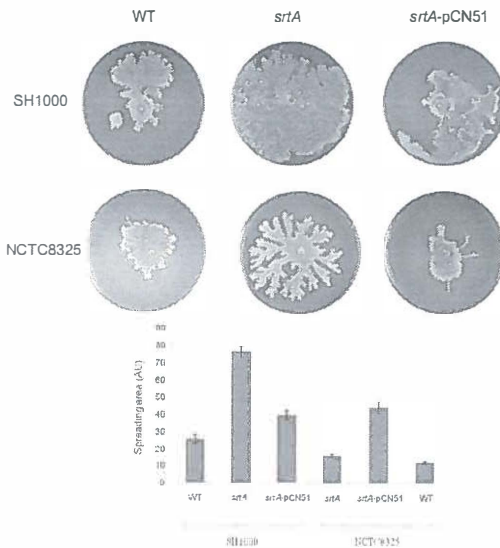
Figure 1. Hyper-spreading phenotype of *srtA* mutant *S. aureus* strains. From an overnight culture, an aliquot of 2 µl was spotted in the middle of a TSA plate, which was then incubated overnight at 37°C. The analyses include the laboratory strains *S. aureus* SH1000 and NCTC8325 (both labeled WT), as well as their *srtA* mutant derivatives (labeled *srtA*) and *srtA* mutants complemented with a plasmid pCN51-borne copy of *S. aureus srtA* (labeled *srtA*-pCN51). The spreading areas of the investigated mutant and parental strains were determined by ImageJ. The graphs show the areas covered in arbitrary units (AU) and respective standard deviations.

The sortase A substrates FnbpA, FnbpB, ClfA and ClfB antagonize colony spreading of *Staphylococcus aureus*

Many LPxTG proteins belong to the MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) and promote bacterial attachment to the extracellular matrix of host tissues. Some of these MSCRAMMs, such as FnbpA and FnbpB, have been implicated in biofilm formation and other MSCRAMMs, such as ClfA and ClfB, have been implicated in cell-cell interactions. Since spreading motility on the one hand and biofilm formation or cell aggregation on the other hand are processes with opposite effects, we investigated whether the individual deletion of the *fnbpA*, *fnbpB*, *clfA* or *clfB* genes would result in enhanced spreading. None of these single mutant strains had a major impact on colony spreading, although the *fnbpA* and *fnbpB* mutant cells did cover slightly larger areas than the corresponding parental strain or *clfA* mutant cells (Fig. 2). Since this suggested that the absence of only one of these proteins might not be sufficient for an increased spreading phenotype, double, triple and quadruple mutant strains were constructed that lacked *fnbpA*, *fnpbB*, *clfA* and/or *clfB*. As shown in Figure 2, the mutant lacking all four of these genes showed a significantly enhanced spreading phenotype that was comparable to the phenotype of the *srtA* single mutant strain. Thus, the two fibronectin-binding proteins FnbpA and FnbpB and the two clumping factors ClfA and ClfB counteract spreading. While we cannot exclude the possibility that other LPxTG proteins also counteract spreading, the observed effect of the quadruple *fnbpA fnbpB clfA clfB* mutation is fully sufficient to explain the hyper-spreading phenotype of the *srtA* mutant. It should be noted that FnbpA, FnbpB, ClfA and ClfB do not block colony spreading as evidenced by the spreading of the parental strains used in the present studies as well as a range of clinical isolates that readily spread on soft agar (61). Thus, it seems that in the absence of FnbpA, FnbpB, ClfA and ClfB the cells are less tightly associated with each other and, consequently, they can cover larger areas on soft agar plates by means of their spreading motility.

The FnbpA, FnbpB, ClfA and ClfB proteins can promote adhesion of *S. aureus* cells to a variety of molecules and surfaces and they have been implicated in cell-cell adhesion. In relation to our present findings, it is interesting to note that the *fnpbB* gene is less common in highly virulent *S. aureus* isolates, and that the presence of *fnbpB* is associated with reduced transmission of staphylococcal skin infections in a rabbit model (36, 64). This seems to suggest that spreading activity and transmission of *S. aureus* could perhaps be linked. Furthermore, FnbpA is a highly variable surface protein. The *fnbpA* gene has a mosaic structure, which indicates that this gene is evolving not only through point mutations, but also through recombination events (33). ClfA mediates attachment to plasma clots, to platelets and to plastic biomaterial used for medical implants. Lastly, ClfB promotes cell clumping in the presence of fibrinogen. However, ClfB is not only able to bind to fibrinogen itself, but also to proteins present in the envelope of squamous cells and to desquamated nasal epithelial cells (5, 13, 15). Interestingly, the production of FnbpA, FnbpB, ClfA and ClfB in different *S. aureus* strains seems to be highly variable (6, 7, 61). This may at least partly explain our previous observation that the spreading abilities of different *S. aureus* clinical isolates are highly variable (7, 61). This view is further supported by the observation that strain Newman, which produces truncated forms of FnbpA and FnbpB, is a very efficient spreader (Fig. 2). These truncated FnbPs are no longer anchored to the cell surface

but secreted, which leads to a loss of their function (17). In fact the high spreading activity of strain Newman is comparable to that of the *srtA* mutant or the *fnbpA fnbpB clfA clfB* quadruple mutant derivatives of strain SH1000 (Fig. 2). Notably, the mutations in *fnbpA* and *fnbpB* may not be sufficient to explain the increased spreading of strain Newman, but our previous studies suggest that this strain also produces very low levels of ClfB, if any (7). This may contribute to the hyper-spreading phenotype of strain Newman. Consistent with these considerations, a *srtA* deletion did not further increase the spreading capacity of strain Newman (not shown).
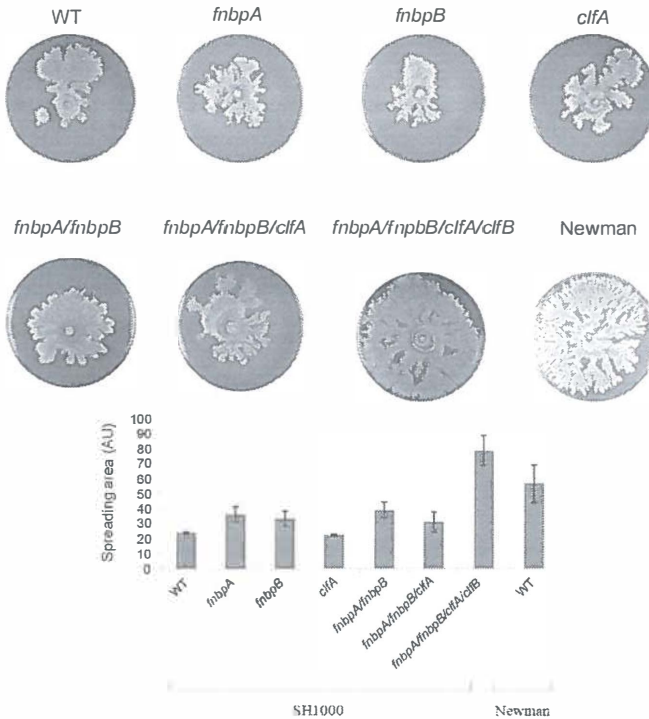
Figure 2. The influence of *fnbpA*, *fnbpB*, *clfA* and *clfB* mutations on colony spreading of *S. aureus*. Spreading motility of *S. aureus* SH1000-derived *fnbpA*, *fnbpB*, *clfA* and/or *clfB* mutant strains and the *S. aureus* Newman strain was assayed as described for Figure 1.

Depending on the strain and growth condition, the *fnbpA* and *fnbpB* genes are negatively regulated by the Agr system (9, 34, 67). On the other hand, the Agr system positively regulates the synthesis of PSMs that are critical for spreading motility (61). The differential Agr-regulated production of FnbpAB and the PSMs is thus fully compatible with our present findings that FnbpAB counteract spreading. Though the *clfA* and *clfB* genes are not regulated by Agr, but by SarA, they are highly expressed during the early exponential growth phase and barely during the late exponential or stationary growth phases (3, 9, 39). The production of ClfA and ClfB thus

The sortase A substrates FnbpA, FnbpB, ClfA and ClfB antagonize colony spreading of *Staphylococcus aureus*

correlates positively with that of FnbpAB and negatively with PSM production, which is also fully consistent with the presently observed negative role of ClfA and ClfB in spreading.

Lastly, to investigate whether surface-attached proteins also set a limit to spreading motility in other staphylococci, we turned to *Staphylococcus epidermidis*. This bacterium is renowned for its high capacity to form biofilms on medical implants (43). Nevertheless, *S. epidermidis* does produce phenol-soluble modulins (4, 68), which should provide it with an intrinsic capacity for spreading motility. As shown in Figure 3, wild-type cells of *S. epidermidis* strain 1457 did indeed spread on soft agar plates, albeit to a lesser extent than cells of *S. aureus* SH1000. As predicted on the basis of our experiments with *S. aureus*, the *srtA* mutant of *S. epidermidis* displayed a massively increased spreading over soft agar plates (Fig. 3). Furthermore, this hyper-spreading phenotype of the *S. epidermidis srtA* mutant was completely reversed to the low-level spreading of the parental strain upon ectopic expression of the *S. epidermidis srtA* gene from plasmid $srtA^{Se}$-pCN51 (Fig. 3). We therefore conclude that, also in *S. epidermidis*, the sortase-mediated cell wall anchoring of proteins sets a limit to spreading motility. Thus, this seems to be a conserved feature of staphylococcal spreading motility, which is fully consistent with the previously shown role of covalently anchored cell wall proteins in the formation of biofilms.
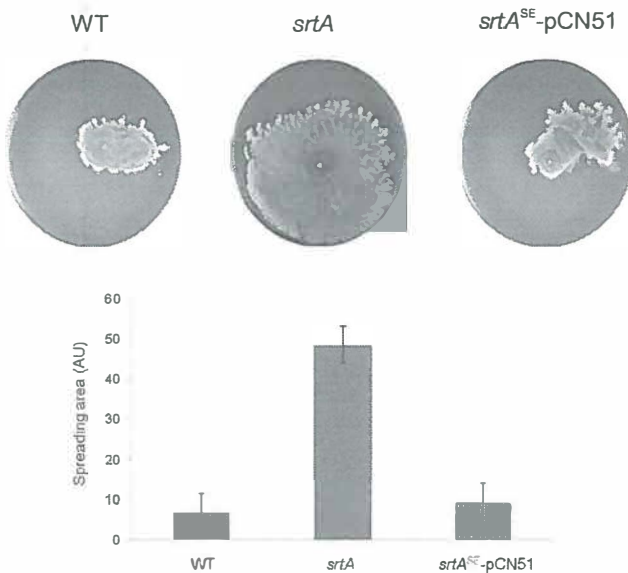

Figure 3. Hyper-spreading phenotype of a *srtA* mutant of *S. epidermidis* 1457. Spreading motility of *S. epidermidis* 1457 (WT), a *srtA* mutant derivative of this strain (*srtA*), and a complemented derivative of the *srtA* mutant (*srtA^Se*-pCN51) was assayed as described for Figure 1.

Chapter 4

Acknowledgements

The sortase A substrates FnbpA, FnbpB, ClfA and ClfB antagonize colony spreading of *Staphylococcus aureus*

References

1. **Arnaud, M., A. Chastanet, and M. Debarbouille.** 2004. New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, Gram-positive bacteria. Appl. Environ. Microbiol. **70:**6887-6891.

2. **Charpentier, E., A. I. Anton, P. Barry, B. Alfonso, Y. Fang, and R. P. Novick.** 2004. Novel cassette-based shuttle vector system for Gram-positive bacteria. Appl. Environ. Microbiol. **70:**6076-6085.

3. **Cheung, A. L., J. M. Koomey, C. A. Butler, S. J. Projan, and V. A. Fischetti.** 1992. Regulation of exoprotein expression in *Staphylococcus aureus* by a locus (*sar*) distinct from *agr*. Proc. Natl. Acad. Sci. U. S. A. **89:**6462-6466.

4. **Cheung, G. Y., K. Rigby, R. Wang, S. Y. Queck, K. R. Braughton, A. R. Whitney, M. Teintze, F. R. DeLeo, and M. Otto.** 2010. *Staphylococcus epidermidis* strategies to avoid killing by human neutrophils. PLoS Pathog. **6:**e1001133.

5. **Corrigan, R. M., H. Miajlovic, and T. J. Foster.** 2009. Surface proteins that promote adherence of *Staphylococcus aureus* to human desquamated nasal epithelial cells. BMC Microbiol. **9:**22.

6. **Dreisbach, A., J. M. van Dijl, and G. Buist.** 2011. The cell surface proteome of *Staphylococcus aureus*. Proteomics **11:**3154-3168.

7. **Dreisbach, A., K. Hempel, G. Buist, M. Hecker, D. Becher, and J. M. van Dijl.** 2010. Profiling the surfacome of *Staphylococcus aureus*. Proteomics **10:**3082-3096.

8. **Driessen, A. J. and N. Nouwen.** 2008. Protein translocation across the bacterial cytoplasmic membrane. Annu. Rev. Biochem. **77:**643-667.

9. **Dunman, P. M., E. Murphy, S. Haney, D. Palacios, G. Tucker-Kellogg, S. Wu, E. L. Brown, R. J. Zagursky, D. Shlaes, and S. J. Projan.** 2001. Transcription profiling-based identification of *Staphylococcus aureus* genes regulated by the agr and/or sarA loci. J. Bacteriol. **183:**7341-7353.

10. **Duthie, E. S. and L. L. Lorenz.** 1952. Staphylococcal coagulase; mode of action and antigenicity. J. Gen. Microbiol. **6:**95-107.

11. **Fischetti, V. A., V. Pancholi, and O. Schneewind.** 1990. Conservation of a hexapeptide sequence in the anchor region of surface proteins from gram-positive cocci. Mol. Microbiol. **4:**1603-1605.

12. **Fitzpatrick, F., H. Humphreys, and J. P. O'Gara.** 2005. Evidence for *icaADBC*-independent biofilm development mechanism in methicillin-resistant *Staphylococcus aureus* clinical isolates. J. Clin. Microbiol. **43:**1973-1976.

13. **Foster, T. J. and M. Hook.** 1998. Surface protein adhesins of *Staphylococcus aureus*. Trends Microbiol. **6:**484-488.

14. **Frankel, B. A., R. G. Kruger, D. E. Robinson, N. L. Kelleher, and D. G. McCafferty.** 2005. *Staphylococcus aureus* sortase transpeptidase SrtA: insight into the kinetic mechanism and evidence for a reverse protonation catalytic mechanism. Biochemistry **44:**11188-11200.

15. **George, N. P., Q. Wei, P. K. Shin, K. Konstantopoulos, and J. M. Ross.** 2006. *Staphylococcus aureus* adhesion via Spa, ClfA, and SdrCDE to immobilized platelets demonstrates shear-dependent behavior. Arterioscler. Thromb. Vasc. Biol. **26:**2394-2400.

16. **Greene, C., D. McDevitt, P. Francois, P. E. Vaudaux, D. P. Lew, and T. J. Foster.** 1995. Adhesion properties of mutants of *Staphylococcus aureus* defective in fibronectin-binding proteins and studies on the expression of *fnb* genes. Mol. Microbiol. **17:**1143-1152.

17. **Grundmeier, M., M. Hussain, P. Becker, C. Heilmann, G. Peters, and B. Sinha.** 2004. Truncation of fibronectin-binding proteins in *Staphylococcus aureus* strain Newman leads to deficient adherence and host cell invasion due to loss of the cell wall anchor function. Infect. Immun. **72:**7155-7163.

18. **Hanada, M., K. Nishiyama, and H. Tokuda.** 1996. SecG plays a critical role in protein translocation in the absence of the proton motive force as well as at low temperature. FEBS Lett. **381:**25-28.

19. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557-580.

Chapter 4

20. **Horsburgh, M. J., J. L. Aish, I. J. White, L. Shaw, J. K. Lithgow, and S. J. Foster.** 2002. σ^B modulates virulence determinant expression and stress resistance: characterization of a functional *rsbU* strain derived from *Staphylococcus aureus* 8325-4. J. Bacteriol. **184:**5457-5467.

21. **House-Pompeo, K., Y. Xu, D. Joh, P. Speziale, and M. Hook.** 1996. Conformational changes in the fibronectin binding MSCRAMMs are induced by ligand binding. J. Biol. Chem. **271:**1379-1384.

22. **Josefsson, E., K. W. McCrea, D. Ni Eidhin, D. O'Connell, J. Cox, M. Hook, and T. J. Foster.** 1998. Three new members of the serine-aspartate repeat protein multigene family of *Staphylococcus aureus.* Microbiology **144 (Pt 12):**3387-3395.

23. **Kaito, C. and K. Sekimizu.** 2007. Colony spreading in *Staphylococcus aureus.* J. Bacteriol. **189:**2553-2557.

24. **Komatsuzawa, H., G. H. Choi, T. Fujiwara, Y. Huang, K. Ohta, M. Sugai, and H. Suginaka.** 2000. Identification of a *fmtA*-like gene that has similarity to other PBPs and beta-lactamases in *Staphylococcus aureus.* FEMS Microbiol. Lett. **188:**35-39.

25. **Kouwen, T. R., E. N. Trip, E. L. Denham, M. J. Sibbald, J. Y. Dubois, and J. M. van Dijl.** 2009. The large mechanosensitive channel MscL determines bacterial susceptibility to the bacteriocin sublancin 168. Antimicrob. Agents Chemother. **53:**4702-4711.

26. **Kreiswirth, B. N., S. Lofdahl, M. J. Betley, M. O'Reilly, P. M. Schlievert, M. S. Bergdoll, and R. P. Novick.** 1983. The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. Nature **305:**709-712.

27. **Kruger, R. G., B. Otvos, B. A. Frankel, M. Bentley, P. Dostal, and D. G. McCafferty.** 2004. Analysis of the substrate specificity of the *Staphylococcus aureus* sortase transpeptidase SrtA. Biochemistry **43:**1541-1551.

28. **Lowy, F. D.** 1998. *Staphylococcus aureus* infections. N. Engl. J. Med. **339:**520-532.

29. **Mack, D., N. Siemssen, and R. Laufs.** 1992. Parallel induction by glucose of adherence and a polysaccharide antigen specific for plastic-adherent *Staphylococcus epidermidis:* evidence for functional relation to intercellular adhesion. Infect. Immun. **60:**2048-2057.

30. **Marraffini, L. A., A. C. Dedent, and O. Schneewind.** 2006. Sortases and the art of anchoring proteins to the envelopes of Gram-positive bacteria. Microbiol. Mol. Biol. Rev. **70:**192-221.

31. **Mazmanian, S. K., G. Liu, H. Ton-That, and O. Schneewind.** 1999. *Staphylococcus aureus* sortase, an enzyme that anchors surface proteins to the cell wall. Science **285:**760-763.

32. **Mazmanian, S. K., G. Liu, E. R. Jensen, E. Lenoy, and O. Schneewind.** 2000. *Staphylococcus aureus* sortase mutants defective in the display of surface proteins and in the pathogenesis of animal infections. Proc. Natl. Acad. Sci. U. S. A. **97:**5510-5515.

33. **McCarthy, A. J. and J. A. Lindsay.** 2010. Genetic variation in *Staphylococcus aureus* surface and immune evasion genes is lineage associated: implications for vaccine design and host-pathogen interactions. BMC Microbiol. **10:**173.

34. **McGavin, M. J., C. Zahradka, K. Rice, and J. E. Scott.** 1997. Modification of the *Staphylococcus aureus* fibronectin binding phenotype by V8 protease. Infect. Immun. **65:**2621-2628.

35. **Merino, N., A. Toledo-Arana, M. Vergara-Irigaray, J. Valle, C. Solano, E. Calvo, J. A. Lopez, T. J. Foster, J. R. Penades, and I. Lasa.** 2009. Protein A-mediated multicellular behavior in *Staphylococcus aureus.* J. Bacteriol. **191:**832-843.

36. **Meulemans, L., K. Hermans, L. Duchateau, and F. Haesebrouck.** 2007. High and low virulence *Staphylococcus aureus* strains in a rabbit skin infection model. Vet. Microbiol. **125:**333-340.

37. **Naik, M. T., N. Suree, U. Ilangovan, C. K. Liew, W. Thieu, D. O. Campbell, J. J. Clemens, M. E. Jung, and R. T. Clubb.** 2006. *Staphylococcus aureus* Sortase A transpeptidase. Calcium promotes sorting signal binding by altering the mobility and structure of an active site loop. J. Biol. Chem. **281:**1817-1826.

38. **Nandakumar, R., M. P. Nandakumar, M. R. Marten, and J. M. Ross.** 2005. Proteome analysis of membrane and cell wall associated proteins from *Staphylococcus aureus.* J. Proteome Res. **4:**250-257.

39. **Ni Eidhin, D., S. Perkins, P. Francois, P. Vaudaux, M. Hook, and T. J. Foster.** 1998. Clumping factor B (ClfB), a new surface-located fibrinogen-binding adhesin of *Staphylococcus aureus.* Mol. Microbiol. **30:**245-257.

40. **Norrander, J., T. Kempe, and J. Messing.** 1983. Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. Gene **26:**101-106.

41. **Novick, R.** 1967. Properties of a cryptic high-frequency transducing phage in *Staphylococcus aureus.* Virology **33:**155-166.

42. **Novick, R. P.** 1991. Genetic systems in staphylococci. Methods Enzymol. **204:**587-636.

43. **O'Gara, J. P. and H. Humphreys.** 2001. *Staphylococcus epidermidis* biofilms: importance and implications. J. Med. Microbiol. **50:**582-587.

44. **O'Neill, E., H. Humphreys, and J. P. O'Gara.** 2009. Carriage of both the *fnbA* and *fnbB* genes and growth at 37 degrees C promote FnBP-mediated biofilm development in meticillin-resistant *Staphylococcus aureus* clinical isolates. J. Med. Microbiol. **58:**399-402.

45. **O'Neill, E., C. Pozzi, P. Houston, H. Humphreys, D. A. Robinson, A. Loughman, T. J. Foster, and J. P. O'Gara.** 2008. A novel *Staphylococcus aureus* biofilm phenotype mediated by the fibronectin-binding proteins, FnBPA and FnBPB. J. Bacteriol. **190:**3835-3850.

46. **Paetzel, M., R. E. Dalbey, and N. C. Strynadka.** 2000. The structure and mechanism of bacterial type I signal peptidases. A novel antibiotic target. Pharmacol. Ther. **87:**27-49.

47. **Papanikou, E., S. Karamanou, and A. Economou.** 2007. Bacterial protein secretion through the translocase nanomachine. Nat. Rev. Microbiol. **5:**839-851.

48. **Peacock, S. J., I. de Silva, and F. D. Lowy.** 2001. What determines nasal carriage of *Staphylococcus aureus*? Trends Microbiol. **9:**605-610.

49. **Periasamy, S., H. S. Joo, A. C. Duong, T. H. Bach, V. Y. Tan, S. S. Chatterjee, G. Y. Cheung, and M. Otto.** 2012. How *Staphylococcus aureus* biofilms develop their characteristic structure. Proc. Natl. Acad. Sci. U. S. A.

50. **Roche, F. M., R. Massey, S. J. Peacock, N. P. Day, L. Visai, P. Speziale, A. Lam, M. Pallen, and T. J. Foster.** 2003. Characterization of novel LPXTG-containing proteins of *Staphylococcus aureus* identified from genome sequences. Microbiology **149:**643-654.

51. **Rusch, S. L. and D. A. Kendall.** 2007. Interactions that drive Sec-dependent bacterial protein transport. Biochemistry **46:**9665-9673.

52. **Sardis, M. F. and A. Economou.** 2010. SecA: a tale of two protomers. Mol. Microbiol. **76:**1070-1081.

53. **Savolainen, K., L. Paulin, B. Westerlund-Wikstrom, T. J. Foster, T. K. Korhonen, and P. Kuusela.** 2001. Expression of *pls*, a gene closely associated with the *mecA* gene of methicillin-resistant *Staphylococcus aureus*, prevents bacterial adhesion in vitro. Infect. Immun. **69:**3013-3020.

54. **Scott, J. R. and T. C. Barnett.** 2006. Surface proteins of Gram-positive bacteria and how they get there. Annu. Rev. Microbiol. **60:**397-423.

55. **Sibbald, M. J., A. K. Ziebandt, S. Engelmann, M. Hecker, A. de Jong, H. J. Harmsen, G. C. Raangs, I. Stokroos, J. P. Arends, J. Y. Dubois, and J. M. van Dijl.** 2006. Mapping the pathways to staphylococcal pathogenesis by comparative secretomics. Microbiol. Mol. Biol. Rev. **70:**755-788.

56. **Sibbald, M. J., T. Winter, M. M. van der Kooi-Pol, G. Buist, E. Tsompanidou, T. Bosma, T. Schafer, K. Ohlsen, M. Hecker, H. Antelmann, S. Engelmann, and J. M. van Dijl.** 2010. Synthetic effects of *secG* and *secY2* mutations on exoproteome biogenesis in *Staphylococcus aureus*. J. Bacteriol. **192:**3788-3800.

57. **Suree, N., C. K. Liew, V. A. Villareal, W. Thieu, E. A. Fadeev, J. J. Clemens, M. E. Jung, and R. T. Clubb.** 2009. The structure of the *Staphylococcus aureus* sortase-substrate complex reveals how the universally conserved LPXTG sorting signal is recognized. J. Biol. Chem. **284:**24465-24477.

58. **Toledo-Arana, A., N. Merino, M. Vergara-Irigaray, M. Debarbouille, J. R. Penades, and I. Lasa.** 2005. *Staphylococcus aureus* develops an alternative, *ica*-independent biofilm in the absence of the *arlRS* two-component system. J. Bacteriol. **187:**5318-5329.

59. **Ton-That, H., S. K. Mazmanian, K. F. Faull, and O. Schneewind.** 2000. Anchoring of surface proteins to the cell wall of *Staphylococcus aureus*. Sortase catalyzed in vitro transpeptidation reaction using LPXTG peptide and NH(2)-Gly(3) substrates. J. Biol. Chem. **275:**9876-9881.

Chapter 4

60. **Ton-That, H., G. Liu, S. K. Mazmanian, K. F. Faull, and O. Schneewind.** 1999. Purification and characterization of sortase, the transpeptidase that cleaves surface proteins of *Staphylococcus aureus* at the LPXTG motif. Proc. Natl. Acad. Sci. U. S. A. **96:**12424-12429.
61. **Tsompanidou, E., M. J. Sibbald, M. A. Chlebowicz, A. Dreisbach, J. W. Back, J. M. van Dijl, G. Buist, and E. L. Denham.** 2011. Requirement of the *agr* locus for colony spreading of *Staphylococcus aureus*. J. Bacteriol. **193:**1267-1272.
62. **Tuteja, R.** 2005. Type I signal peptidase: an overview. Arch. Biochem. Biophys. **441:**107-111.
63. **van Roosmalen, M. L., N. Geukens, J. D. Jongbloed, H. Tjalsma, J. Y. Dubois, S. Bron, J. M. van Dijl, and J. Anne.** 2004. Type I signal peptidases of Gram-positive bacteria. Biochim. Biophys. Acta **1694:**279-297.
64. **Vancraeynest, D., K. Hermans, and F. Haesebrouck.** 2004. Genotypic and phenotypic screening of high and low virulence *Staphylococcus aureus* isolates from rabbits for biofilm formation and MSCRAMMs. Vet. Microbiol. **103:**241-247.
65. **Veenendaal, A. K., C. van der Does, and A. J. Driessen.** 2004. The protein-conducting channel SecYEG. Biochim. Biophys. Acta **1694:**81-95.
66. **Vergara-Irigaray, M., J. Valle, N. Merino, C. Latasa, B. Garcia, I. Ruiz de Los Mozos, C. Solano, A. Toledo-Arana, J. R. Penades, and I. Lasa.** 2009. Relevant role of fibronectin-binding proteins in *Staphylococcus aureus* biofilm-associated foreign-body infections. Infect. Immun. **77:**3978-3991.
67. **Wolz, C., D. McDevitt, T. J. Foster, and A. L. Cheung.** 1996. Influence of *agr* on fibrinogen binding in *Staphylococcus aureus* Newman. Infect. Immun. **64:**3142-3147.
68. **Yao, Y., D. E. Sturdevant, and M. Otto.** 2005. Genomewide analysis of gene expression in *Staphylococcus epidermidis* biofilms: insights into the pathophysiology of *S. epidermidis* biofilms and the role of phenol-soluble modulins in formation of biofilms. J. Infect. Dis. **191:**289-298.

*"To live in the world without becoming aware of the meaning of the world
is like wandering about in a great library without touching the books."*

Dan Brown – The Lost Symbol

Chapter 5

Partially overlapping substrate specificities of staphylococcal group A sortases

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Chapter 5

Abstract

Sortases catalyze the covalent attachment of proteins with a C-terminal LPxTG motif to the cell walls of Gram-positive bacteria. Here we show that deletion of the *srtA* genes of *Staphylococcus aureus* and *Staphylococcus epidermidis* resulted in the dislocation of several LPxTG proteins from the cell wall to the growth medium. Nevertheless, proteomics and Western blotting analyses revealed that substantial amounts of the identified proteins remained cell wall-bound through non-covalent interactions. The protein dislocation phenotypes of *srtA* mutants of *S. aureus* and *S. epidermidis* were reverted by ectopic expression of *srtA* genes of either species. Interestingly, *S. epidermidis* contains a second sortase A, which was previously annotated as 'SrtC'. Ectopic expression of this SrtC in *srtA* mutant cells reverted the dislocation of some, but not all, cell wall-associated proteins. Similarly, defects in biofilm formation were reverted by ectopic expression of SrtC in some, but not all, tested *srtA* mutant strains. Finally, overexpression of SrtA resulted in increased levels of biofilm formation in some tested strains. Taken together, these findings show that the substrate specificities of SrtA and SrtC overlap partially, and that sortase levels may be limiting for biofilm formation in some staphylococci.

Partially overlapping substrate specificities of staphylococcal group A sortases

Introduction

Staphylococcus aureus and *Staphylococcus epidermidis* are both part of the normal human microbiota (6, 49, 51, 65). However, both organisms have the potential to cause life-threatening infections, especially when they become invasive and reach the blood stream. In addition, *S. aureus* and especially *S. epidermidis* are notorious for their ability to form biofilms on medical devices and implants (21, 50). Cell surface-associated proteins play crucial roles in the colonization and invasion of host tissues by staphylococci, and such proteins also have important roles in biofilm formation. The surface-exposed proteins can either be covalently or non-covalently linked to the bacterial cell wall. Covalent protein linkage to the peptidoglycan moiety of the cell wall is catalyzed by specific transpeptidases known as sortases (39, 55).

Gram-positive bacteria, such as *S. aureus* and *S. epidermidis*, but also various bacilli, streptococci and corynebacteria have one or more sortase-encoding genes (17, 57). For several pathogens, such as *S. aureus*, *Listeria monocytogenes* and *Streptococcus pneumoniae*, it has been shown that the deletion of sortase genes results in decreased virulence (7, 24, 28, 42). This underscores the importance of covalent cell wall attachment of particular proteins in the pathogenicity of these Gram-positive bacteria. Based on structural and functional criteria, Dramsi *et al.* (17) classified sortases into four different groups (i.e. A-D; Fig. 1). Sortase A (SrtA) enzymes link several proteins with LPxTG or LPxAG motifs to the cell wall. Such proteins are mainly involved in adhesion to specific organ tissues, survival during phagocytosis, and invasion of host cells. The LPxTG or LPxAG motifs are cleaved by sortase between the Thr/Ala and Gly residues (39, 55). Formation of an amide bond between the Thr or Ala residue of the surface protein and the pentaglycine cross-bridge of branched lipid II completes the covalent protein attachment to the cell wall. In contrast to sortase A, most sortase B (SrtB) enzymes are involved in iron metabolism. In *S. aureus* the *srtB* gene is part of the *isd* operon, which contains several genes *(isdA-G)* that are important for iron-acquisition (43). SrtB recognizes the NPQTN motif in the C-terminus of the IsdC protein, and cleaves this motif C-terminally of the Thr residue thereby linking IsdC to the cell wall (41). Class C sortases (SrtC) are found in several different Gram-positive bacteria (17, 38, 55, 62). In *Corynebacterium diphtheriae* it has been shown that SrtC is necessary for the formation of pili (61). SrtC is responsible for the processing of pilus subunits and their linkage to a neighboring subunit (17)(28). Whereas SrtA seems to handle a wide range of different substrates with LPxTG motifs, SrtC seems to be dedicated to the processing of only a few specific substrates, in some cases even only one substrate (3, 28, 29, 61). Several Gram-positive bacteria have a class D sortase (17). The class D sortase of *Streptomyces coelicolor* links several proteins to the surfaces of aerial hyphae and spores (12, 20). *Bacillus subtilis* 168 also has a SrtD-encoding gene named *yhcS* (23).

All sequenced *Staphylococcus* species contain multiple genes encoding proteins with LPxTG motifs for cell wall sorting. In total, twenty-one proteins with this motif have been identified in *S. aureus* and twelve in *S. epidermidis* (56). One of the proteins with a C-terminal LPxTG motif

is the *S. aureus* surface protein G (SasG; (52)). This protein is involved in adhesion to nasal epithelial cells (52). Interestingly, SasG is also involved in protein-based biofilm formation, independent of the *ica*-encoded polysaccharide for intercellular adhesion (PIA) or poly-N-acetyl glucosamine (PNAG) (15). The homologue of SasG in *S. epidermidis* is the accumulation-associated protein (Aap), which has been implicated in the formation of both polysaccharide- (32) and protein-based biofilms (4, 53, 54). Specifically, the so-called G5 domains of SasG and Aap are required for biofilm formation (14, 15, 53). In addition to SasG, also other SrtA substrates of *S. aureus* like Bap, fibronectin-binding proteins (FnbPs) and protein A have been implicated in protein-dependent biofilm formation (36, 40, 45, 64). Accordingly, SrtA was shown to be necessary for protein-dependent biofilm formation of *S. aureus* (36).

The *S. epidermidis* ATCC12228 strain contains a *srtC* gene in addition to the *srtA* gene (13). This *srtC* gene lies on a genomic island (*v*Se2) that also contains the genes for the LPxTG surface proteins SesJ and SesK (25). These findings raised the question to what extent staphylococcal SrtC can complement the function of SrtA. To answer this question and to increase our understanding of sortase function in *S. aureus* and *S. epidermidis*, we first performed a phylogenetic analysis which revealed that the *S. epidermidis* 'SrtC' groups with the known SrtA proteins. This SrtC should therefore be regarded as a group A sortase (Fig. 1). We then investigated whether *srtA* mutations were complemented by different sortases from the related Firmicutes *S. aureus*, *S. epidermidis* and *B. subtilis*. Interestingly, our results show that SrtC of *S. epidermidis* can partially complement the absence of SrtA in both *Staphylococcus* species. This implies that these group A sortases have partially overlapping substrate specificities.

Partially overlapping substrate specificities of staphylococcal group A sortases
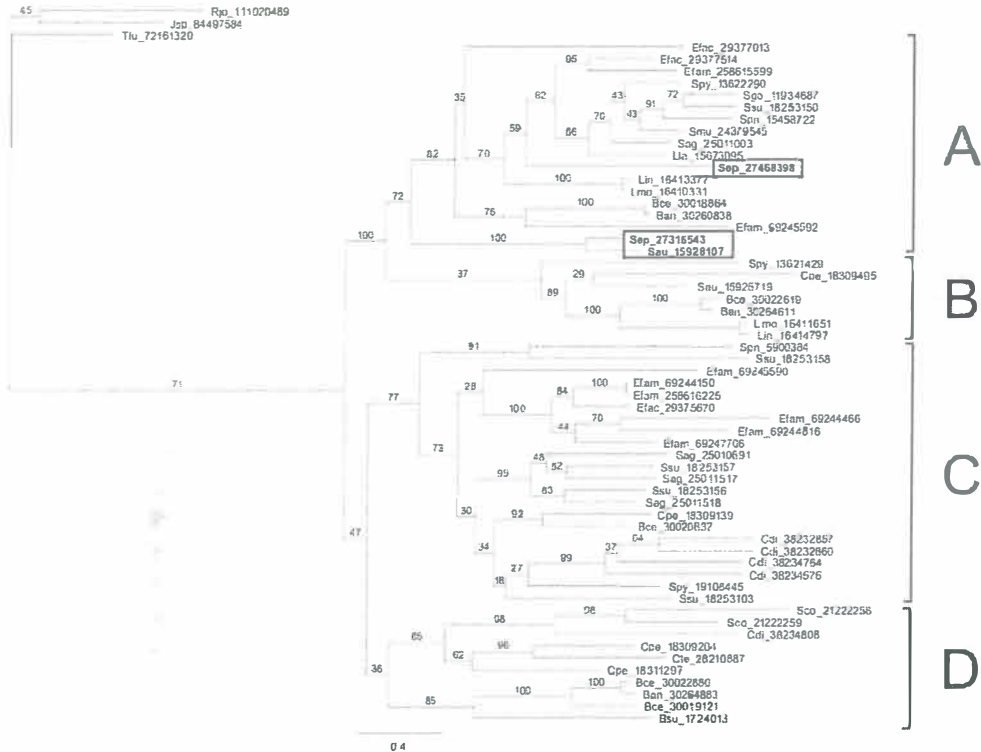
Figure 1. Maximum likelihood tree of sortases from Gram-positive bacteria. Names of bacteria are abbreviated as follows: Ban, *Bacillus anthracis*; Bce, *Bacillus cereus*; Bsu, *Bacillus subtilis*; Cdi, *Corynebacterium diphtheriae*; Cpe, *Clostridium perfringens*; Cte, *Clostridium tetani*; Efac, *Enterococcus faecalis*; Efam, *Enterococcus faecium*; Lin, *Listeria innocua*; Lmo, *Listeria monocytogenes*; Lla, *Lactococcus lactis*; Sau, *S. aureus*; Sep, *S. epidermidis*; Sag, *S. agalactiae*; Sgo, *Streptococcus gordonii*; Smu, *Streptococcus mutans*; Spn, *S. pneumoniae*; Spy, *Streptococcus pyogenes*; Ssu, *Streptococcus suis*; Sco, *S. coelicolor*. Sequence accession numbers are shown for all aligned proteins. The outgroup is represented by *Janibacter* sp. HTCC2649, *T. fusca* YX, and *R. jostii* RHA1. The sortase classes (A-D) as proposed by Dramsi *et al.* (17) are indicated. Sortases used in these studies are boxed and bootstrap values are indicated at the branches. *S. epidermidis* SrtA has the accession number Sep_27316543, and *S. epidermidis* SrtC has the accession number Sep_27468398.

Chapter 5

Material and Methods

Bacterial strains

All strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown in Luria-Bertani broth (LB) at 37°C. Unless stated otherwise, *S. aureus* and *S. epidermidis* strains were grown on tryptic soy broth (TSB), tryptic soy agar (TSA) or brain heart infusion (BHI) at 37°C. Where necessary, antibiotics were added as follows: ampicillin, 100 µg/ml; erythromycin for *E. coli* 100 µg/ml, and for *S. aureus* or *S. epidermidis* 5 µg/ml; kanamycin, 20 µg/ml; chloramphenicol, 15 µg/ml. To monitor β-galactosidase activity in cells of *E. coli*, *S. aureus* or *S. epidermidis*, 80 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) was added to the plates.

Sequence alignment and tree reconstruction

Sortase sequences were retrieved from NCBI using GenBank as the database. These protein sequences were aligned using the MUSCLE software (19) that is implemented in the Geneious Pro program, version 4.8.2 (18) (www.geneious.com). Minor changes were introduced manually in the alignment, such as the removal of multiple insertions and deletions. Phylogenetic trees were constructed by the maximum likelihood method using PhyML (26), which is implemented in the Geneious program. Bootstrap values were calculated with 100 replications. Three homologous sortase sequences of the bacteria *Janibacter* sp. HTCC2649, *Thermobifida fusca* YX, and *Rhodococcus jostii* RHA1 were included in the analysis as outgroups to root the tree.

Construction of sortase mutants

Mutants of *S. aureus* and *S. epidermidis* were constructed using the temperature-sensitive plasmid pMAD (2) and previously described procedures (34). Primers (Table 2) were designed using the genome sequences of *S. aureus* NCTC8325 and *S. epidermidis* RP62A. All mutant strains were checked by isolation of genomic DNA using the GenElute™ Bacterial Genomic DNA Kit (Sigma) and PCR with specific primers.

Partially overlapping substrate specificities of staphylococcal group A sortases

Table 1.: Bacterial strains and plasmids used

Plasmids	Properties	Reference
TOPO	pCR®-Blunt II-TOPO® vector; Km^R	Invitrogen Life technologies
"*srtA*"-TOPO	TOPO plasmid containing the flanking regions of *S. aureus srtA*, Kan^R	This work
"*srtA*"SE-TOPO	TOPO plasmid containing the flanking regions of *S. epidermidis srtA*, Kan^R	This work
pUC18	Amp^R, ColE1, F80dLacZ, *lac* promoter	(47)
srtC-pUC18	pUC18 plasmid with the *S. epidermidis srtC* gene, Amp^R	This work
pMAD	*E. coli* - *S. aureus* shuttle vector that is temperature-sensitive in *S. aureus* and contains the *bgaB* gene, Ery^R, Amp^R	(2)
pCN51	*E. coli* - *S. aureus* shuttle vector that contains a cadmium-inducible promoter	(10)
Sa-srtA-pCN51	pCN51 with *S. aureus srtA* gene, Amp^R; Ery^R	This work
Se-srtA-pCN51	pCN51 with *S. epidermidis srtA* gene, Amp^R; Ery^R	This work
Se-srtC-pCN51	pCN51 with *S. epidermidis* ATCC12228 *srtC* gene, Amp^R; Ery^R	This work
Bs-yhcS-pCN51	pCN51 with *B. subtilis yhcS* gene, Amp^R; Ery^R	This work
pRIT5H	*E. coli* – *S. aureus* shuttle vector that contains the *spa*-promoter, Cm^R	(46)
Sa-srtA-pRIT5H	pRIT5H with *S. aureus srtA* gene, Cm^R	This work
Se-srtC-pRIT5H	pRIT5H with *S. epidermidis srtC* gene, Cm^R	This work

Strains	Genotype	Reference
E. coli		
DH5α	*supE44*; *hsdR17*; *recA1*; *gyrA96*; thi-1; *relA1*	(27)
TOP10	Cloning host for TOPO vector; F *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *recA1 araD139* Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *endA1 nup*G	Invitrogen Life technologies
S. aureus RN4220		
Parental strain	Restriction-deficient derivative of NCTC 8325, cured of all known prophages	(35)
Δ*srtA*	*srtA*	This work
Δ*srtA* Δ*spa*	*srtA spa*; replacement of *spa* by kanamycin resistance marker; Kan^R	This work
Δ*srtA*-comp-Sa	Δ*srtA* strain complemented with *S. aureus srtA* through *Sa-srtA*-pCN51	This work
Δ*srtA*-comp-Se	Δ*srtA* strain complemented with *S. epidermidis srtA* through *Se-srtA*-pCN51	This work
Δ*yhcS*-comp-Bs	Δ*srtA* strain complemented with *B. subtilis yhcS* through *Bs-yhcS*-pCN51	This work
Δ*srtA*-comp-*srtC*	Δ*srtA* strain complemented with *S. epidermidis srtC* through *Se-srtC*-pCN51	This work

Chapter 5

S. aureus SH1000		
Parental strain	Functional *rsbU+* derivative of 8325-4 *rsbU+*, *agr+*	(31)
Δ*srtA*	*srtA*	This work
Δ*srtA* Δ*spa*	*srtA spa*; replacement of *spa* by kanamycin resistance marker; Kan^R	This work
Δ*srtA*-comp-Sa	Δ*srtA* complemented with *S. aureus srtA* through *Sa-srtA*-pCN51	This work
Δ*srtA*-comp-Se	Δ*srtA* complemented with *S. epidermidis srtA* through *Se-srtA*-pCN51	This work
Δ*yhcS*-comp-Bs	Δ*srtA* complemented with *B. subtilis yhcS* through *Bs-yhcS*-pCN51	This work
Δ*srtA*-comp-*srtC*	Δ*srtA* complemented with *S. epidermidis srtC* through *Se-srtC*-pCN51	This work
sasG-pMUTIN4	Overexpression of SasG due to integrated pMUTIN4 plasmid; Ery^R	(15)
sasG-pMUTIN4 Δ*srtA*	Δ*srtA*; overexpression of SasG due to integrated pMUTIN4 plasmid; Ery^R	This Work
sasG-pMUTIN4 Δ*srtA* comp-*srtA*	Δ*srtA*; overexpression of SasG due to integrated pMUTIN4 plasmid; complemented with *S. aureus srtA* through *Sa-srtA*-pRIT5H; Ery^R, Cm^R	This Work
sasG-pMUTIN4 Δ*srtA* comp-*srtC*	Δ*srtA*; overexpression of SasG due to integrated pMUTIN4 plasmid; complemented with *S. epidermidis srtC* through *Se-srtC*-pRIT5H; Ery^R, Cm^R	This Work
S. epidermidis 1457		
Parental	Biofilm positive strain	(37)
Δ*srtA*	*srtA*	This work
Δ*srtA*-comp-Sa	Δ*srtA*; complemented with *S. aureus srtA* through *Sa-srtA*-pCN51	This work
Δ*srtA*-comp-Se	Δ*srtA*; complemented with *S. epidermidis srtA* through *Se-srtA*-pCN51	This work
Δ*yhcS*-comp-Bs	Δ*srtA*; complemented with *B. subtilis yhcS* through *Bs-yhcS*-pCN51	This work
Δ*srtA*-comp-*srtC*	Δ*srtA*; complemented with *S. epidermidis srtC* through *Se-srtC*-pCN51	This work

Partially overlapping substrate specificities of staphylococcal group A sortases

Table 2. Primers used in these studies

Primer	Sequence (5'→3')
	Construction of *S. aureus srtA* mutant
srtA-F1	AATGGTGTAGTAATTGACTAG
srtA-R1	**TTACGTCAGTCAGTCACCATGGC**AACGTTAAGGCTCCTTTTATAC
srtA-F2	**TGCCATGGTGACTGACTGACGTAA**TCTATTACGCTAATGGATGAA
srtA-R2	CTCACATTACTTACTATTAAT
	Construction of *S. epidermidis srtA* mutant
esrtA-F1	AACTTTGTTCTTTAGCGTAACGAAT
esrtA-R1	**TGCCATGGTGACTGACTGACGTAA**TTATGTTACTCCTTTATATTTATT
esrtA-F2	**TTACGTCAGTCAGTCACCATGGCA**TATTCTTATAAGTGAAAGATACGTA
esrtA-R2	CTTTATAGATGACTGCTCCAT
	Complementation in *S. aureus*
srtA-F3	CAGCC<u>GGATCC</u>AATGTATAAAAGGAGCCTTAACGT (*Bam*HI)
srtA-R3	<u>CGGAATTCTT</u>ATTTGACTTCTGTAGCTACAAA (*Eco*RI)
srtA-F4	GGGGG<u>GGATCC</u>TTAACAGGCATTGTGAAATGT (*Bam*HI)
srtA-R4	GGGGG<u>GTCGAC</u>CCTTATTTGACTTCTGTAGCT (*Sal*I)
esrtA-F3	CAGCC<u>GGATCC</u>AATGTATAAAAGGAGCCTTAACGTATGAAGCAGTGGATGAATAGA (*Bam*HI)
esrtA-R3	CG<u>GAATTC</u>TTAGTTAATTTGTGTAGCTATGAA (*Eco*RI)
yhcS-F1	AAAA<u>CTGCAG</u>AATGTATAAAAGGAGCCTTAACGTATGAAAAAAGTTATTCCACTA (*Pst*I)
yhcS-R1	CAGCC<u>GGATCC</u>TTAAGTCACTCGTTTTCCATATAT (*Bam*HI)
esrtC-F1	GGGGG<u>GTCGAC</u>TGAGGAGGTACATATGAGTGC (*Sal*I)
esrtC-R1	GGGGG<u>GGATCC</u>ATTTATAATTTGAAAATACCA (*Bam*HI)
esrtC-F2	GGGGG<u>GGATCC</u>TGAGGAGGTACATATGAGTGC (*Bam*HI)
esrtC-R2	GGGGG<u>GTCGAC</u>ATTTATAATTTGAAAATACCA (*Sal*I)
	Complementation in *S. epidermidis*
srtA-F5	CAGCC<u>GGATCC</u>*AAATAAATATAAAGG*AGTAACATAAATGAAAAAATGGACAAATCG (*Bam*HI)
srtA-R5	<u>CGGAATTCTT</u>ATTTGACTTCTGTAGCTACAAA (*Eco*RI)
esrtA-F4	GGGGG<u>GGATCC</u>AAATAAATATAAAGGAGTAACATAA (*Bam*HI)
esrtA-R4	GGGGG<u>GAATTC</u>TTAGTTAATTTGTGTAGCTATGA (*Eco*RI)
yhcS-F2	AAAA<u>CTGCAG</u> *AAATAAATATAAAGG*AGTAACATAAATGAAAAAAGTTATTCCACTA (*Pst*I)
yhcS-R2	CAGCC<u>GGATCC</u>TTAAGTCACTCGTTTTCCATATAT (*Bam*HI)

Overlapping parts are shown in bold and restriction sites are underlined and shown in parentheses

To delete the *srtA* genes, primer pairs with the designations F1/R1 and F2/R2 were used for PCR amplification of the respective upstream and downstream regions (each ~500 bp), and their fusion with 21-24 bp linkers. The fused flanking regions were cloned in pMAD, and the resulting plasmids were used to transform *S. aureus* RN4220. To delete the *srtA* gene from the *S. aureus* SH1000 genome, the respective pMAD construct was transferred from the RN4220 strain to the SH1000 strain by transduction with phage φ85 (48). To delete *srtA* from *S. epidermidis*, the respective pMAD construct was transferred from the RN4220 strain to *S. epidermidis* 1457 by electrotransformation.

Chapter 5

Complementation of srtA mutations

Primer pairs for PCR amplification of *srtA* from *S. aureus*, *srtA* from *S. epidermidis*, *yhcS* from *B. subtilis* and *srtC* from *S. epidermidis* are listed in Table S1. For expression in *S. aureus* the RBS and start codon of *S. aureus srtA* were used, and for expression in *S. epidermidis* the RBS and start codon of *S. epidermidis srtA* were used. PCR products were purified using the PCR Purification Kit (Roche), and ligated into the pUC18 plasmid (47). The cloned sortase genes were then excised from the resulting constructs with restriction enzymes as specified in Table S1, and ligated into plasmids pCN51 (10) or pRIT5H (46) that were cut with the same enzymes. The obtained plasmids were introduced in *S. aureus* RN4220 by electro-transformation and colonies were selected on TSA plates containing erythromycin. Subsequently, the plasmids were transferred from *S. aureus* RN4220 to *S. aureus* SH1000 strains via transduction as described above, or to *S. epidermidis* 1457 strains via electro-transformation of competent cells with the purified plasmids.

Under standard laboratory growth conditions, no SasG production is detectable in *S. aureus* (52). To study the localization of SasG, we used strains in which the expression of SasG is directed by the IPTG-inducible Pspac-promoter of plasmid pMUTIN4 (kindly provided by T. Foster (15)). The *sasG*-pMUTIN4 plasmid was transferred to the *S. aureus* SH1000 Δ*srtA* strain by transduction as described above. Since *sasG*-pMUTIN4 carries an erythromycin resistance marker, strains containing this plasmid cannot be transformed with derivatives of plasmid pCN51. Therefore, the pRIT5H plasmid (46) was used for complementation experiments with *S. aureus srtA* or *S. epidermidis srtC* in the *S. aureus* Δ*srtA sasG*-pMUTIN4 strain. Genes cloned in pRIT5H are transcribed from the *S. aureus spa* promoter. The *srtA* and *srtC* genes were PCR amplified with primer pairs *srtA*-F4/R4 and *srtC*-F2/R2, respectively, and cloned into the pRIT5H plasmid using restriction enzymes specified in Table S1. The resulting plasmids were introduced into *S. aureus* RN4220 strains containing *sasG*-pMUTIN4 via electro-transformation, and they were subsequently transferred to *sasG*-pMUTIN4-containing *S. aureus* SH1000 strains via transduction. Transformants and transductants were selected on TSA plates with erythromycin and chloramphenicol.

Cell fractionation, SDS-PAGE, and Western blotting

Overnight cultures were diluted to an OD_{600} of 0.05 and grown in 25 ml TSB under vigorous shaking. For complementation of mutant strains with pCN51-based plasmids, $CdSO_4$ was added after three hours of growth to a final concentration of 0.25 µM. For complementation of mutant strains with pRIT5H-based plasmids, IPTG was added after 3 h of growth to a final concentration of 1 mM. Samples were taken after 6 h of growth and separated in growth medium, whole cell and non-covalently cell wall-bound protein fractions. Cells were separated from the growth medium by centrifugation of 1 ml of the culture. The proteins in the growth medium were precipitated with 250 µl 50% trichloroacetic acid (TCA), washed with acetone and dissolved in 100 µl Loading Buffer (Invitrogen). Cells were resuspended in 300 µl Loading

Partially overlapping substrate specificities of staphylococcal group A sortases

Buffer and disrupted with glass beads using the Precellys®24 bead beating homogenizer (Bertin Technologies). From the same culture 20 ml was used for the extraction of non-covalently bound cell wall proteins using KSCN. Cells were collected by centrifugation, washed with PBS, and incubated for 10 min with 1M KSCN on ice. After centrifugation the non-covalently cell wall-bound proteins were precipitated from the supernatant fraction with TCA, washed with acetone and dissolved in 100 µl Loading Buffer. Upon addition of Reducing Agent (Invitrogen), the samples were incubated at 95°C. Proteins were separated by SDS-PAGE using precast NuPage gels (Invitrogen) and subsequently blotted onto a nitrocellulose membrane (Protran®, Schleicher & Schuell). The presence of Aap, SasG or Clumping factor A (ClfA) was monitored by immunodetection with specific polyclonal antibodies raised in rabbits at 1:10.000 dilution. These antibodies were kind gifts from D. Mack (Aap) (53) and T. Foster (SasG and ClfA) (44, 52). Bound primary antibodies were visualized using fluorescent IgG secondary antibodies (IRDye 800 CW goat anti-rabbit from LiCor Biosciences). Membranes were scanned for fluorescence at 800 nm using the Odyssey Infrared Imaging System (LiCor Biosciences).

Protein identification by mass spectrometry

Proteins were separated by SDS-PAGE and gels were stained with SimplyBlue™ SafeStain (Invitrogen). Protein bands were cut from the gels (E. Tsompanidou, unpublished data available on request) and in-gel digestion of the proteins was performed as described by Eymann *et al.* 2004 (22). All peptides obtained from an in-gel digestion were separated by liquid chromatography and measured online by ESI mass spectrometry. LC-MS/MS analyses were performed using a nanoACQUITY UPLC™ system (Waters) coupled to an LTQ Orbitrap™ or LTQ-FTICR mass spectrometer (Thermo Fisher Scientific,Waltham, MA) creating an electro spray by the application of 1.5 kV between Picotip™ Emitter (SilicaTip™, FS360-20-10 Coating P200P, New Objective) and transfer capillary. Peptides were loaded onto a trap column (nanoAcquity UPLC ™ column, Symmetry® C18, 5 µm, 180 µm inner diameter x 20 mm, Waters) and washed 3 min with 99% buffer A (0.1% (v/v) acetic acid) with a flow rate of 10 µl/min. Elution was performed onto an analytical column (nanoAcquity UPLC ™ column, BEH130 C18 1.7 µm, 100 µm inner diameter x 100 mm, Waters) by a binary gradient of buffer A and B (100% (v/v) acetonitrile, 0.1% (v/v) acetic acid) over a period of 80 min with a flow rate of 400 nl/ min.

For MS/MS analysis full survey scans were performed in the Orbitrap or FTICR (m/z 300–2000) with resolutions of 30,000 in the Orbitrap or 50,000 for the FTICR respectively. The full scan was followed by MS/MS experiments of the five most abundant precursor ions acquired in the LTQ via CID. Precursors were dynamically excluded for 30 s, and unassigned charge states and singly charged ions were rejected.

For protein identification tandem mass spectra were extracted using Sorcerer™ v3.5 (Sage-N Research, Inc. Milpitas, CA). Charge state deconvolution and deisotoping were not performed. All MS/MS samples were analyzed using Sequest (ThermoFinnigan, San Jose, CA; version v.27,

rev. 11), applying the following search parameters: peptide tolerance, 10 ppm; tolerance for fragmentions, 1 amu; b- and y-ion series; an oxidation of methionine (15.99 Da) was considered as variable modification (maximal three modifications per peptide). Sequest was set up to search the *S. epidermidis* RP62A database (extracted from NCBI, including concatenated reverse database, 4600 entries) assuming the digestion enzyme trypsin. For *S. aureus* samples, the *S. aureus* NCTC 8325-4 database (extracted from NCBI, including concatenated reverse database, 5784 entries) was used. Scaffold (version Scaffold 3.4.3, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they exceeded specific database search engine thresholds. Sequest identifications required at least deltaCn scores of greater than 0.10 and XCorr scores of greater than 2.2, 3.3 and 3.8 for doubly, triply and quadruply charged peptides. Protein identifications were accepted if at least 2 identified peptides were detected with the above mentioned filter criteria. With these filter parameter no false positive hit was obtained. The proteomics data are available on request.

Biofilm formation

Biofilm assays were performed based on the method of Christensen (11). *S. aureus* and *S. epidermidis* strains were grown overnight at 37°C in TSB supplemented with 0.25% glucose (TSBg). Each culture was diluted 1:200 in TSBg and 200 µl of this cell suspension was used to inoculate sterile 96-well polystyrene microtiter plates. After 24 h of growth at 37°C in standing conditions, the plates were washed twice with 200 µl sterile PBS, air-dried in an inverted position, and stained with 0.1% safranin for 30 s. Wells were rinsed and the absorbance was determined at 490 nm with the BIOTEK® ELx800™ Universal Microplate Reader (BioTek Instruments, Inc.). Every strain was tested in triplicate and all assays were performed at least three times.

Results

Protein export in srtA mutants of S. aureus

In the absence of functional SrtA, proteins with LPxTG or LPxAG motifs will not be covalently anchored to the cell wall of *S. aureus*. Therefore, one might expect at least a partial release of these proteins into the growth medium of *srtA* mutant cells, especially if they are subject to the "shaving activity" of exported proteases. As a first approach to investigate how a *srtA* mutation impacts on the localization of exported proteins of *S. aureus*, the *srtA* gene was deleted from strain RN4220. As shown by SDS-PAGE, the banding pattern of extracellular proteins of the *srtA* mutant strain displayed major differences compared to the parental strain since the intensity of several bands was strongly increased (Fig. 2A; compare lanes 1 and 2). In contrast, the differences observed for the non-covalently cell wall-bound proteins of both strains were much less pronounced (Fig. 2B, lanes 1 and 2). Importantly, deletion of *srtA* affected neither the growth rate of *S. aureus* RN4220, nor the optical density reached in the stationary phase, and no

obvious morphological differences between cells of the *srtA* mutant and the parental strain were detectable by electron microscopy (results not shown). To identify the proteins in bands of which the relative amounts were increased in the medium or cell wall fractions of the *S. aureus srtA* mutant (Fig. 2), 27 bands were cut from gels and analyzed by mass spectrometry (E. Tsompanidou, unpublished data available on request). This led to the identification of known *S. aureus* cell wall proteins in the different samples (E. Tsompanidou, unpublished data available on request: Table S2). Of the identified proteins, SdrC, SdrD, SA2855, ClfB and protein A contain an LPxTG motif (Table 3). Interestingly, all these proteins contain a YSIRK(R) motif in their signal peptides, as does the LipA protein, which was also secreted in higher amounts by the *srtA* mutant strain. This motif serves in the targeting of proteins to the cross wall of *S. aureus* [31].

A very prominent effect of the *srtA* deletion was observed for protein A (Fig. 2A; marked Spa). Moreover, SdrC and SdrD were identified in several bands of the medium fraction of the *S. aureus srtA* mutant. Although these proteins were also identified in medium samples of the parental strain, the numbers of identified unique peptides for these proteins were increased in medium samples from the *srtA* mutant (Fig. 2A; Table S2). The absence of *srtA* also caused some clear changes in the relative amounts of proteins that remained non-covalently cell wall-bound proteins, including increased amounts of the bifunctional autolysin, and decreased amounts of the 5'-nucleotidase SA0295 (Fig. 2B; E. Tsompanidou, unpublished data available on request: Table S2).

Since several well-studied LPxTG proteins like SasG (15) and the clumping factor A (ClfA; (33, 58, 60)) were not detectable in the Coomassie-stained gels, we studied the localization of these proteins by Western blotting and subsequent immunodetection with specific antibodies. To reduce background levels of lgG that was bound by protein A, we used *S. aureus* SH1000 for all Western blotting experiments as this strain produces less protein A than the RN4220 strain. Furthermore, to detect SasG, we used strains that expressed *sasG* from the pMUTIN4 plasmid upon induction with IPTG (15). In cells of the parental strain SH1000, SasG was detected predominantly as a band of very high molecular weight (Fig. 3A, lane C).

Chapter 5

Table 3. Extracellular proteins identified in sortase mutants of *S. aureus* and *S. epidermidis*

Protein	GI Accession #	Function	Mw mature protein (kD)	Cell Wall binding domain
S. aureus				
Spa (YSIRK)	88193885	protein A	49.7	LPETG, LysM (1x)
SA0295	88194087	5'-nucleotidase, lipoprotein e(P4) family	30.2	-
LipA (YSIRK)	88194101	Lipase	72.3	-[a]
SdrC (YSIRK)	88194324	SdrC protein, putative	98.8	LPETG
SdrD (YSIRK)	88194325	SdrD protein, putative	136.9	LPETG
Atl	88194750	Bifunctional autolysin	134.3	GW-repeats (3x)
Hla	88194865	hemolysin A	33.3	-
SA2285 (YSIRK)	88196433	Hypothetical protein	172.9	LPKTG
ClfB (YSIRR)	88196585	Clumping factor B	88.7	LPETG
Aly	88196599	N-acetylmuramoyl-L-alanine amidase	66.3	CHAP[b]
S. epidermidis				
GehC (YSIRK)	57865740	lipase	73.7	-
Aap (YSIRK)	57865793	accumulation associated protein	246.3	LPDTG, G5 (7x)
SERP0100	57866082	also known as ScaA or Aae; LysM domain protein	32.5	LysM (3x), CHAP[c]
SERP0270	57866259	hypothetical protein	16.2	-
AtlE	57866522	bifunctional autolysin	145.2	GW-repeats (3x)

[a] Cell wall binding has been shown, but no particular wall-binding domain has been identified (8).

[b] Peptidoglycan hydrolase with a cysteine/histidine-dependent amidohydrolase/peptidase domain (CHAP).

[c] Autolysin with a C-terminal cysteine, histidine-dependent amidohydrolase/peptidase (CHAP) domain; binding to fibrinogen, fibronectin and vitronectin (30).

Partially overlapping substrate specificities of staphylococcal group A sortases
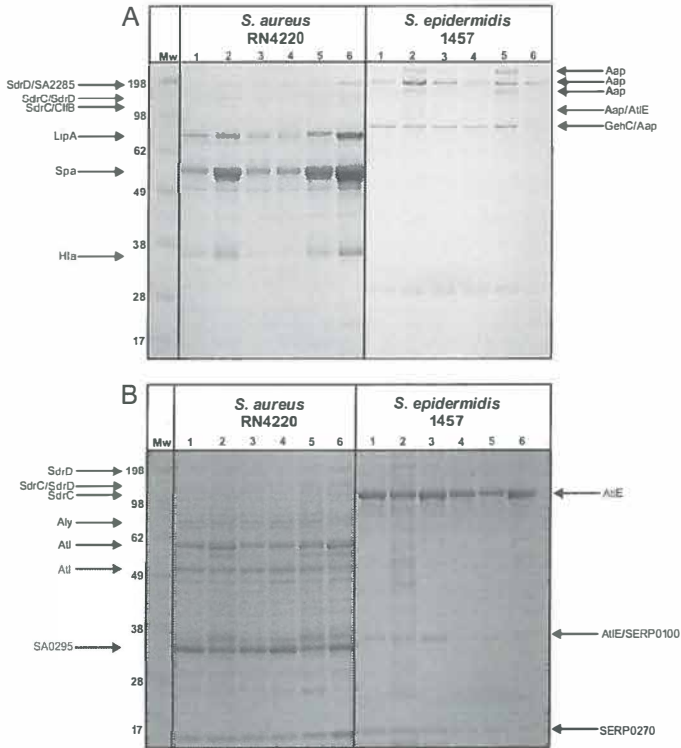
Figure 2. SDS-PAGE analysis of exported proteins of *S. aureus* and *S. epidermidis*. Sortase A mutants of *S. aureus* RN4220 and *S. epidermidis* 1457 were grown in TSB medium at 37°C till the early stationary growth phase. Proteins in the growth medium (**A**) and non-covalently cell wall-bound proteins (**B**) were collected and separated by SDS-PAGE. Gels were stained with Coomassie. Samples were loaded as follows: lane 1, parental strain; lane 2, *srtA* mutant; lane 3, *srtA* mutant complemented with plasmid-borne *S. aureus srtA*; lane 4, *srtA* mutant complemented with plasmid-borne *S. epidermidis srtA*; lane 5, *srtA* mutant complemented with plasmid-borne *B. subtilis yhcS*; and lane 6, *srtA* mutant complemented with plasmid-borne *S. epidermidis srtC*. Protein bands were cut from the gel and identified by mass spectrometry (E. Tsompanidou, unpublished data available on request). Molecular weight (Mw) reference markers are indicated.

Furthermore, the growth medium of the parental strain contained multiple SasG-specific protein species that were detectable in the Western blots as a ladder of discrete protein bands with molecular weights that were much lower than the molecular weight of the SasG detected in cells (Fig. 3A, lane M). In contrast, the high molecular weight band of SasG was not detectable in cells of the *srtA* mutant, and much higher amounts of the low molecular weight bands were detectable not only in the growth medium fraction, but also in the fraction of non-covalently cell wall-bound proteins (Fig. 3A, lane CW). Similar to what we observed for SasG, a high molecular mass species of the ClfA protein was detectable in cells of the parental strain SH1000, whereas the growth medium contained two dominant low molecular ClfA-specific protein species (Fig.

3B, lanes CW and M, respectively). The high molecular weight species of ClfA was not detectable in the *srtA* mutant cells, and substantial amounts of at least three low molecular weight species of ClfA were detectable in the cell wall-bound protein fractions of these cells (Fig. 3B, lane CW). Furthermore, the ratio between the two low molecular weight extracellular ClfA species was changed in the *srtA* mutant. Taken together, these findings show that in the absence of SrtA there are still substantial amounts of SasG and ClfA bound to the cell wall of *S. aureus*, albeit non-covalently. Furthermore, these proteins are to greater or lesser extents released into the growth medium of cells lacking SrtA, which is also consistent with the absence of their covalent cell wall binding.

Figure 3. Localization of LPxTG proteins in *srtA* mutants of *S. aureus* and *S. epidermidis* complemented with different *srtA* or *srtC* genes. Sortase A mutants of *S. aureus* SH1000 and *S. epidermidis* 1457 were grown in TSB medium at 37°C till the early stationary growth phase. Samples of extracellular proteins isolated from the growth medium (M), non-covalently cell wall-bound proteins (CW) and total cells (C) were used for Western blotting and immunodetection with antibodies against *S. aureus* SasG (panel A), *S. aureus* ClfA (panel B) or *S. epidermidis* Aap (panel C). The positions of SasG, ClfA, and Aap are marked with arrows. Constructs used for complementation of Δ*srtA* mutations are indicated on top of each gel. Lanes with samples from the respective parental strain are marked 'WT'.

Partially overlapping substrate specificities of staphylococcal group A sortases

Protein export in a srtA mutant of S. epidermidis

To investigate how a *srtA* mutation influences the localization of proteins in *S. epidermidis*, the extracellular and non-covalently cell wall-bound proteins of *S. epidermidis* 1457 and a *srtA* mutant derivative of this strain were analyzed by SDS-PAGE (Fig. 2). In total 23 bands corresponding to proteins of *S. epidermidis* 1457 were excised from the gel and analyzed by mass spectrometry (Fig. S1). A major difference in localization was observed for the Aap protein, which was dislocated to the growth medium of the *srtA* mutant in higher amounts compared to the parental strain 1457 (Fig. 2A, lane 2). Furthermore, while only two Aap-specific bands were detectable in the growth medium of the parental strain, which probably correspond to the 220 kD and 180 kD forms of Aap described by Rohde *et al.* (53), a third Aap-containing band of ~140 kD was detectable in the medium of the *srtA* mutant. In the non-covalently cell wall-bound protein fraction of the *S. epidermidis srtA* mutant (Fig. 2B), Aap was also identified, but no major differences with the parental strain were detectable.

The subcellular localization of Aap was further examined by Western blotting and immunodetection with specific antibodies. As shown in Figure 3C, two dominant Aap-specific bands of ~220 kD and ~180 kD, and a faint band of ~140 kDa, were detectable in growth medium samples of the parental strain 1457. These bands probably correspond to the 220 kDa, 180 kDa and 140 kDa bands identified by Rohde *et al.* (53). Remarkably, no Aap was detectable in the total cell fraction. In this respect, the behavior of Aap differs from that of its *S. aureus* homologue SasG, which was detectable in cell fractions as a high molecular weight band (Fig. 3A). Due to the *srtA* mutation, the extracellular amounts of the 220 kDa, 180 kDa and 140 kDa forms of Aap were significantly increased, and substantial amounts of the 220 kDa and 180 kDa forms were also detectable in the fraction of non-covalently cell wall-bound proteins (Fig. 3C). To a lesser extent the 220 kDa and 180 kDa forms were also detectable in the cellular fraction of the *srtA* mutant. These findings imply that, in the absence of SrtA, substantial amounts of Aap remain bound to the cell wall, but in a non-covalent manner.

Complementation analysis of srtA mutant strains with sortases A, 'C' and D

Deletion of the *srtA* genes of *S. aureus* and *S. epidermidis* resulted for both organisms in clear changes in the localization of several proteins to the cell wall and growth medium. To distinguish between the effects of the *srtA* mutation and possible unwanted second-site mutations, a complementation analysis was performed with *srtA* genes expressed from a plasmid. Full reversion of the observed protein localization phenotypes was achieved by ectopic expression of the *S. aureus* or *S. epidermidis* s*rtA* genes in the *S. aureus* or *S. epidermidis srtA* mutant strains (Fig. 2, lanes 3 and 4; Fig. 3, A and B). This raised the question to what extents the phenotypes of *srtA* mutant strains could be reverted by ectopic expression of other sortases that also seem to recognize the LPxTG motif, like 'SrtC' of *S. epidermidis* or SrtD of *B. subtilis*. To address this question, the *S. epidermidis srtC* gene or the *B. subtilis yhcS* gene were expressed from the same plasmid-borne promoters that were used for the complementation analyses with *srtA* genes. As

shown in Figure 2 (lanes 5) and Figure 3C, none of the phenotypes observed in *S. aureus* or *S. epidermidis srtA* mutant strains were reversed by expression of *yhcS* from *B. subtilis*. In contrast, expression of *srtC* did lead to the complementation of some, but not all phenotypes of *srtA* mutant strains. As shown in Figure 2 (lane 6), expression of *srtC* in the *S. aureus srtA* mutant did not result in lowered extracellular levels of the LPxTG proteins SdrC, sdrD and protein A as would have been expected to be the case if *srtC* were able to fully complement for the absence of *srtA*. However, *srtC* expression did result in a partial complementation of the localization defect observed for SasG in the *S. aureus srtA* mutant, where clearly lowered amounts of non-covalently cell wall-bound forms of SasG were observed (Fig. 3A, lane CW), as well as an increased amount of the high molecular weight cellular form of SasG (Fig. 3A, lane C). Similarly, *srtC* expression in the *S. aureus srtA* mutant resulted in a partial restoration of the localization of ClfA, the most prominent effect being the re-appearance of the high molecular weight form of ClfA in the cellular fraction (Fig. 3B, lane C). Furthermore, *srtC* expression substantially reduced the amounts of low molecular weight forms of ClfA in the fraction of non-covalently cell wall-bound proteins, but not to the extent that was observed when the *srtA* mutant was complemented with *srtA* of *S. aureus* (Fig. 3B).

Consistent with the observations for SasG in *S. aureus* (Fig. 3A), expression of *srtC* in the *S. epidermidis srtA* mutant resulted in a significant reversion of the dislocation phenotype of the SasG homologue Aap (Fig. 2, lanes labeled 6; Fig. 3C). Upon *srtC* expression, the amounts of the ~220 kDa and ~140 kDa forms of Aap were clearly reduced in the growth medium of *S. epidermidis* Δ*srtA*, the strongest effect being observed for the ~220 kDa species, which virtually disappeared. Taken together, these findings show that SrtA and SrtC have at least partially overlapping substrate specificities.

Biofilm formation by complemented srtA mutants of S. aureus and S. epidermidis

Surface proteins like SasG and protein A of *S. aureus* (15, 45) and Aap of *S. epidermidis* (32, 53, 54) have been implicated in biofilm formation. Therefore, we analyzed the biofilm-forming capacity of complemented *srtA* mutants of *S. aureus* and *S. epidermidis*. The results are summarized in Figure 4. As previously reported for *S. aureus* 312, the biofilm-forming capacity of *srtA* mutants of the *S. aureus* RN4220 and SH1000 strains was significantly reduced compared to the respective parental strains (64). The same turned out to be the case for the *S. epidermidis* 1457 strain. Biofilm formation by the *srtA* mutants of *S. aureus* RN4220 and SH1000, or *S. epidermidis* 1457 was largely restored by expression of the *srtA* genes from *S. aureus* or *S. epidermidis* (Fig. 4). Furthermore, YhcS of *B. subtilis* was unable to complement the defects in biofilm formation of the tested *srtA* mutant *S. aureus* or *S. epidermidis* strains. Interestingly, SrtC of *S. epidermidis* was unable to restore biofilm formation in the *srtA* mutant of *S. aureus* RN4220, but it did restore biofilm formation to wild-type levels in the *srtA* mutants of *S. aureus* SH1000 and *S. epidermidis* 1457 (Fig. 4, A and B). Interestingly, the negative effect of the *srtA* mutation on biofilm formation by the *S. aureus* SH1000 Δ*srtA* strain was largely suppressed by SasG overproduction (Fig. 4C), which indicates that, despite the absence of SrtA,

sufficient SasG was correctly localized to have a stimulating effect on biofilm formation. Notably, biofilm formation was enhanced to levels that exceeded the biofilm formation by the SasG-overproducing parental strain SH1000 when the *S. aureus srtA* gene or the *S. epidermidis srtC* gene were ectopically expressed (Fig. 4C). This shows that SrtA is a limiting factor for the correct localization and functionality of overproduced SasG, and that this particular function of SrtA can also be fulfilled by SrtC.
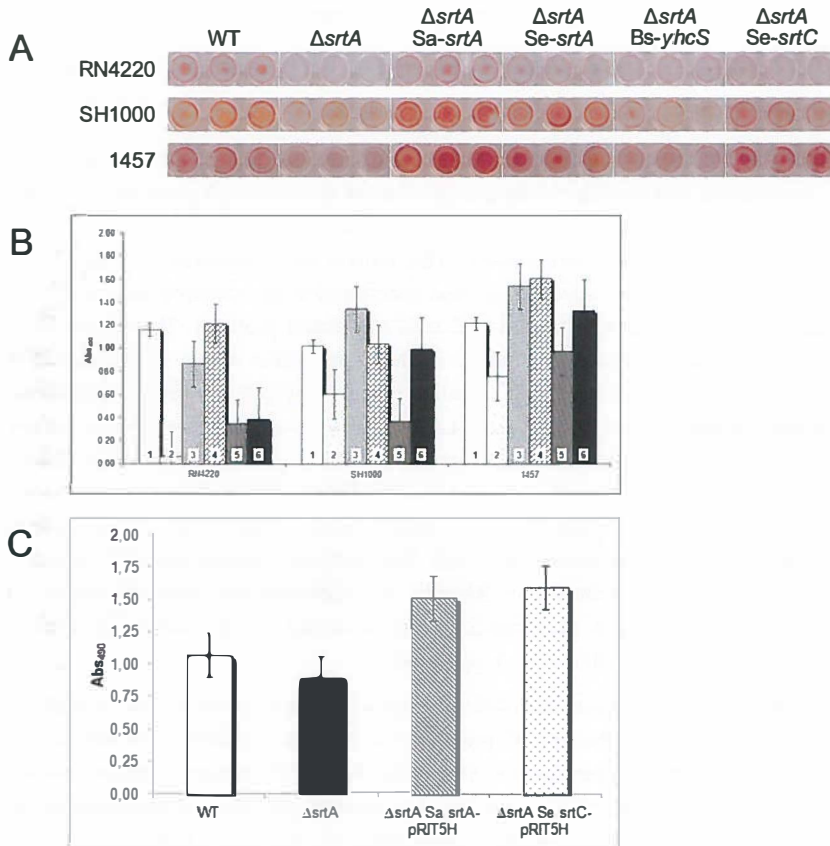
Figure 4. Biofilm formation by *srtA* mutants of *S. aureus* and *S. epidermidis* complemented with different *srtA* or *srtC* genes. Cells were grown in TSB medium with 0.25% glucose for 24 h at 37°C using 96-well microtiter plates. Biofilms were stained with safranin and the absorbance at 490 nm (A_{490}) was measured. Per assay each strain was tested in triplicate, and the assay was performed at least three times. (**A**) Biofilms of indicated strains stained with safranin. (**B**) A_{490} measurements for $\Delta srtA$ mutants of *S. aureus* RN4220, *S. aureus* SH1000 and *S. epidermidis* 1457. 1, parental strain (WT); 2, $\Delta srtA$ mutant; 3, $\Delta srtA$ mutant complemented with *srtA* from *S. aureus*; 4, $\Delta srtA$ mutant complemented with *srtA* from *S. epidermidis*; 5, $\Delta srtA$ mutant complemented with *yhcS* of *B. subtilis*; 6. $\Delta srtA$ mutant complemented with *srtC* of *S. epidermidis*. (**C**) A_{490} measurements for the *srtA* mutant of *S. aureus* SH1000 overproducing SasG and complemented with *srtA* from *S. aureus*, or *srtC* of *S. epidermidis*.

105

Interestingly, the ectopic expression of *srtA* from *S. aureus* or *S. epidermidis* in the *S. epidermidis srtA* mutant resulted in a significant increase in the biofilm-forming capacity of *S. epidermidis* (Fig. 4, A and B), similar to what was observed for SasG-overproducing *S. aureus* SH1000 strains (Fig. 4C). Taken together, our present findings show that the activities of SrtA and SrtC are crucial for biofilm formation not only in *S. aureus* but also in *S. epidermidis*. Intriguingly, under the tested conditions SrtA seems to be produced in limiting amounts for biofilm formation as evidenced by the fact that stronger biofilms are produced by SrtA-overproducing cells.

Discussion

Surface proteins of the Gram-positive bacterial pathogens *S. aureus* and *S. epidermidis* serve important roles in virulence and biofilm formation. Several of these surface proteins are linked covalently to the cell wall by the sortase SrtA. In the present studies we report for the first time the construction of a *srtA* mutant of *S. epidermidis*. This mutant and equivalent *srtA* mutants of *S. aureus* were used to address three aspects of sortase function. Firstly, we investigated to what extent *srtA* mutations affect the localization of cell wall-associated proteins. The results show that the absence of SrtA causes substantial changes in the composition of the *S. aureus* and *S. epidermidis* exoproteomes, mainly due to the dislocation of normally cell wall-attached proteins. Nevertheless, substantial amounts of the different LPxTG proteins remain attached to the cell wall in a non-covalent manner. Secondly, the *srtA* mutants were used to study whether there is any overlap in the substrate specificities of SrtA, SrtC and SrtD from *Staphylococcus* species and *B. subtilis*, all of which recognize proteins with LPxTG motifs. Our results show that the substrate specificities of the staphylococcal SrtA and SrtC proteins overlap partially, which is consistent with their phylogenetic relatedness. Thirdly, we addressed the roles of sortases in biofilm formation by *S. aureus* and *S. epidermidis*, which revealed that the sortase production level sets a limit to this process in particular in *S. epidermidis*.

Three possible effects on the localization of covalently cell wall-bound proteins can be expected when SrtA is not expressed. Firstly, the LPxTG proteins may remain anchored to the cell surface through their C-terminal transmembrane domain. Secondly, the LPxTG proteins may be released into the growth medium through proteolytic 'shaving' by exported proteases, a phenomenon that was previously observed for many unprocessed lipoproteins (1, 59, 63). Thirdly, the LPxTG proteins may remain attached to the cell surface via non-covalent interaction with components of the cell wall. Clearly, all LPxTG proteins investigated in the present studies were released into the growth medium of *srtA* mutant strains, which implies that they had lost their C-terminal transmembrane domains. Nevertheless, we cannot exclude the possibility that a subfraction of these proteins remained attached to the membrane via an uncleaved C-terminal transmembrane domain. Furthermore, substantial amounts of the LPxTG proteins remained attached to the cell wall in a non-covalent manner. This can be explained by the fact that several of these proteins have repeated cell wall-binding domains. For example, LysM domains for peptidoglycan-binding are present in the protein A of *S. aureus* (9), and G5 repeats for N-acetylglucosamine-binding are

Partially overlapping substrate specificities of staphylococcal group A sortases

present in SasG of *S. aureus* and Aap of *S. epidermidis* (4). Interestingly, high molecular weight species of SasG were observed in *srtA*-proficient cells of *S. aureus*. These might represent SasG proteins interacting with each other through their G5 domains as was previously proposed for the homologous Aap protein of *S. epidermidis* (14). However, the high molecular weight species may also represent SasG molecules covalently attached to the cell wall of *S. aureus*. Similar explanations can be entertained for the presence of a high molecular weight form of ClfA in *srtA*-proficient cells of *S. aureus*. Clearly, in absence of *srtA* these proteins are not linked covalently to the cell wall and, in agreement with this notion, no high molecular weight species of SasG and ClfA were detectable in *srtA* mutant cells.

Interestingly, all LPxTG proteins of *S. aureus* and *S. epidermidis* that were found to be dislocated in the respective *srtA* mutant strains contain a YSIRK/GS domain within their signal peptide. It has been shown that the proteins with this YSIRK/GS motif, such as ClfA, protein A, fibronectin-binding protein B (FnbpB), and the serine-aspartate repeat proteins SdrC and SdrD display a ring-like distribution on the *S. aureus* cell surface (16). This has led to the proposal that proteins with the YSIRK/GS motif are site-specifically translocated to the cross wall. Our finding that in particular proteins with the YSIRK/GS motif in their signal peptides are dislocated to the growth medium when SrtA is absent could suggest that the release of these proteins from the cell wall is related to their site of secretion or surface display. This could also be a possible explanation for the observed increased release of the lipase LipA by the *srtA* mutant of *S. aureus*. However, it has to be noted that secretion of the lipase GehC of *S. epidermidis*, which also has the YSIRK/GC motif in its signal peptide, was not detectably influenced by the *srtA* mutation. Most likely, the observed effects of *srtA* mutations on the localization of non-LPxTG proteins, such as LipA, Hla and Atl of *S. aureus*, or AtlE of *S. epidermidis* are indirectly caused by the absence of SrtA. This could relate to as yet unidentified alterations in the cell wall composition of *srtA* mutant strains, or perhaps even to altered interactions with LPxTG proteins that are dislocated due to the *srtA* mutations.

Within the low-GC Gram-positive bacteria (Firmicutes), which include *Staphylococcus* and *Bacillus* species, the function of group A sortases has been studied in much more detail than the function of sortases belonging to other groups that also recognize the LPxTG motif. Therefore, we decided to study the complementation of *S. aureus* and *S. epidermidis srtA* mutants with *srtC* from *S. epidermidis* ATCC12228 or *yhcS* (*srtD*) from *B. subtilis* 168. Notably however, upon phylogenetic analyses it became immediately clear that the *S. epidermidis* gene was erroneously annotated as *srtC* since the encoded protein clusters with known SrtA proteins (Fig. 1B). SrtC of *S. epidermidis* was nevertheless included in the analyses, because its primary structure is significantly different from that of other SrtA proteins (not shown). No complementation was observed upon introduction of *yhcS* in any of the *srtA* mutant strains tested. This may either mean that YhcS does not recognize the staphylococcal LPxTG proteins monitored in the present studies, or that the cells contained insufficient amounts of active YhcS. The latter possibility could, for example, be due to inefficient translation, membrane insertion or post-translational degradation of YhcS. In contrast, a partially restored localization of SasG and ClfA was observed

in *srtA* mutant strains of *S. aureus* expressing *srtC*, and for Aap and AtlE in the *srtA* mutant strain of *S. epidermidis* expressing *srtC*. The molecular basis for this apparently partial overlap in the specificities of the staphylococcal SrtA and SrtC proteins is presently not completely clear. Firstly, the 'LPxTG' sites of SasG (LPKTG), ClfA (LPDTG) and Aap (LPDTG) differ only in the non-conserved central 'x residue' with the LPxTG sites of SdrC, SdrD and protein A (LPETG). This could mean that a Glu residue at the x position is not acceptable for *S. epidermidis* SrtC, whereas Lys or Asp residues at this position are acceptable both for SrtA and SrtC. Based on bioinformatics analyses, Comfort and Clubb (13) have classified various LPxTG sites for recognition by different classes of sortases. Their results suggest that a central Lys residue in the LPxTG motif, as encountered in SasG, would be acceptable to several different groups of sortases. This is consistent with our present finding that SasG is a substrate both for SrtA and SrtC. In contrast, this bioinformatics-based classification did not predict LPxTG motifs with an Asp residue at the central x position as SrtA substrates. Even so, our present analyses indicate that proteins, like ClfA and Aap, which have an LPDTG motif, are SrtA substrates that are also recognized by *S. epidermidis* SrtC. Clearly, at this stage we cannot rule out the possibility that other features of SasG, ClfA and Aap are responsible for the fact that these LPxTG proteins are substrates for SrtA and SrtC, while SdrC, SdrD and protein A are only substrates for SrtA. In this context, it should be noted that SrtC displays several structural differences to class A sortases. Specifically, SrtA of *S. aureus* and SrtC of *S. epidermidis* 12228 merely share 34% amino acid sequence identity. Even though the key residues involved in catalysis (His-120, Cys-184 and Arg-197) and substrate recognition (Val-168 and Leu-169) are conserved in both sortases, the differences between both proteins are large enough to allow for specific differences in the geometry of their active sites. Similarly, a stretch of amino acids in the *β6/β7* loop was shown to determine the substrate specificity of SrtB (5). Intriguingly, Aap appears to be conserved also in *S. epidermidis* 12228 from which the *srtC* gene was derived. This suggests that the SrtC of this *S. epidermidis* strain may not only be dedicated to cell wall attachment of the LPxTG proteins SesJ and SesK as was previously suggested (25), but it may also be involved in covalent cell wall attachment of Aap.

The results of our comparative analyses on the roles of sortases in biofilm formation by *S. aureus* and *S. epidermidis* clearly show that SrtA is important for biofilm formation by *S. aureus* and *S. epidermidis*. Merino *et al.* have shown the involvement of protein A in the formation of protein-dependent biofilms in *S. aureus* (45) and a major dislocating effect of the *srtA* mutation was observed for protein A in the present studies. In addition, Vergera *et al.* (64) showed the importance of the SrtA substrates FnbpA and FnbpB in biofilm formation of *S. aureus* 312. However, also other LPxTG proteins may be involved in this phenomenon. Furthermore, SasG overexpression was able to compensate partially for the absence of SrtA, underpinning the importance of SasG for protein-dependent biofilm formation. Also in this case, the levels of biofilm formation were even further increased upon ectopic expression of SrtA or SrtC, which indicates that sortase activity is present in limiting amounts for biofilm formation under the conditions tested. The finding that SrtC production in SasG-overproducing cells did also

Partially overlapping substrate specificities of staphylococcal group A sortases

stimulate biofilm formation is consistent with the finding that SrtC was able to revert the SasG dislocation phenotype of *srtA* mutant cells at least in part. Accordingly, the role of SrtC in biofilm formation needs to be investigated in more detail in future studies. Consistent with the findings in *S. aureus* cells producing SasG, the ectopic expression of SrtA in *S. epidermidis* had a significant stimulating effect on biofilm formation. This shows that at least in *S. epidermidis* strain 1457, SrtA is a limiting determinant for biofilm formation. Whether this is also the case in other *S. epidermidis* strains remains to be shown.

Acknowledgements

Chapter 5

References

1. **Antelmann, H., H. Tjalsma, B. Voigt, S. Ohlmeier, S. Bron, J. M. van Dijl, and M. Hecker.** 2001. A proteomic view on genome-based signal peptide predictions. Genome Res. **11:**1484-1502.

2. **Arnaud, M., A. Chastanet, and M. Debarbouille.** 2004. New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, gram-positive bacteria. Appl. Environ. Microbiol. **70:**6887-6891.

3. **Barnett, T. C., A. R. Patel, and J. R. Scott.** 2004. A novel sortase, SrtC2, from *Streptococcus pyogenes* anchors a surface protein containing a QVPTGV motif to the cell wall. J. Bacteriol. **186:**5865-5875.

4. **Bateman, A., M. T. Holden, and C. Yeats.** 2005. The G5 domain: a potential N-acetylglucosamine recognition domain involved in biofilm formation. Bioinformatics **21:**1301-1303.

5. **Bentley, M. L., H. Gaweska, J. M. Kielec, and D. G. McCafferty.** 2007. Engineering the substrate specificity of *Staphylococcus aureus* Sortase A. The $\beta6/\beta7$ loop from SrtB confers NPQTN recognition to SrtA. J. Biol. Chem. **282:**6571-6581.

6. **Bibel, D. J., D. J. Lovell, and R. J. Smiljanic.** 1976. Effects of occlusion upon population dynamics of skin bacteria. Br. J. Dermatol. **95:**607-612.

7. **Bierne, H., S. K. Mazmanian, M. Trost, M. G. Pucciarelli, G. Liu, P. Dehoux, L. Jansch, P. F. Garcia-del, O. Schneewind, and P. Cossart.** 2002. Inactivation of the *srtA* gene in *Listeria monocytogenes* inhibits anchoring of surface proteins and affects virulence. Mol. Microbiol. **43:**869-881.

8. **Bowden, M. G., L. Visai, C. M. Longshaw, K. T. Holland, P. Speziale, and M. Höök.** 2002. Is the GehD lipase from *Staphylococcus epidermidis* a collagen binding adhesin? J. Biol. Chem. **277:**43017-43023.

9. **Buist, G., A. Steen, J. Kok, and O. P. Kuipers.** 2008. LysM, a widely distributed protein motif for binding to (peptido)glycans. Mol. Microbiol. **68:**838-847.

10. **Charpentier, E., A. I. Anton, P. Barry, B. Alfonso, Y. Fang, and R. P. Novick.** 2004. Novel cassette-based shuttle vector system for gram-positive bacteria. Appl. Environ. Microbiol. **70:**6076-6085.

11. **Christensen, G. D., W. A. Simpson, J. J. Younger, L. M. Baddour, F. F. Barrett, D. M. Melton, and E. H. Beachey.** 1985. Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. J. Clin. Microbiol. **22:**996-1006.

12. **Claessen, D., R. Rink, J. W. de, J. Siebring, V. P. de, F. G. Boersma, L. Dijkhuizen, and H. A. Wosten.** 2003. A novel class of secreted hydrophobic proteins is involved in aerial hyphae formation in *Streptomyces coelicolor* by forming amyloid-like fibrils. Genes Dev. **17:**1714-1726.

13. **Comfort, D. and R. T. Clubb.** 2004. A comparative genome analysis identifies distinct sorting pathways in gram-positive bacteria. Infect. Immun. **72:**2710-2722.

14. **Conrady, D. G., C. C. Brescia, K. Horii, A. A. Weiss, D. J. Hassett, and A. B. Herr.** 2008. A zinc-dependent adhesion module is responsible for intercellular adhesion in staphylococcal biofilms. Proc. Natl. Acad. Sci. U. S. A. **105:**19456-19461.

15. **Corrigan, R. M., D. Rigby, P. Handley, and T. J. Foster.** 2007. The role of *Staphylococcus aureus* surface protein SasG in adherence and biofilm formation. Microbiology **153:**2435-2446.

16. **DeDent, A., T. Bae, D. M. Missiakas, and O. Schneewind.** 2008. Signal peptides direct surface proteins to two distinct envelope locations of *Staphylococcus aureus*. EMBO J.

17. **Dramsi, S., P. Trieu-Cuot, and H. Bierne.** 2005. Sorting sortases: a nomenclature proposal for the various sortases of Gram-positive bacteria. Res. Microbiol. **156:**289-297.

18. **Drummond, A. J., B. Ashton, M. Cheung, J. Heled, M. Kearse, R. Moir, S. Stones-Havas, T. Thierer, and A. Wilson.** 2009. Geneious v4.8.2.

19. **Edgar, R. C.** 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. **32:**1792-1797.

20. **Elliot, M. A., N. Karoonuthaisiri, J. Huang, M. J. Bibb, S. N. Cohen, C. M. Kao, and M. J. Buttner.** 2003. The chaplins: a family of hydrophobic cell-surface proteins involved in aerial mycelium formation in *Streptomyces coelicolor*. Genes Dev. **17:**1727-1740.

Partially overlapping substrate specificities of staphylococcal group A sortases

21. **Escher, A. and G. W. Characklis.** 1990. Modeling the initial events in biofilm accumulation, p. 445-486. In G. W. Characklis (ed.), Biofilms. Wiley Press, New York, NY, USA.

22. **Eymann, C., A. Dreisbach, D. Albrecht, J. Bernhardt, D. Becher, S. Gentner, T. Tam le, K. Büttner, G. Buurman, C. Scharf, S. Venz, U. Völker, and M. Hecker.** 2004. A comprehensive proteome map of growing *Bacillus subtilis* cells. Proteomics **4:**2849-2876.

23. **Fasehee, H., H. Westers, A. Bolhuis, H. Antelmann, M. Hecker, W. J. Quax, A. F. Mirlohi, J. M. van Dijl, and G. Ahmadian.** 2011. Functional analysis of the sortase YhcS in *Bacillus subtilis*. Proteomics **11:**3905-3913.

24. **Garandeau, C., H. Réglier-Poupet, I. Dubail, J. L. Beretti, P. Berche, and A. Charbit.** 2002. The sortase SrtA of *Listeria monocytogenes* is involved in processing of internalin and in virulence. Infect. Immun. **70:**1382-1390.

25. **Gill, S. R., D. E. Fouts, G. L. Archer, E. F. Mongodin, R. T. Deboy, J. Ravel, I. T. Paulsen, J. F. Kolonay, L. Brinkac, M. Beanan, R. J. Dodson, S. C. Daugherty, R. Madupu, S. V. Angiuoli, A. S. Durkin, D. H. Haft, J. Vamathevan, H. Khouri, T. Utterback, C. Lee, G. Dimitrov, L. Jiang, H. Qin, J. Weidman, K. Tran, K. Kang, I. R. Hance, K. E. Nelson, and C. M. Fraser.** 2005. Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant *Staphylococcus aureus* strain and a biofilm-producing methicillin-resistant *Staphylococcus epidermidis* strain. J. Bacteriol. **187:**2426-2438.

26. **Guindon, S. and O. Gascuel.** 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. **52:**696-704.

27. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557-580.

28. **Hava, D. L. and A. Camilli.** 2002. Large-scale identification of serotype 4 *Streptococcus pneumoniae* virulence factors. Mol. Microbiol. **45:**1389-1406.

29. **Hava, D. L., C. J. Hemsley, and A. Camilli.** 2003. Transcriptional regulation in the *Streptococcus pneumoniae* rlrA pathogenicity islet by RlrA. J. Bacteriol. **185:**413-421.

30. **Heilmann, C., G. Thumm, G. S. Chhatwal, J. Hartleib, A. Uekötter, and G. Peters.** 2003. Identification and characterization of a novel autolysin (Aae) with adhesive properties from *Staphylococcus epidermidis*. Microbiology **149:**2769-2778.

31. **Horsburgh, M. J., J. L. Aish, I. J. White, L. Shaw, J. K. Lithgow, and S. J. Foster.** 2002. σ^B modulates virulence determinant expression and stress resistance: characterization of a functional rsbU strain derived from *Staphylococcus aureus* 8325-4. J. Bacteriol. **184:**5457-5467.

32. **Hussain, M., M. Herrmann, E. C. von, F. Perdreau-Remington, and G. Peters.** 1997. A 140-kilodalton extracellular protein is essential for the accumulation of *Staphylococcus epidermidis* strains on surfaces. Infect. Immun. **65:**519-524.

33. **Josefsson, E., O. Hartford, L. O'Brien, J. M. Patti, and T. Foster.** 2001. Protection against experimental *Staphylococcus aureus* arthritis by vaccination with clumping factor A, a novel virulence determinant. J. Infect. Dis. **184:**1572-1580.

34. **Kouwen, T. R., E. N. Trip, E. L. Denham, M. J. Sibbald, J. Y. Dubois, and J. M. van Dijl.** 2009. The large mechanosensitive channel MscL determines bacterial susceptibility to the bacteriocin sublancin 168. Antimicrob. Agents Chemother. **53:**4702-4711.

35. **Kreiswirth, B. N., S. Löfdahl, M. J. Betley, M. O'Reilly, P. M. Schlievert, M. S. Bergdoll, and R. P. Novick.** 1983. The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. Nature **305:**709-712.

36. **Lasa, I. and J. R. Penadés.** 2006. Bap: a family of surface proteins involved in biofilm formation. Res. Microbiol. **157:**99-107.

37. **Mack, D., N. Siemssen, and R. Laufs.** 1992. Parallel induction by glucose of adherence and a polysaccharide antigen specific for plastic-adherent *Staphylococcus epidermidis*: evidence for functional relation to intercellular adhesion. Infect. Immun. **60:**2048-2057.

38. **Mandlik, A., A. Swierczynski, A. Das, and H. Ton-That.** 2008. Pili in Gram-positive bacteria: assembly, involvement in colonization and biofilm development. Trends Microbiol. **16:**33-40.

Chapter 5

39. **Marraffini, L. A., A. C. Dedent, and O. Schneewind.** 2006. Sortases and the art of anchoring proteins to the envelopes of gram-positive bacteria. Microbiol. Mol. Biol. Rev. **70:**192-221.

40. **Martí, M., M. P. Trotonda, M. A. Tormo-Más, M. Vergara-Irigaray, A. L. Cheung, I. Lasa, and J. R. Penadés.** 2010. Extracellular proteases inhibit protein-dependent biofilm formation in *Staphylococcus aureus.* Microbes Infect. **12:**55-64.

41. **Mazmanian, S. K., H. Ton-That, K. Su, and O. Schneewind.** 2002. An iron-regulated sortase anchors a class of surface protein during *Staphylococcus aureus* pathogenesis. Proc. Natl. Acad. Sci. U. S. A. **99:**2293-2298.

42. **Mazmanian, S. K., G. Liu, E. R. Jensen, E. Lenoy, and O. Schneewind.** 2000. *Staphylococcus aureus* sortase mutants defective in the display of surface proteins and in the pathogenesis of animal infections. Proc. Natl. Acad. Sci. U. S. A. **97:**5510-5515.

43. **Mazmanian, S. K., E. P. Skaar, A. H. Gaspar, M. Humayun, P. Gornicki, J. Jelenska, A. Joachmiak, D. M. Missiakas, and O. Schneewind.** 2003. Passage of heme-iron across the envelope of *Staphylococcus aureus.* Science **299:**906-909.

44. **McDevitt, D., P. Francois, P. Vaudaux, and T. J. Foster.** 1995. Identification of the ligand-binding domain of the surface-located fibrinogen receptor (clumping factor) of *Staphylococcus aureus.* Mol. Microbiol. **16:**895-907.

45. **Merino, N., A. Toledo-Arana, M. Vergara-Irigaray, J. Valle, C. Solano, E. Calvo, J. A. Lopez, T. J. Foster, J. R. Penadés, and I. Lasa.** 2009. Protein A-mediated multicellular behavior in *Staphylococcus aureus.* J. Bacteriol. **191:**832-843.

46. **Morikawa, K., Y. Inose, H. Okamura, A. Maruyama, H. Hayashi, K. Takeyasu, and T. Ohta.** 2003. A new staphylococcal sigma factor in the conserved gene cassette: functional significance and implication for the evolutionary processes. Genes Cells **8:**699-712.

47. **Norrander, J., T. Kempe, and J. Messing.** 1983. Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. Gene **26:**101-106.

48. **Novick, R. P.** 1991. Genetic systems in staphylococci. Methods Enzymol. **204:**587-636.

49. **Otto, M.** 2009. *Staphylococcus epidermidis*--the 'accidental' pathogen. Nat. Rev. Microbiol. **7:**555-567.

50. **Otto, M.** 2008. Staphylococcal biofilms. Curr. Top. Microbiol. Immunol. **322:**207-228.

51. **Peacock, S. J., I. de Silva, and F. D. Lowy.** 2001. What determines nasal carriage of *Staphylococcus aureus*? Trends Microbiol. **9:**605-610.

52. **Roche, F. M., R. Massey, S. J. Peacock, N. P. Day, L. Visai, P. Speziale, A. Lam, M. Pallen, and T. J. Foster.** 2003. Characterization of novel LPXTG-containing proteins of *Staphylococcus aureus* identified from genome sequences. Microbiology **149:**643-654.

53. **Rohde, H., C. Burdelski, K. Bartscht, M. Hussain, F. Buck, M. A. Horstkotte, J. K. Knobloch, C. Heilmann, M. Herrmann, and D. Mack.** 2005. Induction of *Staphylococcus epidermidis* biofilm formation via proteolytic processing of the accumulation-associated protein by staphylococcal and host proteases. Mol. Microbiol. **55:**1883-1895.

54. **Rohde, H., E. C. Burandt, N. Siemssen, L. Frommelt, C. Burdelski, S. Wurster, S. Scherpe, A. P. Davies, L. G. Harris, M. A. Horstkotte, J. K. Knobloch, C. Ragunath, J. B. Kaplan, and D. Mack.** 2007. Polysaccharide intercellular adhesin or protein factors in biofilm accumulation of *Staphylococcus epidermidis* and *Staphylococcus aureus* isolated from prosthetic hip and knee joint infections. Biomaterials **28:**1711-1720.

55. **Scott, J. R. and T. C. Barnett.** 2006. Surface proteins of gram-positive bacteria and how they get there. Annu. Rev. Microbiol. **60:**397-423.

56. **Sibbald, M. J., A. K. Ziebandt, S. Engelmann, M. Hecker, A. de Jong, H. J. Harmsen, G. C. Raangs, I. Stokroos, J. P. Arends, J. Y. Dubois, and J. M. van Dijl.** 2006. Mapping the pathways to staphylococcal pathogenesis by comparative secretomics. Microbiol. Mol. Biol. Rev. **70:**755-788.

57. **Sibbald, M. J. J. B. and J. M. van Dijl.** 2009. Secretome mapping in Gram-positive pathogens. In K. Wooldridge (ed.), Bacterial protein secretion systems. Horizon Scientific Press, Norwich, UK.

58. **Siboo, I. R., A. L. Cheung, A. S. Bayer, and P. M. Sullam.** 2001. Clumping factor A mediates binding of *Staphylococcus aureus* to human platelets. Infect. Immun. **69:**3120-3127.

112

Partially overlapping substrate specificities of staphylococcal group A sortases

59. **Stoll, H., J. Dengjel, C. Nerz, and F. Götz.** 2005. *Staphylococcus aureus* deficient in lipidation of prelipoproteins is attenuated in growth and immune activation. Infect. Immun. **73:**2411-2423.

60. **Sullam, P. M., A. S. Bayer, W. M. Foss, and A. L. Cheung.** 1996. Diminished platelet binding *in vitro* by *Staphylococcus aureus* is associated with reduced virulence in a rabbit model of infective endocarditis. Infect. Immun. **64:**4915-4921.

61. **Ton-That, H. and O. Schneewind.** 2003. Assembly of pili on the surface of *Corynebacterium diphtheriae*. Mol. Microbiol. **50:**1429-1438.

62. **Ton-That, H., L. A. Marraffini, and O. Schneewind.** 2004. Sortases and pilin elements involved in pilus assembly of *Corynebacterium diphtheriae*. Mol. Microbiol. **53:**251-261.

63. **Venema, R., H. Tjalsma, J. M. van Dijl, A. de Jong, K. Leenhouts, G. Buist, and G. Venema.** 2003. Active lipoprotein precursors in the Gram-positive eubacterium *Lactococcus lactis*. J. Biol. Chem. **278:**14739-14746.

64. **Vergara-Irigaray, M., J. Valle, N. Merino, C. Latasa, B. García, I. Ruiz de Los Mozos, C. Solano, A. Toledo-Arana, J. R. Penadés, and I. Lasa.** 2009. Relevant role of fibronectin-binding proteins in *Staphylococcus aureus* biofilm-associated foreign-body infections. Infect. Immun. **77:**3978-3991.

65. **Wertheim, H. F., M. C. Vos, A. Ott, A. van Belkum, A. Voss, J. A. Kluytmans, P. H. van Keulen, C. M. Vandenbroucke-Grauls, M. H. Meester, and H. A. Verbrugh.** 2004. Risk and outcome of nosocomial *Staphylococcus aureus* bacteraemia in nasal carriers versus non-carriers. Lancet **364:**703-705.

"A scientist in his laboratory is not a mere technician: he is also a child confronting natural phenomena that impress him as though they were fairy tales."

Marie Curie

Chapter 6

What is required for motility and pellicle formation in *Bacillus subtilis*

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Chapter 6

Abstract

Many bacteria have the ability to undergo lifestyle changes that allow them to survive under a large number of conditions. Biofilm formation and the ability of bacteria to move over wet surfaces are just two of these properties. Due to the increased incidence of biofilm-associated infections and the resistance of biofilms to the subsequent treatment, there is a pronounced requirement for understanding the mechanisms involved in biofilm formation, maturation and detachment. *Bacillus subtilis* is able to form robust biofilms and is easily genetically manipulated, therefore making it an ideal organism to study the biology of biofilms. Bacterial motility is another property of *B. subtilis* cells, which is tightly correlated with biofilm formation. Here we have analyzed 286 large genomic deletion strains of *B. subtilis* for their ability to form biofilms and to move over wet surfaces. We found that the CssRS two-component system, the Pst phosphate transport system and two genes of unknown function *yeaC* and *yqgC* are all essential for swarming motility and biofilm formation.

What is required for motility and pellicle formation in *Bacillus subtilis*

Introduction

Biofilms are considered to be the predominant lifestyle of bacteria in their natural environment, in clinical settings and in engineered ecosystems (14). The involvement of biofilms in human infections and numerous biotechnological and ecological processes, together with the increased resistance of biofilms to antibiotics, our immune system and other environmental stresses pinpoints the importance of understanding the mechanisms used during biofilm formation, maturation and detachment (15, 20, 35, 47, 52).). Biofilms are dynamic communities and several different cell types can co-exist in a mature biofilm. Early in biofilm formation the majority of the cells are often motile. During the process of biofilm maturation, motile cells differentiate into matrix-producing cells. As shown for bacteria of the genus Bacillus, the matrix-producing cells can transform into sporulating cells at the site of aerial structures (57). The Gram-positive soil bacterium *Bacillus subtilis* has been widely used as a model organism for biofilm-related studies and many genes have been identified that are involved in the process (4, 6, 8, 31, 38, 41). *B. subtilis* is able to form biofilms on solid surfaces and at air-liquid interfaces. These biofilms consist of long chains of cells in parallel patterns that are tightly bound together by the extracellular matrix (4). At the early stages of biofilm formation a subpopulation of *eps* and *tapA* expressing cells supply matrix to the entire community (9). Flagella-driven motility and to a lesser extent chemotaxis, have been shown to be important for biofilm formation (23). Initially, motile bacteria attach to a surface and also to each other thus becoming immobilized and able to initiate biofilm formation.

Surface translocation is another common feature of the bacterial world (23, 25).). Many bacteria (including *B. subtilis*) are capable of flagella-dependent swimming and surfactant-dependent swarming (29, 50, 51). Surfactants are amphipathic molecules that lower the surface tension, thereby allowing bacteria to move over surfaces. Surfactants can be seen as a transparent halo that precedes the expanding colony (1, 28, 29, 34, 39, 48, 51). In *B. subtilis* swarming is dependent on the production of a surfactant called surfactin and on the presence of flagella (7, 29). Interestingly, swarming motility shares many control mechanisms with biofilm formation (13). The *B. subtilis* laboratory strain 168, is defective in the production of both surfactin and SwrA, and consequently it is unable to swarm (30, 41, 46).). SwrA is part of a dicistronic operon and is required for swarming differentiation (7). Notably, motility and surfactin production are important for the formation of robust biofilms by *B. subtilis*, which is why usually wild-type undomesticated strains of *B. subtilis* are included in studies on biofilm formation. Many genes have been shown to be important for biofilm formation in *B. subtilis*. Among them is the *epsA-O* operon for exopolysaccharide (EPS) production, the *sfp* gene involved in surfactin production, regulatory genes such as *spo0A*, *spo0H* and *abrB* that are also implicated in the early stages of sporulation, *sipW* which encodes a signal peptidase necessary for processing TapA and TasA, *yqek* encoding for a putative phosphatase, an ABC transporter subunit coding gene (*ecsB*), the enzymatic products of *gltAB* and *ampS* genes, and two genes with unknown function, *ylbF* and *ymcA* (3, 5, 8, 10, 11, 21, 22, 31, 53).

117

Chapter 6

The goal of the present work was to identify novel genes involved in biofilm formation and motility in *B. subtilis*, to determine which genes fall into common pathways and to finally elucidate how these pathways cooperate. We analyzed a collection of 286 strains of *B. subtilis* with large genomic deletion for their ability to swim, swarm and form pellicle. The initial screening was carried out in *B. subtilis* 168 and the observed phenotypes were verified by transferring the selected mutations to the undomesticated *B. subtilis* strains 3610 and *B. subtilis* 200.

Materials and Methods

Bacterial strains and growth condition

B. subtilis 168, 3610 and *B. subtilis* 200 were routinely grown in Luria Bertani (LB) or LB plates supplemented with 1.5% agar. All strains used in the present studies are listed in Table 1. Where necessary, phleomycin (Phl; 4 µg/ml), erythromycin (Em; 2 µg/ml), and/or isopropyl β-D-1-thiogalactopyranoside (IPTG; 100 µM) were added. For transformation, strain *B. subtilis* 200 was grown in Paris Minimal (PM) medium consisting of 10.7 mg/ml K2HPO4, 6 mg/ml KH2PO4, 1 mg/ml trisodium citrate, 0.02 mg/ml MgSO4, 1% glucose, 0.1% casamino acids (Difco), 20 µg/ml L-tryptophan, 2.2 µg/ml ferric ammonium citrate and 20 mM potassium glutamate.

Table 1: *B. subtilis* **strains used in these studies**

B. subtilis strains	Description	Reference
168	*trpC2*	(36)
168-JJS-DIn153	*sspA, thiI, iscS, braB, ezrA, hisJ,yttP,ytsP*	BaSynthec
168-JJS-DIn084	*yqzC,pstBB, pstBA, pstA, pstC,pstS,pbpA, yqgE, sodA, yqgC, yqgB*	BaSynthec
168-JJS-DIn087	*rimO, rsmE, prmA, dnaJ, dnaK, grpE, hrcA, hemN, lepA, yqxA*	BaSynthec
168-JJS-DIn129	*rpoE, acdA, fadF, clsB, ywjD, ywjC, ywjB, ywjA, ywiE, narI, narJ, narH, narG, arfM, ywjC, fnr, narK*	BaSynthec
168-JJS-DIn077	*ypzK, ribH, ribAB, ribE, ribD, ypuD, sipS, ypzC, ypzJ, ypuC, ypuC, ypuB, ypzD, ppiB, ypuA, lysA, spoVAF, spoVAEA, spoVAEB, spoVAD, spoVAC, spoVAB, spoVAA, sigF, spoIIAB, spoIIAA, dacF, pupG, drm, ripX, yqzK, fur, spoIIM, yqkK, mleA, mleN, ansB, ansA, ansR, yqxK, nudF, yqkF, yqkE, yqkD, yqkC*	BaSynthec
168-JJS-DIn169	*gabP, ydzX, yeaB, yeaC, yeaD, yebA, guaA, pbuG, yebC, yebD, yebE*	BaSynthec
168-JJS-DIn142	*yjkA, yjkB, yjlA, yjlB, yjlC, ndh, uxaC, exuM, yjmC, yjmD, uxuA, uxuB, exuT, exuR, uxaB, uxaA, yjnA, yjoA, yjoB, rapA, phrA, yjpA, xlyB, yjqA, yjqB, yjqC, xkdA, xrE, yjzJ, xkdB, xkdC, ykzK, xkdD, xtrA, xpf, xtmA, xtmB, xkdE, xkdF, xkdG, ykzL, xkdH, xkdI, xkdJ, ykzM, xkdK, xkdM, xkdN, xkzB, xkdO, xkdP, xkdQ, xkdR, xkdS, xkdT, xkdU, xkzA, xkdV, xkdW, xkdX, xepA, xhlA, xhlB, xlyA, spoIISB, spoIISA, pit, ykaA*	BaSynthec
168-JJS-DIn174	*purE, purK, purB, purC, purS, purQ, purL, purF, purM, purN, purH, purD*	BaSynthec
168-JJS-DIn186	*yqhO, mntR, yqhM, yqhL*	BaSynthec
168-JJS-DIn191	*yutC, lipA, lytH*	BaSynthec

What is required for motility and pellicle formation in *Bacillus subtilis*

168-JJS-DIn203	htrB, cssR, cssS, yuzO, yuxN, fumC, yvzF, gerAA, gerAB, gerAC, liar, liaS, liaF, liaG, liaH, lial, yvqJ, yvqK, yvrA, yvrB, yvr, yvrD	BaSynthec
168-JJS-DIn200	yusD, yusE, yusF, yusG, yusH, yusI	BaSynthec
168-JJS-DIn151	ytkL, ytkK, ytzD, argH, argG, moaB, ackA, ytxK	BaSynthec
3610	wt	(12)
3610-JJS-DIn153	sspA, thiI, iscS, braB, ezrA, hisJ,yttP,ytsP	This work
3610-JJS-DIn084	yqzC,pstBB, pstBA, pstA, pstC,pstS,pbpA, yqgE, sodA, yqgC, yqgB	This work
3610-JJS-DIn087	rimO, rsmE, prmA, dnaJ, dnaK, grpE, hrcA, hemN, lepA, yqxA	This work
3610-JJS-DIn129	rpoE, acdA, fadF, clsB, ywjD, ywjC, ywjB, ywjA, ywiE, narI, narJ, narH, narG, arfM, , fnr, narK	This work
3610-JJS-DIn077	ypzK, ribH, ribAB, ribE, ribD, ypuD, sipS, ypzC, ypzJ, ypuC, ypuC, ypuB, ypzD, ppiB, ypuA, lysA, spoVAF, spoVAEA, spoVAEB, spoVAD, spoVAC, spoVAB, spoVAA, sigF, spoIIAB, spoIIAA, dacF, pupG, drm, ripX, yqzK, fur, spoIIM, yqkK, mleA, mleN, ansB, ansA, ansR, yqxK, nudF, yqkF, yqkE, yqkD, yqkC	This work
3610-JJS-DIn169	gabP, ydzX, yeaB, yeaC, yeaD, yebA, guaA, pbuG, yebC, yebD, yebE	This work
3610-JJS-DIn142	yjkA, yjkB, yjlA, yjlB, yjlC, ndh, uxaC, exuM, yjmC, yjmD, uxuA, uxuB, exuT, exuR, uxaB, uxaA, yjnA, yjoA, yjoB, rapA, phrA, yjpA, xlyB, yjqA, yjqB, yjqC, xkdA, xrE, yjzJ, xkdB, xkdC, ykzK, xkdD, xtrA, xpf, xtmA, xtmB, xkdE, xkdF, xkdG, ykzL, xkdH, xkdI, xkdJ, ykzM, xkdK, xkdM, xkdN, xkzB, xkdO, xkdP, xkdQ, xkdR, xkdS, xkdT, xkdU, xkzA, xkdV, xkdW, xkdX, xepA, xhlA, xhlB, xlyA, spoIISB, spoIISA, pit, ykaA	This work
3610-JJS-DIn174	purE, purK, purB, purC, purS, purQ, purL, purF, purM, purN, purH, purD	This work
3610-JJS-DIn186	yqhO, mntR, yqhM, yqhL	This work
3610-JJS-DIn191	yutC, lipA, lytH	This work
3610-JJS-DIn203	htrB, cssR, cssS, yuzO, yuxN, fumC, yvzF, gerAA, gerAB, gerAC, liar, liaS, liaF, liaG, liaH, lial, yvqJ, yvqK, yvrA, yvrB, yvr, yvrD	This work
3610-JJS-DIn200	yusD, yusE, yusF, yusG, yusH, yusI	This work
3610-JJS-DIn151	ytkL, ytkK, ytzD, argH, argG, moaB, ackA, ytxK	This work
200	wt	(2)
200-JJS-DIn153	sspA, thiI, iscS, braB, ezrA, hisJ,yttP,ytsP	This work
200-JJS-DIn084	yqzC,pstBB, pstBA, pstA, pstC,pstS,pbpA, yqgE, sodA, yqgC, yqgB	This work
200-JJS-DIn087	rimO, rsmE, prmA, dnaJ, dnaK, grpE, hrcA, hemN, lepA, yqxA	This work
200-JJS-DIn129	rpoE, acdA, fadF, clsB, ywjD, ywjC, ywjB, ywjA, ywiE, narI, narJ, narH, narG, arfM, , fnr, narK	This work
200-JJS-DIn077	ypzK, ribH, ribAB, ribE, ribD, ypuD, sipS, ypzC, ypzJ, ypuC, ypuC, ypuB, ypzD, ppiB, ypuA, lysA, spoVAF, spoVAEA, spoVAEB, spoVAD, spoVAC, spoVAB, spoVAA, sigF, spoIIAB, spoIIAA, dacF, pupG, drm, ripX, yqzK, fur, spoIIM, yqkK, mleA, mleN, ansB, ansA, ansR, yqxK, nudF, yqkF, yqkE, yqkD, yqkC	This work
200-JJS-DIn169	gabP, ydzX, yeaB, yeaC, yeaD, yebA, guaA, pbuG, yebC, yebD, yebE	This work
200-JJS-DIn142	yjkA, yjkB, yjlA, yjlB, yjlC, ndh, uxaC, exuM, yjmC, yjmD, uxuA, uxuB, exuT, exuR, uxaB, uxaA, yjnA, yjoA, yjoB, rapA, phrA, yjpA, xlyB, yjqA, yjqB, yjqC, xkdA, xrE, yjzJ, xkdB, xkdC, ykzK, xkdD, xtrA, xpf, xtmA, xtmB, xkdE, xkdF, xkdG, ykzL, xkdH, xkdI, xkdJ, ykzM, xkdK, xkdM, xkdN, xkzB, xkdO, xkdP, xkdQ, xkdR, xkdS, xkdT, xkdU, xkzA, xkdV, xkdW, xkdX, xepA, xhlA, xhlB, xlyA, spoIISB, spoIISA, pit, ykaA	This work
200-JJS-DIn174	purE, purK, purB, purC, purS, purQ, purL, purF, purM, purN, purH, purD	This work

200-JJS-DIn186	*yqhO, mntR, yqhM, yqhL*	This work
200-JJS-DIn191	*yutC, lipA, lytH*	This work
200-JJS-DIn203	*htrB, cssR, cssS, yuzO, yuxN, fumC, yvzF, gerAA, gerAB, gerAC, liar, liaS,*	This work
	liaF, liaG, liaH, liaI, yvqJ, yvqK, yvrA, yvrB, yvr, yvrD	
200-JJS-DIn200	*yusD, yusE, yusF, yusG, yusH, yusI*	This work
200-JJS-DIn151	*ytkL, ytkK, ytzD, argH, argG, moaB, ackA, ytxK*	This work
3610 *ΔcssS*	3610 lacking the *cssS* gene	This work
200 *ΔcssS*	*B. subtilis* 200 lacking the *cssS* gene	This work
3610 *ΔcssR*	3610 lacking the *cssR* gene	This work
200 *ΔcssR*	*B. subtilis* 200 lacking the *cssR* gene	This work
3610 *ΔpstA*	3610 lacking the *pstA* gene	This work
200 *ΔpstC*	*B. subtilis* 200 lacking the *pstC* gene	This work
3610 *ΔpstS*	3610 lacking the *pstS* gene	This work
200 *ΔpstS*	*B. subtilis* 200 lacking the *pstS* gene	This work

Swimming and swarming

Swimming was assayed on LB supplemented with 0.26% agar, and swarming on LB supplemented with 0.6% of agar. Each plate (10 ml) was dried for approximately 10 min. From an overnight culture of *B. subtilis*, 2 µl were spotted in the center of a plate, which was subsequently dried for an additional 5 min. When strain *B. subtilis* 168 was tested for swarming, 2 µl of 1 µg/ml pure surfactin (Sigma-Aldrich) from *B. subtilis* was spotted on the plate prior to the inoculation of the bacteria. The plates were then incubated overnight at 37°C. Images were recorded with a G:box (Syngene, Leusden, the Netherlands).

Pellicle formation

Bacteria were grown overnight at 30°C in Tryptic Soy Broth (TSB), with the appropriate antibiotics, under vigorous shaking. 10 µl of the overnight-grown bacteria were transferred to 2 ml fresh TSB in 24-well plates and incubated at 30°C with no shaking for 48 h (6). Digital images of the pellicles were taken using a Sony cyber-shot camera.

SPP1 phage lysate and phage transduction

SPP1 phage stock was serial-diluted and 100 µl of each dilution was added to 200 µl of dense culture grown in TY broth (LB supplemented after autoclaving with 10 mM $MgSO_4$ and 100 µM $MnSO_4$) and statically incubated for 15 minutes at 37°C. To each mixture 3 ml of TYSA (TY supplemented with 0.6% agar) was added, poured over fresh TY plates and incubated overnight at 37°C. The top agar from the plate containing almost confluent plaques was harvested by scraping into a 15 ml conical tube, vortexed and centrifuged for 10 minutes at 5000 g. The supernatant was transferred to a fresh tube and 10 µl of a 25 µg/ml DNase solution was added. After vortexing the supernatant was incubated at room temperature for 10 minutes before being passed through a 0.45µm syringe filter and stored at 4°C (31).

What is required for motility and pellicle formation in *Bacillus subtilis*

Recipient 3610 cells were grown to stationary phase in 2 ml TY broth at 37°C. 1 ml of cells was mixed with 10 µl of SPP1 donor phage stock in a 15 ml conical tube. 9 ml of TY broth was added to the mixture and incubated statically for 15 minutes at 37°C. Subsequently, the transduction mixture was centrifuged for 10 minutes at 5000 g, the supernatant was discarded and the pellet was resuspended in the remaining volume. 100 µl of the cell suspension was plated on TY supplemented with 1.5% agar, 5% sheep blood, the appropriate antibiotic and 10 mM sodium citrate, and incubated overnight at 37°C (31).

Transformation of B. subtilis strain B. subtilis 200

Strain *B. subtilis* 200 was grown overnight in 500 µl PM. Subsequently, 10 µl from the overnight culture were transferred to 500 µl fresh PM and grown for 3 h. 10 µl of extracted genomic DNA was added to the culture and grown for an additional 5 h. Bacteria were plated on LB agar plates containing the appropriate antibiotic.

Results and Discussion

Screen of BaSynthec deletion strains

In the context of the European project BaSynthec, a collection of 286 large deletion mutants were constructed that cover the majority of the non-essential genes of the *B. subtilis* genome (except those known to be involved in competence and those that share an operon with an essential gene). During the present studies, we have screened this collection for mutants that are deficient in motility and pellicle formation. Notably, the BaSynthec deletion strains were created in the *B. subtilis* strain 168 background, which carries a frameshift mutation in the *sfp* gene (43). Consequently, strain 168 is unable to produce surfactin and therefore to swarm. Pure surfactin was therefore added to facilitate swarming motility in all experiments carried out with the strain168-derived mutants. The addition of surfactin proved to be sufficient to promote swarming motility and to allow discrimination between swarming-proficient and deficient strains (Fig.1).

Many genes are already known to be involved in the swimming and swarming processes of *B. subtilis* (7, 29, 30, 37, 45, 46) and we were able to identify all of these genes that had been deleted from strains in the BaSynthec mutant collection during our initial screening. Such known genes included for example *sigD* for the motility sigma factor σ^D, genes involved in flagellar biogenesis, and the *efp* and the *degSU* genes (data not shown). Only strains containing novel genes involved in these processes, as well as in pellicle formation are discussed in this chapter. Furthermore, mutant strains that showed a difference in growth rate when compared to the majority of the other strains in the collection were discarded from further analyses as altered growth rates can account for differences in swarm patterns (17, 46). From the 286 strains that were tested, four strains were found to be affected in swimming motility, seven were affected in swarming motility and twelve in the ability to form pellicles (Table 2).
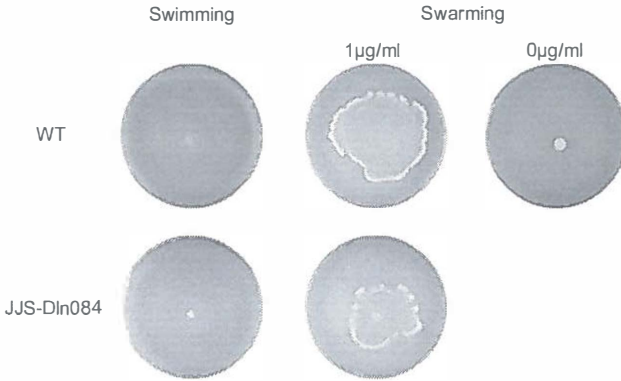
Chapter 6

Figure 1. Primary swimming and swarming screens with mutants from the BaSynthec collection The BaSynthec *B. subtilis* 168 parental strain can readily swim, but is unable to swarm without the external addition of surfactin. The deletion strain JJS-Dln084 can swim as well as the parental strain, but its ability to swarm even after addition of surfactin is decreased in comparison to the parental strain. Strains with similar phenotype to strain JJS-Dln084 were selected for further analyses.

Table 2: phenotypic characteristics of the minimal strains constructed for the present studies

Name	Swimming		Swarming		Pellicle	
	3610	*B. subtilis* 200	3610	*B. subtilis* 200	3610	*B. subtilis* 200
WT	+	+	+	+	+	+
JJS-Dln153	+	-	-	--	+	+
JJS-Dln084	--	-	lost completely	--	--	--
JJS-Dln087	+	+	-	-	different morphology	-
JJS-Dln129	--	+	--	+	+	-
JJS-Dln077	+	+	+	+	--	-
JJS-Dln169	+	+	-	+	-	+
JJS-Dln142	+	++	-	-	-	-
JJS-Dln174	+	+	+	+	-	--
JJS-Dln186	+	+	+	+	--	--
JJS-Dln191	+	+	+	+	--	--
JJS-Dln203	+	+	+	+	--	-
JJS-Dln200	+	+	+	+	--	-
JJS-Dln151	-	+	lost completely	+	-	+

+ wild-type phenotype

++ stronger phenotype in comparison to the wild-type

- slightly impaired

-- severely impaired

What is required for motility and pellicle formation in *Bacillus subtilis*

Transfer of large genome deletions to undomesticated B. subtilis strains

Genomic regions that were deleted from BaSynthec strains showing phenotypes in the initial screens, were deleted also from the genomes of *B. subtilis* 3610 and *B. subtilis B. subtilis* 200 for the verification of these phenotypes. Notably, when we studied the effects of the transferred mutations in these two strains on swimming, swarming and pellicle formation, differing results were obtained as summarized in Table 2. For example, when deletions JJS-DIn084, JJS-DIn129 or JJS-DIn151 were introduced in strain 3610, the resulting mutants were significantly affected in swimming (Fig. 2 and Fig. 3A).
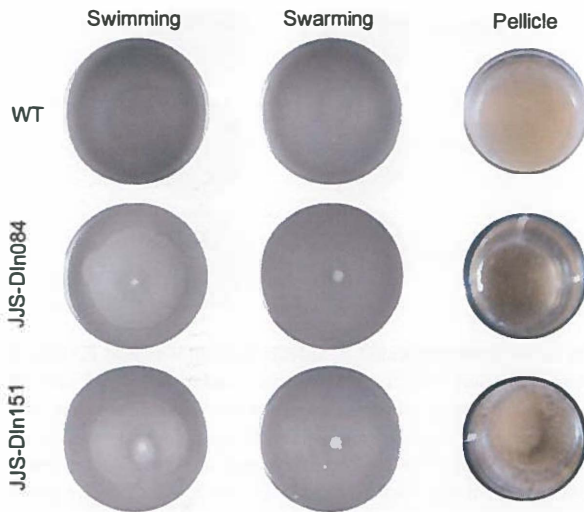

Figure 2. Effects of large genomic deletions in *B. subtilis* 3610-derived strains. The JJS-Dln084 and JJS-Dln151 deletions were introduced in the genome of *B. subtilis* 3610. The resulting mutants (i) were slightly affected in swimming motility, (ii) had completely lost the ability to swarm, and (iii) showed reduced pellicle formation.

In the *B. subtilis* 200 strain, only the JJS-DIn084 deletion caused a mild decrease in swimming whereas the two other deletions had no swimming phenotype in this strain (not shown). Furthermore, the JJS-DIn084 and JJS-DIn151 deletions in strain 3610 blocked swarming and reduced pellicle formation (Fig. 2). The same was true for the JJS-DIn084 deletion in strain *B. subtilis* 200, while the JJS-DIn151 deletion in strain *B. subtilis* 200 affected neither swarming nor pellicle formation (Table 2). These different phenotypes imply that the effects of large genomic deletions on swimming, swarming and pellicle formation depend on the genomic context of the strain in which they are introduced. Nevertheless, the confirmation of a phenotype in the 3610 or *B. subtilis* 200 strains was interpreted as a clear lead for further investigations on the specific individual genes that were responsible for the observed phenotypes relating to motility or biofilm formation.
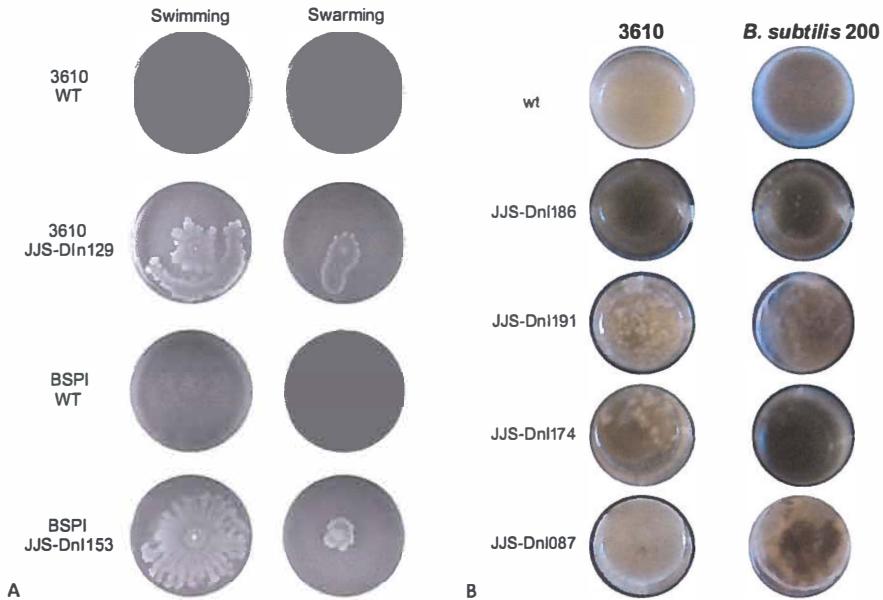
Figure 3. Differential effects of large deletions in the undomesticated *B. subtilis* strains 3610 and *B. subtilis* 200. A. The phenotypes observed in the *B. subtilis* 168 strain were transferable to the undomesticated *B. subtilis* strains 3610 and *B. subtilis* 200. However, differences were observed between the two undomesticated strains used in the present studies. The JJS-DIn129 deletion affected swimming motility only in the 3610 strain, whereas deletion JJS-DIn153 resulted in a decreased ability to swim only in the *B. subtilis* 200 background. **B.** Many mutant strains were impaired in their ability to form pellicles. Some of the tested deletions like JJS-DIn186 had the same impact in both the 3610 and the *B. subtilis* 200 background. In contrast, other deletions such as JJS-DIn187 yielded differential phenotypes.

Individual genes and operons affecting swimming, swarming and pellicle formation

In the strains tested from the BaSynthec collection, a large number of genes were deleted. In order to pinpoint which specific genes impact on swimming, swarming or pellicle formation, the respective mutant strains created in the *Bacillus* functional analysis (BFA) program were analyzed for these properties (33). Mutations in individual genes that were found to cause a different phenotype compared to the parental strain 168 were transferred to the undomesticated *B. subtilis* strains 3610 and *B. subtilis* 200. In what follows, the identification of particular genes required for swimming, swarming or pellicle formation is discussed per large deleted region.

Deletion JJS-DIn084 caused defects in swimming, swarming and pellicle formation in the two undomesticated *B. subtilis* strains. Specifically, it covers 11 genes including the operonic *pstS,C,A,BA,BB* genes. Unexpectedly, none of the individual mutations in this region affected swimming motility, suggesting that the swimming defect caused by the JJS-DIn084deletion is due to the absence of at least two genes. However, *B. subtilis* strains with single mutations in

What is required for motility and pellicle formation in *Bacillus subtilis*

pstA, *pstC*, *pstS* or *pstBB* had completely lost the ability to swarm and their ability to form pellicles was severely affected (Fig 4). Interestingly, strains of *Pseudomonas aureofaciens* with mutations in the phosphate-specific transport (Pst) system are unable to form biofilms (42), and our data imply that this feature of the Pts system is conserved in *B. subtilis*. In addition, the JJS-Din084 deletion covers the *yqgC* gene. This gene stood out in the analysis of the transcriptome of *B. subtilis* 168 under 104 different biological conditions, because it was highly expressed during swarming (44). We therefore, assessed the effects of the *yqgC* mutation in the undomesticated strains. Indeed, the *yqgC* mutation resulted in decreased pellicle formation and the loss of swarming (Fig 4). To date, the precise function of YqgC is unknown, but it is a predicted integral membrane protein that is conserved across *Bacillus* species.
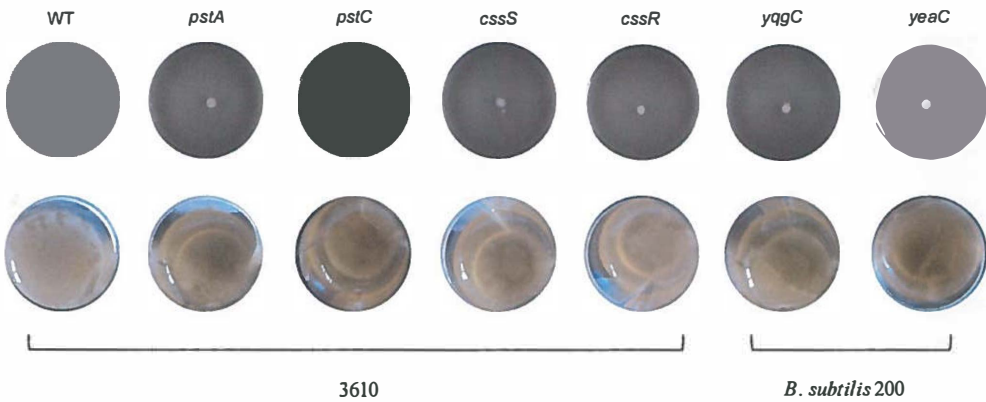

Figure 4. Swarming motility and pellicle formation of individual mutants in the 3610 and *B. subtilis* 200 strain. The BaSynthec strains contain large deletions of genomic regions covering multiple genes. To pinpoint the individual gene(s) responsible for the observed phenotypes, relevant strains from the BFA collection were analyzed. The single gene mutations were transferred to the undomesticated *B. subtilis* strains 3610 and *B. subtilis* 200, and the resulting mutants were analyzed for swimming, swarming and pellicle formation. We identified a limited number of genes that affect both swarming and pellicle formation in the same manner in the 3610 and *B. subtilis* 200 strains as is shown in this Figure. Here the impact of *pstA*, *pstC*, *cssS*, *cssR*, *yqgC* and *yeaC* on swarming and pellicle formation of strain 3610 or *B. subtilis* 200 is shown.

Deletion JJS-DIn151, which covers the *ytkL*, *ytkK*, *ytzD*, *argH*, *argG*, *moaB*, *ackA*, and *ytxK* genes, caused a reduction in swimming, swarming and pellicle formation in the 3610 strain. In contrast, no phenotype was observed in the *B. subtilis* 200 strain. In *Escherichia coli* K-12, mutations in *ackA* and *argG* strongly repress swarming (27). Furthermore, it was shown that a *Listeria monocytogenes ΔackA Δpta* mutant, which could no longer synthesize acetyl phosphate, was strongly affected in motility, biofilm formation and chemotaxis (19). This suggests that the equivalent genes of *B. subtilis* may have similar functions. However, at the present time we do not know which of the eight genes within the region covered by the JJS-DIn151 deletion are responsible for the observed phenotypes. Based on studies with the BFA mutations, we can only

125

Chapter 6

conclude that strains lacking *ytkL*, *ytkK* or *moaB* have wild-type phenotypes with respect to motility and biofilm formation.

Deletion JJS-DIn087 caused reduced swarming motility and pellicle formation in both undomesticated *B. subtilis* strains (Fig. 3B). Ten genes are covered by this deletion including *dnaK* and *hrcA*. In *L. monocytogenes*, the expression of both *dnaK* and *hrcA* is activated during continuous-flow biofilm formation. Furthermore, a Δ*dnaK* mutant of *L. monocytogenes* was deficient in the formation of static biofilms (55). We are now in the process of constructing single mutant strains for *dnaK* and *hrcA*. However, at present we cannot exclude that one or more of the other genes in the region covered by the JJS-DIn087 deletion have a role in swarming and /or pellicle formation of *B. subtilis*.

Purine biosynthesis has been shown to be important during biofilm formation in a number of different bacterial species, including *Bacillus cereus* where deletion of the *purA*, *purC* and *purL* genes led to decreased biofilm formation (56). In *Staphylococcus* species *purL* was shown to play a role in oral biofilm formation (40). In *Photorhabdus temperate* the *purL* gene and the purine biosynthesis pathway were found to be essential for biofilm formation (49). In *Escherichia coli* K-12 deletion of *purC* and *purK* lead to an impaired swarming phenotype(27). In our screen of the large deletion mutants of *B. subtilis*, we identified the *purEKBCSQLFMNHD* (covered by the JJS-DIn174 deletion) as being important for pellicle formation. Intriguingly, it has previously been shown that deletion of the *purR* gene encoding the transcriptional repressor of purine biosynthesis operon affects pellicle formation in *B. subtilis* (32). We therefore conclude that the purine biosynthesis pathway and the properly regulated expression of the corresponding genes is probably important for bacterial biofilm formation in general. For this reason we did not further analyze this region.

The JJS-DIn129 deletion, which covers 16 genes, severely affected swimming and swarming in the 3610 strain, but not in the *B. subtilis* 200 strain. One of the genes included in this region is *narH*. Mutation of the *narH* gene in *E. coli* K-12 strongly represses swarming motility, but has no effect on swimming (27). Furthermore, analysis of the transcriptome of *B. subtilis* 168 under 104 conditions revealed that the deleted *acdA*, *fadF*, *ywjB*, *ywjA* and *ywiE* genes are most highly expressed in swarming cells, suggesting that they are important for this process (44).). Interestingly, the JJS-DIn129 deletion has a negative impact on pellicle formation in the *B. subtilis* 200 strain, but not the 3610 strain (Table 2). Further investigations are required to determine which of these gene(s) are responsible for the observed phenotypes in strains *B. subtilis* 200 and 3610.

Further differences between strains 3610 and *B. subtilis* 200 are observed in relation to the JJS-DIn169 deletion, which covers eleven genes. Deletion of these genes affects both swarming and pellicle formation in strain 3610, but not in the *B. subtilis* 200 strain. Recent tilling array data of *B. subtilis* grown under swarming conditions show that *yeaC*, a gene with an unknown function, is highly expressed under these conditions (44). Therefore we deleted *yeaC* from the genome of the 3610 and *B. subtilis* 200 strains and tested the resulting mutants for swimming, swarming and

pellicle formation. In accordance with the tilling array data the mutant strains were unable to swarm although they were able to produce flagella and swim like the corresponding parental strains. Interestingly, the *yeaC* mutant strains are defective in the production or secretion of the surfactant surfactin (data not shown). This was observed through the absence of the surfactant halo around the mother colonies. In addition, when the strains were grown on blood agar plates, no hemolysis was observed. The hypothesis that *yeaC* may be involved in surfactin production or secretion still needs to be verified. Furthermore, we currently cannot explain why the single *yeaC* mutant has a more severe phenotype than the 168 strain with the JJS-DIn169 deletion, and why the *B. subtilis* 200 strain with the JJS-DIn169 deletion strain exhibits no phenotype.

Furthermore, deletion JJS-DIn191, severely affect pellicle formation in both undomesticated strains (Fig. 3B). This deletion covers the *yutC*, *lipA* and *lytH* genes. A single mutant of *yutC* showed no difference in swimming, swarming or pellicle formation when compared to the parental strain. There are some data indicating that extracellular enzymes such as lipases can accumulate in the biofilm matrix and interact with the EPS (58). For example, *Pseudomonas aeruginosa* biofilms contain significant levels of lipase, protease, esterase and phosphatase activities (54). LytH is a peptidoglycan hydrolase and cell separation is dependent on the activity of cell wall hydrolases. A feature of pellicle formation is the degradation of cell chains via the control of cell separation. Mutation in the peptidoglycan hydrolase *cwlS* has an effect on the later stages of pellicle formation (32). CwlS has been shown to play a role in cell separation together with LytE and LytF in *B. subtilis* (18). Thus more cell wall hydrolases could have an effect on pellicle formation. Currently we are investigating which of these genes are responsible for the observed phenotypes.

Deletion JJS-Din203, which covers 22 genes, caused a reduction in pellicle formation in the 3610 and *B. subtilis* 200 strains. The deleted region includes the genes encoding for the two-component system CssRS, which consists of the membrane-embedded sensor kinase CssS and the response regulator CssR (26). This two-component system responds to high-level protein secretion and heat stress via the phosphorylation of CssR. The phosphorylated CssR activates the transcription of *htrA* and *htrB*, which encode for the membrane-bound serine proteases that are involved in degradation of misfolded and aggregated proteins (16). Interestingly, the *cssS* mutant strains have reduced expression of the *flgB* operon (38), which is involved in flagellar synthesis and chemotaxis (24). Therefore, we analyzed *cssS* and *ccsR* mutant strains for their ability to swim, swarm and form pellicles. These mutant strains were not only unable to form pellicles, but they were also unable to swarm. No impact on swimming was observed (Fig 4).

Another large deletion that seems to affect strains 3610 and *B. subtilis* 200 in a different manner is deletion JJS-DIn153. This deletion has an impact on both swimming and swarming in the *B. subtilis* 200strain (Table 2). This deletion covers 8 genes, including the *iscS* gene. Deletion of this gene has been shown to affect swarming motility in *E. coli* (27). Deletion JJS-DIn153 caused a swarming defect also in 3610 strain.

Chapter 6

There are several other deletions identified during this work that caused alteration in pellicle formation. These deletions were JJS-DIn077, JJS-DIn142, JJS-DIn186 and JJS-DIn200. Currently, we do not know and cannot hypothesize which of the genes within these relatively large deleted regions are responsible for the observed phenotypes. Therefore, further mutagenesis experiments will need to be carried out to genetically dissect all the swimming, swarming and biofilm phenotypes reported in this chapter. Even more so will it be interesting to find out why the 1610 and *B. subtilis* 200 strains frequently respond differently to the tested deletion. Irrespective of the work that remains to be done, the present studies have already pinpointed new roles for CssRS, the Pst proteins, and the YeaC and YqgC proteins in the motile and sessile lifestyles of *B. subtilis*.

Acknowledgements

What is required for motility and pellicle formation in *Bacillus subtilis*

References

1. **Alberti, L. and R. M. Harshey.** 1990. Differentiation of *Serratia marcescens* 274 into swimmer and swarmer cells. J. Bacteriol. **172:**4322-4328.

2. **Barbosa, T. M., C. R. Serra, R. M. La Ragione, M. J. Woodward, and A. O. Henriques.** 2005. Screening for bacillus isolates in the broiler gastrointestinal tract. Appl. Environ. Microbiol. **71:**968-978.

3. **Branda, S. S., F. Chu, D. B. Kearns, R. Losick, and R. Kolter.** 2006. A major protein component of the *Bacillus subtilis* biofilm matrix. Mol. Microbiol. **59:**1229-1238.

4. **Branda, S. S., J. E. Gonzalez-Pastor, S. Ben-Yehuda, R. Losick, and R. Kolter.** 2001. Fruiting body formation by *Bacillus subtilis*. Proc. Natl. Acad. Sci. U. S. A. **98:**11621-11626.

5. **Branda, S. S., J. E. Gonzalez-Pastor, E. Dervyn, S. D. Ehrlich, R. Losick, and R. Kolter.** 2004. Genes involved in formation of structured multicellular communities by *Bacillus subtilis*. J. Bacteriol. **186:**3970-3979.

6. **Bridier, A., D. Le Coq, F. Dubois-Brissonnet, V. Thomas, S. Aymerich, and R. Briandet.** 2011. The spatial architecture of *Bacillus subtilis* biofilms deciphered using a surface-associated model and in situ imaging. PLoS One **6:**e16177.

7. **Calvio, C., F. Celandroni, E. Ghelardi, G. Amati, S. Salvetti, F. Ceciliani, A. Galizzi, and S. Senesi.** 2005. Swarming differentiation and swimming motility in *Bacillus subtilis* are controlled by *swrA*, a newly identified dicistronic operon. J. Bacteriol. **187:**5356-5366.

8. **Chagneau, C. and M. H. Saier Jr.** 2004. Biofilm-defective mutants of *Bacillus subtilis*. J. Mol. Microbiol. Biotechnol. **8:**177-188.

9. **Chai, Y., F. Chu, R. Kolter, and R. Losick.** 2008. Bistability and biofilm formation in *Bacillus subtilis*. Mol. Microbiol. **67:**254-263.

10. **Chu, F., D. B. Kearns, S. S. Branda, R. Kolter, and R. Losick.** 2006. Targets of the master regulator of biofilm formation in *Bacillus subtilis*. Mol. Microbiol. **59:**1216-1228.

11. **Chu, F., D. B. Kearns, A. McLoon, Y. Chai, R. Kolter, and R. Losick.** 2008. A novel regulatory protein governing biofilm formation in *Bacillus subtilis*. Mol. Microbiol. **68:**1117-1127.

12. **Conn, H. J.** 1930. The identity of *Bacillus subtilis*. J. Infect **46:**341-350.

13. **Connelly, M. B., G. M. Young, and A. Sloma.** 2004. Extracellular proteolytic activity plays a central role in swarming motility in *Bacillus subtilis*. J. Bacteriol. **186:**4159-4167.

14. **Costerton, J. W.** 2007. The biofilm primer, p. 5-6. In Anonymous Springer series on biofilms

15. **Costerton, J. W., P. S. Stewart, and E. P. Greenberg.** 1999. Bacterial biofilms: a common cause of persistent infections. Science **284:**1318-1322.

16. **Darmon, E., D. Noone, A. Masson, S. Bron, O. P. Kuipers, K. M. Devine, and J. M. van Dijl.** 2002. A novel class of heat and secretion stress-responsive genes is controlled by the autoregulated CssRS two-component system of *Bacillus subtilis*. J. Bacteriol. **184:**5661-5671.

17. **Fall, R., D. B. Kearns, and T. Nguyen.** 2006. A defined medium to investigate sliding motility in a *Bacillus subtilis* flagella-less mutant. BMC Microbiol. **6:**31.

18. **Fukushima, T., A. Afkham, S. Kurosawa, T. Tanabe, H. Yamamoto, and J. Sekiguchi.** 2006. A new D,L-endopeptidase gene product, YojL (renamed CwlS), plays a role in cell separation with LytE and LytF in *Bacillus subtilis*. J. Bacteriol. **188:**5541-5550.

19. **Gueriri, I., S. Bay, S. Dubrac, C. Cyncynatus, and T. Msadek.** 2008. The Pta-AckA pathway controlling acetyl phosphate levels and the phosphorylation state of the DegU orphan response regulator both play a role in regulating *Listeria monocytogenes* motility and chemotaxis. Mol. Microbiol. **70:**1342-1357.

20. **Hall-Stoodley, L. and P. Stoodley.** 2009. Evolving concepts in biofilm infections. Cell. Microbiol. **11:**1034-1043.

21. **Hamon, M. A. and B. A. Lazazzera.** 2001. The sporulation transcription factor Spo0A is required for biofilm development in *Bacillus subtilis*. Mol. Microbiol. **42:**1199-1209.

Chapter 6

22. **Hamon, M. A., N. R. Stanley, R. A. Britton, A. D. Grossman, and B. A. Lazazzera.** 2004. Identification of AbrB-regulated genes involved in biofilm formation by *Bacillus subtilis.* Mol. Microbiol. **52:**847-860.

23. **Harshey, R. M.** 1994. Bees aren't the only ones: swarming in Gram-negative bacteria. Mol. Microbiol. **13:**389-394.

24. **Heinzerling, H. F., M. Olivares, and R. A. Burne.** 1997. Genetic and transcriptional analysis of *flgB* flagellar operon constituents in the oral spirochete *Treponema denticola* and their heterologous expression in enteric bacteria. Infect. Immun. **65:**2041-2051.

25. **Henrichsen, J.** 1972. Bacterial surface translocation: a survey and a classification. Bacteriol. Rev. **36:**478-503.

26. **Hyyrylainen, H. L., A. Bolhuis, E. Darmon, L. Muukkonen, P. Koski, M. Vitikainen, M. Sarvas, Z. Pragai, S. Bron, J. M. van Dijl, and V. P. Kontinen.** 2001. A novel two-component regulatory system in *Bacillus subtilis* for the survival of severe secretion stress. Mol. Microbiol. **41:**1159-1172.

27. **Inoue, T., R. Shingaki, S. Hirose, K. Waki, H. Mori, and K. Fukui.** 2007. Genome-wide screening of genes required for swarming motility in *Escherichia coli* K-12. J. Bacteriol. **189:**950-957.

28. **Julkowska, D., M. Obuchowski, I. B. Holland, and S. J. Seror.** 2004. Branched swarming patterns on a synthetic medium formed by wild-type *Bacillus subtilis* strain 3610: detection of different cellular morphologies and constellations of cells as the complex architecture develops. Microbiology **150:**1839-1849.

29. **Kearns, D. B. and R. Losick.** 2003. Swarming motility in undomesticated *Bacillus subtilis*. Mol. Microbiol. **49:**581-590.

30. **Kearns, D. B., F. Chu, R. Rudner, and R. Losick.** 2004. Genes governing swarming in *Bacillus subtilis* and evidence for a phase variation mechanism controlling surface motility. Mol. Microbiol. **52:**357-369.

31. **Kearns, D. B., F. Chu, S. S. Branda, R. Kolter, and R. Losick.** 2005. A master regulator for biofilm formation by *Bacillus subtilis*. Mol. Microbiol. **55:**739-749.

32. **Kobayashi, K.** 2007. Gradual activation of the response regulator DegU controls serial expression of genes for flagellum formation and biofilm formation in *Bacillus subtilis*. Mol. Microbiol. **66:**395-409.

33. **Kobayashi, K., S. D. Ehrlich, A. Albertini, G. Amati, K. K. Andersen, M. Arnaud, K. Asai, S. Ashikaga, S. Aymerich, P. Bessieres, F. Boland, S. C. Brignell, S. Bron, K. Bunai, J. Chapuis, L. C. Christiansen, A. Danchin, M. Debarbouille, E. Dervyn, E. Deuerling, K. Devine, S. K. Devine, O. Dreesen, J. Errington, S. Fillinger, S. J. Foster, Y. Fujita, A. Galizzi, R. Gardan, C. Eschevins, T. Fukushima, K. Haga, C. R. Harwood, M. Hecker, D. Hosoya, M. F. Hullo, H. Kakeshita, D. Karamata, Y. Kasahara, F. Kawamura, K. Koga, P. Koski, R. Kuwana, D. Imamura, M. Ishimaru, S. Ishikawa, I. Ishio, D. Le Coq, A. Masson, C. Mauel, R. Meima, R. P. Mellado, A. Moir, S. Moriya, E. Nagakawa, H. Nanamiya, S. Nakai, P. Nygaard, M. Ogura, T. Ohanan, M. O'Reilly, M. O'Rourke, Z. Pragai, H. M. Pooley, G. Rapoport, J. P. Rawlins, L. A. Rivas, C. Rivolta, A. Sadaie, Y. Sadaie, M. Sarvas, T. Sato, H. H. Saxild, E. Scanlan, W. Schumann, J. F. Seegers, J. Sekiguchi, A. Sekowska, S. J. Seror, M. Simon, P. Stragier, R. Studer, H. Takamatsu, T. Tanaka, M. Takeuchi, H. B. Thomaides, V. Vagner, J. M. van Dijl, K. Watabe, A. Wipat, H. Yamamoto, M. Yamamoto, Y. Yamamoto, K. Yamane, K. Yata, K. Yoshida, H. Yoshikawa, U. Zuber, and N. Ogasawara.** 2003. Essential *Bacillus subtilis* genes. Proc. Natl. Acad. Sci. U. S. A. **100:**4678-4683.

34. **Kohler, T., L. K. Curty, F. Barja, C. van Delden, and J. C. Pechere.** 2000. Swarming of *Pseudomonas aeruginosa* is dependent on cell-to-cell signaling and requires flagella and pili. J. Bacteriol. **182:**5990-5996.

35. **Kolter, R. and E. P. Greenberg.** 2006. Microbial sciences: the superficial life of microbes. Nature **441:**300-302.

36. **Kunst, F., N. Ogasawara, I. Moszer, A. M. Albertini, G. Alloni, V. Azevedo, M. G. Bertero, P. Bessieres, A. Bolotin, S. Borchert, R. Borriss, L. Boursier, A. Brans, M. Braun, S. C. Brignell, S. Bron, S. Brouillet, C. V. Bruschi, B. Caldwell, V. Capuano, N. M. Carter, S. K. Choi, J. J. Codani, I. F. Connerton, and A. Danchin.** 1997. The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. Nature **390:**249-256.

What is required for motility and pellicle formation in *Bacillus subtilis*

37. **Leclere, V., R. Marti, M. Bechet, P. Fickers, and P. Jacques.** 2006. The lipopeptides mycosubtilin and surfactin enhance spreading of *Bacillus subtilis* strains by their surface-active properties. Arch. Microbiol. **186**:475-483.

38. **Lemon, K. P., A. M. Earl, H. C. Vlamakis, C. Aguilar, and R. Kolter.** 2008. Biofilm development with an emphasis on *Bacillus subtilis*. Curr. Top. Microbiol. Immunol. **322**:1-16.

39. **Lindum, P. W., U. Anthoni, C. Christophersen, L. Eberl, S. Molin, and M. Givskov.** 1998. N-Acyl-L-homoserine lactone autoinducers control production of an extracellular lipopeptide biosurfactant required for swarming motility of *Serratia liquefaciens* MG1. J. Bacteriol. **180**:6384-6388.

40. **Mack, D., P. Becker, I. Chatterjee, S. Dobinsky, J. K. Knobloch, G. Peters, H. Rohde, and M. Herrmann.** 2004. Mechanisms of biofilm formation in *Staphylococcus epidermidis* and *Staphylococcus aureus*: functional molecules, regulatory circuits, and adaptive responses. Int. J. Med. Microbiol. **294**:203-212.

41. **McLoon, A. L., S. B. Guttenplan, D. B. Kearns, R. Kolter, and R. Losick.** 2011. Tracing the domestication of a biofilm-forming bacterium. J. Bacteriol. **193**:2027-2034.

42. **Monds, R. D., M. W. Silby, and H. K. Mahanty.** 2001. Expression of the Pho regulon negatively regulates biofilm formation by *Pseudomonas aureofaciens* PA147-2. Mol. Microbiol. **42**:415-426.

43. **Nakano, M. M., N. Corbell, J. Besson, and P. Zuber.** 1992. Isolation and characterization of *sfp*: a gene that functions in the production of the lipopeptide biosurfactant, surfactin, in *Bacillus subtilis*. Mol. Gen. Genet. **232**:313-321.

44. **Nicolas, P., U. Mader, E. Dervyn, T. Rochat, A. Leduc, N. Pigeonneau, E. Bidnenko, E. Marchadier, M. Hoebeke, S. Aymerich, D. Becher, P. Bisicchia, E. Botella, O. Delumeau, G. Doherty, E. L. Denham, M. J. Fogg, V. Fromion, A. Goelzer, A. Hansen, E. Hartig, C. R. Harwood, G. Homuth, H. Jarmer, M. Jules, E. Klipp, L. Le Chat, F. Lecointe, P. Lewis, W. Liebermeister, A. March, R. A. Mars, P. Nannapaneni, D. Noone, S. Pohl, B. Rinn, F. Rugheimer, P. K. Sappa, F. Samson, M. Schaffer, B. Schwikowski, L. Steil, J. Stulke, T. Wiegert, K. M. Devine, A. J. Wilkinson, J. M. van Dijl, M. Hecker, U. Volker, P. Bessieres, and P. Noirot.** 2012. Condition-dependent transcriptome reveals high-level regulatory architecture in *Bacillus subtilis*. Science **335**:1103-1106.

45. **Ohgiwari, M., Matsushita, M., and Matsuyama, T.** 1992. Morphological changes in growth phenomena of bacterial colony patterns. J Phys Soc Jpn **61**:816-822.

46. **Patrick, J. E. and D. B. Kearns.** 2009. Laboratory strains of *Bacillus subtilis* do not exhibit swarming motility. J. Bacteriol. **191**:7129-7133.

47. **Potera, C.** 1999. Forging a link between biofilms and disease. Science **283**:1837, 1839.

48. **Rashid, M. H. and A. Kornberg.** 2000. Inorganic polyphosphate is needed for swimming, swarming, and twitching motilities of *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. U. S. A. **97**:4885-4890.

49. **Ruisheng, A. and P. S. Grewal.** 2011. *purL* gene expression affects biofilm formation and symbiotic persistence of *Photorhabdus temperata* in the nematode *Heterorhabditis bacteriophora*. Microbiology **157**:2595-2603.

50. **Senesi, S., E. Ghelardi, F. Celandroni, S. Salvetti, E. Parisio, and A. Galizzi.** 2004. Surface-associated flagellum formation and swarming differentiation in *Bacillus subtilis* are controlled by the *ifm* locus. J. Bacteriol. **186**:1158-1164.

51. **Senesi, S., F. Celandroni, S. Salvetti, D. J. Beecher, A. C. Wong, and E. Ghelardi.** 2002. Swarming motility in *Bacillus cereus* and characterization of a *fliY* mutant impaired in swarm cell differentiation. Microbiology **148**:1785-1794.

52. **Singh, R., D. Paul, and R. K. Jain.** 2006. Biofilms: implications in bioremediation. Trends Microbiol. **14**:389-397.

53. **Stanley, N. R., R. A. Britton, A. D. Grossman, and B. A. Lazazzera.** 2003. Identification of catabolite repression as a physiological regulator of biofilm formation by *Bacillus subtilis* by use of DNA microarrays. J. Bacteriol. **185**:1951-1957.

54. **Tielen, P., F. Rosenau, S. Wilhelm, K. E. Jaeger, H. C. Flemming, and J. Wingender.** 2010. Extracellular enzymes affect biofilm formation of mucoid *Pseudomonas aeruginosa*. Microbiology **156**:2239-2252.

Chapter 6

55. **van der Veen, S. and T. Abee.** 2010. HrcA and DnaK are important for static and continuous-flow biofilm formation and disinfectant resistance in *Listeria monocytogenes*. Microbiology **156:**3782-3790.

56. **Vilain, S., J. M. Pretorius, J. Theron, and V. S. Brozel.** 2009. DNA as an adhesin: *Bacillus cereus* requires extracellular DNA to form biofilms. Appl. Environ. Microbiol. **75:**2861-2868.

57. **Vlamakis, H., C. Aguilar, R. Losick, and R. Kolter.** 2008. Control of cell fate by the formation of an architecturally complex bacterial community. Genes Dev. **22:**945-953.

58. **Wingender, J and Jaeger k.** 2002. Extracellulat enzymes in biofilms, p. 1207-1223. In G. Bitton (ed.), Encyclopedia of Environmental Microbiology, vol. 3. Jonh Wile and Sons, New York.

"All of my memories keep you near
It's all about us, imagine you'd be here"

All of my memories keep you near
The silent whispers, the silent tears..."

Within Temptation - Memories

Chapter 7

Summary and General Discussion

Chapter 7

In every human being the surface tissues, such as the skin and mucous membranes are constantly in contact with microorganisms and can thus be colonized by various microbial species. The cocktail of these microorganisms is known as the 'microbiota'. The normal microbiota includes fungi and archaea, but bacteria are the most numerous. Some of the bacteria that belong to the normal microbiota are also opportunistic pathogens. One such microorganism is *Staphylococcus aureus*, which can be frequently found in the nasopharynx and occasionally on the skin. Around 20% of the healthy population carries *S. aureus* in the nose. However, when the host defenses are breached by trauma, surgery or other means, *S. aureus* can disseminate into almost every tissue and organ system in the human body causing a wide range of diseases. Notably, *S. aureus* can also be encountered outside the human host, for example in livestock and on fresh meat for human consumption. The latter explains why *S. aureus* has also become notorious as a causative agent of food poisoning. The reason why *S. aureus* is such a successful pathogen, even called a superbug, is the arsenal of virulence factors that can be coordinately expressed by this bacterium. In order to regulate all of these virulence and other accessory genes that enhance fitness, *S. aureus* has many global regulators that coordinate the expression of these genes. One such regulator that has been extensively studied is the *a*ccessory *g*ene *r*egulator (*agr*), which is involved in quorum-sensing to allow *S. aureus* to respond appropriately to cell density. The staphylococcal *agr* system acts in such a manner that the cell wall-associated virulence factors, such as protein A, are expressed at low cell densities whereas secreted virulence factors, such as the Phenol-Soluble Modulins (PSMs), are only expressed when higher cell densities are reached. This coordination of virulence gene expression may play an important role in the colonization and spreading of *S. aureus* within the host. Initially, the bacteria are present in small numbers, expressing their cell surface-exposed virulence factors in order to evade the immune system of the host. When the first site of infection becomes depleted of nutrients due to increased bacterial numbers, the organisms increases the production of secreted factors, allowing the bacteria gather nutrients from more distant sites and to spread through the host. The vast amount of virulence factors and the incredible ability of *S. aureus* to acquire resistance to antibiotics have established *S. aureus* as one of the major human pathogens. Thus there is a great need for the development of new antibiotics and other alternative drugs, such as vaccines and target-directed drugs. Proteomic analyses have revealed great heterogeneity and strain-dependent differences between *S. aureus* isolates, which has brought an urgent need for the identification of "invariant" immunogenic determinants (1, 8).

This thesis describes investigations on the mechanisms that allow *S. aureus* to choose between sessile and motile lifestyles. Importantly, several key principles in staphylococcal motility were identified. In particular, the present findings have led to a better understanding of how *S. aureus* can spread over wet surfaces. This provides a knowledge base that is necessary to understand how this pathogen spreads within individual human hosts and the community. Future drugs that target the proteinaceous determinants for spreading could potentially become effective tools to fight *S. aureus* infections.

Summary and General Discussion

Chapter 2 documents the involvement of the staphylococcal quorum-sensing system *agr*, in the colony spreading motility of *S. aureus*. Only when a functional *agr* system is present on the staphylococcal chromosome can colony spreading take place. Mutations in the *agr* system that cause silencing or delay in the activation of the system result in the inhibition of colony spreading (5). During growth under aerobic conditions, mutations arise in *agr* creating a mixed bacterial population. This diverse population of bacteria enhances the likelihood of the subsequent selection of *S. aureus agr* variants most capable of surviving in a specific environment. Evidence of selection for an *agr* negative (*agr*) phenotype in chronic infections is emerging. While most clinical isolates are *agr*⁺, *agr* strains are found in *S. aureus* infections indicating that *agr*⁺ and *agr* variants may have a cooperative interaction in certain types of infections. These data suggest that, whereas secreted virulence factors may be important during the acute phase of infection, loss of *agr* function may enhance the long-term survival of staphylococci in the host and contribute to persistent infections through the formation of biofilms. The *agr* system not only determines the motility status of *S. aureus*, but it has been shown to play a role in biofilm formation. In most areas of a biofilm at most times the *agr* system is not expressed. However, cells that do express *agr* appear to be released from the biofilm (3, 6). Together, the results described in **Chapter 2** imply that the *agr* system plays a decisive role in the choice of *S. aureus* cells between a sessile and a motile lifestyle. This is a function that was not previously attributed to the *agr* system.

The *agr* system is a global regulator of virulence and other accessory genes. The research described in **Chapter 3** shows that *agr* controls colony spreading by regulating the expression of the PSM peptides. The PSMs are short, amphipathic, α-helical peptides with biosurfactant properties, leukocidal activity and antimicrobial activity (2, 7). Proteomic analyses of *agr*⁺ and *agr* variants determined that the only factors commonly secreted by all *agr*⁺ strains are PSM peptides and the staphylococcal lipase. It should be noted here that mainly PSMβ peptides were detected by proteomics. This probably relates to the fact that the PSMα and PSMγ peptides are difficult to detect due to their small size. Subsequent analyses showed that mutations in the *psm* gene loci dramatically decrease the ability of *S. aureus* to spread over wet surfaces. In an *agr* cell, that is unable to spread, PSM peptides are not being expressed and therefore the colony spreading motility cannot take place. There are seven PSM peptides encoded by the *S. aureus* core genome. Mutagenesis experiments showed that not all of these peptides contribute equally to colony spreading. Deletion of the *psmα* operon leads to a dramatic decrease in colony spreading, whereas deletion of the *psmβ* operon has only a minor effect. The use of synthetic PSM peptides helped to further unravel the role of each individual PSM peptide in colony spreading. In accordance with the mutagenesis experiments, not all the peptides have the same ability to induce colony spreading. The peptides with the strongest colony spreading phenotype are the PSMα3 and PSMγ (δ-toxin). As shown for *in vivo* infections, *agr*⁺ and *agr* variants may cooperate to achieve successful invasion and survival in the host. Here it was demonstrated that, when *agr*⁺ and *agr* variants are co-inoculated, the *agr* cells can be found at the edges of the

colony spreading area. This indicates that these cells, which are unable to secrete PSMs and thus to spread away from the inoculation site, are making use of the PSM peptides produced by the neighboring agr^+ variants.

Notably, surfactants are known to influence the adherence of staphylococci to biomaterials. For example, it has been shown that PSMβ peptides can promote both the structuring of *Staphylococcus epidermidis* biofilms and the detachment of cells from such biofilms. As described in **Chapter 3**, we therefore tested the synthetic PSM peptides of *S. aureus* for their ability to inhibit biofilm formation. In accordance with the results obtained for *S. epidermidis*, the PSMβ peptides were shown to strongly decrease biofilm formation of *S. aureus*. However, not all the tested PSM peptides can block biofilm formation to the same extent. Because *S. aureus* is one of the most common causes of catheter-associated infections, we tested the ability of PSM peptides to promote detachment of *S. aureus* from a catheter-associated biofilm and subsequent spreading over a surrounding wet surface. As shown for colony spreading and biofilm formation, some PSM peptides allow *S. aureus* cells to rapidly spread away from a catheter-associated biofilm. Importantly, the studies described in **Chapter 3** show that colony spreading not only takes place on abiotic surfaces, but that it can also occur on biotic surfaces such as fresh meat. As shown by the experiments with fresh meat, spreading cells can colonize larger surfaces in shorter periods of time than non-spreading cells. Thus, it can be hypothesized that spreading is an important parameter at least in food spoilage and, consequently, in food poisoning by *S. aureus*. Potentially, staphylococcal spreading has other clinically relevant roles as well, for example in post-surgical wound infections, but it will remain however a challenge for future studies to verify this hypothesis.

Chapter 4 focuses on the role of some of cellular factors in staphylococcal spreading. Mutation of the *srtA* gene encoding the sortase A (SrtA) was found to lead to an increased colony spreading ability of *S. aureus* and *S. epidermidis*. Notably, SrtA is responsible for the covalent attachment of 'LPxTG proteins' to the cell surface. In the absence of SrtA, the surface display of LPxTG proteins is affected and thus these proteins are less effective in fulfilling their normal functions. Indeed, the results show that the mislocalization of FnbpA, FnbpB, ClfA and ClfB is responsible for the enhanced colony spreading phenotype of the *S. aureus* and *S. epidermidis* *srtA* mutants. Interestingly, previous studies have shown that these factors are also responsible for the attenuated biofilm formation of a *S. aureus srtA* mutant. Thus, the results described in **Chapter 4** show that the same cell surface-associated factors are involved in colony spreading, biofilm formation and adhesion/colonization, but that they have opposing effects. While these cell surface-associated factors are needed for biofilm formation and adherence to host surfaces and host proteins, their absence is beneficial for spreading motility.

Summary and General Discussion

The role of sortases in protein attachment to the cell wall and biofilm formation was further explored in studies documented in **Chapter 5**, especially because *S. aureus* and *S. epidermidis* are the leading causes of biofilm-associated infections. Mutation of the *srtA* genes in both species was shown to lead to the dislocation of ClfA, SasG, SdrC, SdrD and protein-A in *S. aureus* and the Aap protein in *S. epidermidis* from the cell wall to the growth medium. The dislocation of these proteins could be reversed by ectopic expression of the *srtA* gene of either species. Interestingly, ectopic expression of the *srtC* gene from *S. epidermidis* in *S. aureus* and *S. epidermidis* was able to reverse the dislocation of ClfA, SasG and Aap to a significant extent. However, this was not the case for SdrC and SdrD. These results therefore indicate that there is partial overlap in the substrate specificities of SrtA and SrtC. Interestingly, defects in biofilm formation were reverted by ectopic expression of SrtC in some, but not all, tested *srtA* mutant strains. Lastly, overexpression of SrtA resulted in increased levels of biofilm formation in some tested strains, including *S. epidermidis*. Taken together, these findings show that the substrate specificities of SrtA and SrtC overlap partially, and that sortase levels may be limiting for biofilm formation in some staphylococci. In the larger context of the present PhD studies, these findings provide further support for the view that factors that enhance biofilm formation set limits to staphylococcal spreading and vice versa.

Although motility is needed to move from one ecological nice to another, biofilms appear to represent the predominant lifestyle of bacteria. Due to the significant role of biofilms in human infections, it is important to understand the mechanisms of biofilm formation, development and detachment. To obtain new insights into phenomena that govern Gram-positive bacterial motility and biofilm formation in general, a combined systems and synthetic biology approach was followed using the model organism *Bacillus subtilis*. For these studies, *B. subtilis* was more suitable than *S. aureus*, not only because it is the best studied Gram-positive bacterium, but also because *B. subtilis* is highly amenable for genetic modifications at a large scale. Furthermore, *B. subtilis* is able to form robust biofilms and displays flagella-driven motility. **Chapter 6** describes the analyses of 286 *B. subtilis* mutants with large genomic deletions for their performance in swimming, swarming and the formation of biofilms in standing cultures (i.e. pellicles). The laboratory strain *B. subtilis* 168 is most widely used for research but, unfortunately, it contains mutations in loci that are essential for swarming motility and formation of robust biofilms. Therefore, two wild-type undomesticated *B. subtilis* strains, 3610 and BSPI, were included in the analyses. Nevertheless, the initial screen of the mutants had to be carried out with strain 168 in which all large genomic deletions had already been created. Since strain 168 is unable to produce the surfactant known as surfactin, this surfactant was externally supplied to assess swarming motility of the investigated mutant strains. Taken together, the analyses resulted in the identification of more than 50 strains with impaired motility and/or pellicle formation. These large deletions were subsequently transferred to the *B. subtilis* 3610 and BSPI strains for phenotypic verification. The resulting mutants were then tested again for motility and pellicle formation. As observed in the 168 strain, most of the selected mutations also caused impaired

motility and/or pellicle formation in the two undomesticated strains. Interestingly, differences between the 3610 and BSPI strains were observed, more severe phenotypes being observed in the 3610 strain than in the BSPI strain. In total, 13 out of the 286 genomic deletions were found to cause severe motility or biofilm defects in the 3610 and/or BSPI strains. Two of these multi-gene deletions caused a complete loss in the ability to swarm, but only in the 3610 strain. In order to identify which deleted genes were responsible for the observed phenotypes, individual mutants from the *Bacillus* Functional Analysis (BFA) program were analyzed. Thus, it was found that the CssRS two component system, the Pst phosphate transport system and two genes with unknown function *yeaC* and *yqgC* are of major importance for swarming motility and pellicle formation.

In conclusion, this PhD research has unraveled mechanisms that facilitate or counteract colony spreading in *S. aureus*. The *agr*-regulated PSM peptides were found to be the key facilitators of *S. aureus* motility. The *agr* system thus plays a decisive role in the motile lifestyle of *S. aureus* by regulating the expression of PSMs. This is likely to enhance the ability of *S. aureus* to adapt to changing environmental conditions. When present in low numbers, this bacterium forms biofilms in which it is protected from host defenses and antibiotic treatment. Under these conditions, the *agr* system and thus PSM synthesis are down-regulated. When the bacterial population increases, the *agr* system is activated and PSM peptides are produced, whereas the motility-limiting surface proteins FnbpA, FnbpB, ClfA and ClfB are down-regulated. Once this rearrangement has taken place, staphylococcal cells can readily detach from the biofilm and spread to new sites that may be richer in nutrients. While *S. aureus* is translocating to new sites it may encounter other microorganisms that it will be competing with for nutrients. By using the antimicrobial properties of the PSM peptides, *S. aureus* can outcompete these organisms and successfully colonize a new site and/or host. Moreover, PSM peptides can lyse leukocytes and other cell types thereby allowing *S. aureus* to escape major defense mechanisms of the host. Interestingly, community-acquired methicillin resistant *S. aureus* (MRSA) strains, which are highly capable of infecting healthy people outside healthcare settings, display high expression levels of *agr* and therefore also of PSMs (4). The findings described in this thesis therefore suggest that the *agr* system and the PSM peptides strongly contribute to the successful spread and transmission of these strains. However, to validate this idea, it will be necessary to compare PSM production levels in closely related *S. aureus* lineages that differ substantially in their transmissibility. Such studies will be of crucial important for defining future strategies for preventing and fighting staphylococcal infections. The present observations indicate that a combination of different approaches may be needed to successfully combat both the sessile and motile forms of *S. aureus*.

Summary and General Discussion

References

1. **Dreisbach, A., K. Hempel, G. Buist, M. Hecker, D. Becher, and J. M. van Dijl.** 2010. Profiling the surfacome of *Staphylococcus aureus*. Proteomics **10:**3082-3096.
2. **Joo, H. S., G. Y. Cheung, and M. Otto.** 2011. Antimicrobial activity of community-associated methicillin-resistant *Staphylococcus aureus* is caused by phenol-soluble modulin derivatives. J. Biol. Chem. **286:**8933-8940.
3. **Kong, K. F., C. Vuong, and M. Otto.** 2006. Staphylococcus quorum sensing in biofilm formation and infection. Int. J. Med. Microbiol. **296:**133-139.
4. **Otto, M.** 2010. Basis of virulence in community-associated methicillin-resistant *Staphylococcus aureus*. Annu. Rev. Microbiol. **64:**143-162.
5. **Tsompanidou, E., M. J. Sibbald, M. A. Chlebowicz, A. Dreisbach, J. W. Back, J. M. van Dijl, G. Buist, and E. L. Denham.** 2011. Requirement of the *agr* locus for colony spreading of *Staphylococcus aureus*. J. Bacteriol. **193:**1267-1272.
6. **Vuong, C., H. L. Saenz, F. Gotz, and M. Otto.** 2000. Impact of the *agr* quorum-sensing system on adherence to polystyrene in *Staphylococcus aureus*. J. Infect. Dis. **182:**1688-1693.
7. **Wang, R., K. R. Braughton, D. Kretschmer, T. H. Bach, S. Y. Queck, M. Li, A. D. Kennedy, D. W. Dorward, S. J. Klebanoff, A. Peschel, F. R. DeLeo, and M. Otto.** 2007. Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. Nat. Med. **13:**1510-1514.
8. **Ziebandt, A. K., H. Kusch, M. Degner, S. Jaglitz, M. J. Sibbald, J. P. Arends, M. A. Chlebowicz, D. Albrecht, R. Pantucek, J. Doskar, W. Ziebuhr, B. M. Broker, M. Hecker, J. M. van Dijl, and S. Engelmann.** 2010. Proteomics uncovers extreme heterogeneity in the *Staphylococcus aureus* exoproteome due to genomic plasticity and variant gene regulation. Proteomics **10:**1634-1644.

"Music can change the world, because it can change people."

Bono

Chapter 8

Nederlandse samenvatting

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Nederlandse samenvatting

Ons lichaam is gekoloniseerd met micro-organismen zoals schimmels, archaea en bacteriën. Sommige bacteriën zijn nuttig voor ons lichaam. Ze kunnen bijvoorbeeld vitaminen produceren, breken stoffen af die ons lichaam zelf niet kan afbreken, en concurreren met andere ziekteverwekkende bacteriën. Normaal gesproken kunnen deze bacteriën ons lichaam niet aantasten, maar wanneer de natuurlijke barrière doorbroken is vanwege lichamelijke trauma's of wanneer er al een andere infectie in het lichaam aanwezig is, kunnen deze bacteriën een (nieuwe) infectie te veroorzaken. *Staphylococcus aureus* is een van de bacteriën die hierom bekend is. Meer dan 20% van de gezonde mensen draagt deze bacterie met zich mee. Bij deze mensen wordt *S. aureus* meestal in de neus gevonden, maar ook in de oksel en op de huid. Wanneer ons immuun systeem niet goed werkt, kan *S. aureus* zich naar andere weefsels en organen verspreiden en daar nieuwe infecties veroorzaken. De meerderheid van deze infecties zijn onschuldig maar soms kan zo'n infectie ook levensbedreigend zijn. *S. aureus* is zo succesvol in het overleven in ons lichaam, omdat het veel verschillende virulente factoren produceert. Om de expressie van deze virulente factoren te controleren, heeft *S. aureus* verschillende regulatoren.

Een dergelijke regulator is het *agr* regulatiesysteem (*accessoir gene regulator*) dat reageert op veranderingen in de hoeveelheid omliggende bacteriële cellen. Het *agr* systeem reguleert de expressie van verschillende virulente factoren van *S. aureus*. Deze virulentiefactoren worden alleen geproduceerd wanneer er genoeg bacteriën aanwezig zijn. Kort nadat de bacteriën het lichaam zijn binnengedrongen, is de concentratie van bacteriële cellen nog niet erg hoog. Vervolgens worden virulentie factoren geproduceerd die op het oppervlak van de bacterie blijven zitten. Deze aan het oppervlak blootgestelde factoren helpen de bacteriën om ons immuun systeem te ontwijken en vervolgens om verschillende organen te koloniseren. Zodra de bacteriën ons immuun systeem hebben ontweken, kunnen deze bacteriën zich vermenigvuldigen. Wanneer ze in hoge concentraties aanwezig zijn, wordt het *agr* systeem actief.

In **hoofdstuk 2** van dit proefschrift wordt de rol van het *agr* system in de motiliteit van *S. aureus* bekeken. Deze motiliteit wordt 'colony spreading' genoemd en in dit hoofdstuk blijkt dat *S. aureus* een actief *agr* system nodig hebben om te kunnen verspreiden over het oppervlak van een agarplaat. Wanneer het *agr* system wordt uitgeschakeld door middel van mutaties in het DNA blijkt dat de bacteriën zich niet meer kunnen verspreiden over het oppervlak van een agarplaat.

In **hoofdstuk 3** wordt gekeken naar de invloed van het *agr* system op de regulatie van de expressie van gesecreteerde virulentie factoren. Uit deze studies blijkt dat specifieke factoren verantwoordelijk zijn voor 'colony spreading' en in het bijzonder de Phenol-soluble Modulins (PSM) peptiden. Deze peptiden hebben de specifieke eigenschap dat ze de oppervlaktespanning kunnen verminderen en daardoor de bacteriën in staat stellen om zich te kunnen verspreiden over het oppervlak. *S. aureus* produceert acht verschillende peptiden met deze eigenschap: PSMα1-4, PSMβ1-2, PSMγ, en PSM-mec. Om aan te tonen dat deze peptiden verantwoordelijk zijn voor 'colony spreading', zijn er mutaties geïntroduceerd in de genen die coderen voor de PSM peptiden. Als gevolg van de geïntroduceerde mutaties werden de PSMα1-4 peptiden en de PSMβ1-2 peptiden niet meer geproduceerd door *S. aureus*. Uit experimenten met deze mutanten is gebleken dat voornamelijk de PSMα3 peptide en de PSMγ peptide verantwoordelijk zijn voor 'colony spreading' van *S. aureus*.

Hoofdstuk 4 gaat in op de rol van eiwitten die op het oppervlak van de bacterie zitten in 'colony spreading' van *S. aureus*. Een interessante vondst is dat dezelfde factoren verantwoordelijk zijn voor de

Nederlandse samenvatting

kolonisatie van *S. aureus* in ons lichaam, specifiek onze neus. Deze factoren zorgen er voor dat de bacteriën dicht bij elkaar worden gehouden. Dit heeft tot gevolg dat de bacteriën zich niet verder verspreiden naar andere locaties in ons lichaam. Wanneer deze factoren niet aanwezig zijn op het oppervlak van de bacteriën zou dan betekenen dat *S. aureus* zich makkelijker zou kunnen verplaatsen naar andere organen of weefsels in ons lichaam. **Hoofdstuk 5** gaat over hoe deze factoren aan het oppervlak getoond worden. *S. aureus* en *Staphylococcus epidermidis* hebben een eiwit (SrtA) dat deze factoren covalent aan de celwand bindt. Daarnaast hebben sommige stammen van *S. epidermidis* een extra eiwit (SrtC) dat deze functie zou kunnen vervullen. In dit hoofdstuk wordt gekeken naar welke oppervlakte-eiwitten nog aan de celwand gebonden zijn of in het medium terechtkomen in een mutant waar SrtA niet meer geproduceerd wordt. Het blijkt dat een aantal eiwitten in het medium terechtkomen (o.a. ClfA, SasG en Spa), maar ook dat er een groot deel nog aan de celwand is gebonden, waarschijnlijk via andere interacties met de celwand. Daarnaast blijken deze eiwitten ook belangrijk te zijn voor het vormen van een biofilm. Deze situatie kan weer compleet hersteld worden wanneer SrtA van *S. aureus* of *S. epidermidis* tot expressie wordt gebracht in de mutant, en ook gedeeltelijk wanneer SrtC van *S. epidermidis* tot expressie wordt gebracht.

Het laatste hoofdstuk, **Hoofdstuk 6**, beschrijft de rol van verschillende factoren in de bacteriële beweeglijkheid van *Bacillus Subtilis*. *B. subtilis* is een modelorganisme voor Gram-positieve bacteriën en de kennis die we met studies aan deze bacterie verkrijgen kan gebruikt worden om processen in pathogene bacteriën te verklaren. In de studie beschreven in dit hoofdstuk hebben we nieuwe factoren gevonden die *B. subtilis* helpen om over 'natte' oppervlaktes te bewegen.

Tenslotte worden in **Hoofdstuk 7** de onderwerpen die in dit proefschrift besproken in een breder perspectief geplaatst.

"Χρώμα δεν αλλάζουνε τα μάτια
που θυμάσαι και θυμάμαι
τίποτα δε χάθηκε ακόμα
όσο ζούμε και πονάμε
χρώμα δεν αλλάζουνε τα μάτια
μόνο τρόπο να κοιτάνε"

Γεράσιμος Ανδρεάτος - Χρώμα δεν αλλάζουνε τα μάτια

"The eyes you remember and I remember
Do not change color
Nothing is lost yet
As long as we leave and feel pain
The eyes do not change color
Only the way they are looking"

Gerasimos Andreatos - The eyes don't change color

Chapter 9

Περίληψη και γενική συζήτηση

Chapter 9

Περίληψη και γενική συζήτηση

Σε κάθε ανθρώπινο οργανισμό οι επιφανειακοί ιστοί και οι βλεννογόνες μεμβράνες έρχονται σε συνεχή επαφή με περιβαλλοντικούς οργανισμούς και αποικίζονται από διάφορους μικροοργανισμούς. Μεταξύ αυτών μύκητες, πρωτόζωα και κυρίως βακτήρια. Πολλά από αυτά τα βακτήρια είναι ευεργετικά για τον ανθρώπινο οργανισμό. Παράγουν βιταμίνες, διασπούν τροφές που ο ανθρώπινος οργανισμός αδυνατεί να διασπάσει και ανταγωνίζονται παθογόνα βακτήρια. Υπό φυσιολογικές συνθήκες αυτά τα βακτήρια δεν μπορούν να βλάψουν τον οργανισμό μας. Εντούτοις, αν υπάρξει μόλυνση, τραύμα ή κάποια χειρουργική επέμβαση τότε κάποια από αυτά τα βακτήρια μπορούν να προκαλέσουν ασθένεια και να γίνουν ευκαιριακά παθογόνα. Ένα τέτοιο ευκαιριακό παθογόνο είναι και ο σταφυλόκοκκος. Πάνω από το 20% του ανθρώπινου πληθυσμού αποικίζεται με σταφυλόκοκκο στη μύτη. Όταν το ανοσοποιητικό μας σύστημα δεν δουλεύει σωστά τότε ο σταφυλόκοκκος έχει την ευκαιρία να μετατοπιστεί σε άλλους ιστούς και όργανα και να τα αποικίσει, προκαλώντας ασθένειες. Πολλές από τις ασθένειες που προκαλεί είναι ήπιες αλλά μπορεί να προκαλέσει και σοβαρές ασθένειες όπως πνευμονία που μπορεί να επιφέρει και θάνατο. Ο σταφυλόκοκκος έχει τη δυνατότητα να προκαλέσει διαφορετικές ασθένειες γιατί παράγει πολλούς διαφορετικούς παθογόνους παράγοντες. Επίσης, για να μπορέσει να ρυθμίσει την έκφραση όλων αυτών των παθογόνων παραγόντων, ο σταφυλόκοκκος έχει πολλούς ρυθμιστικούς μηχανισμούς. Ένας τέτοιος μηχανισμός είναι το σύστημα το οποίο ανταποκρίνεται στη συγκέντρωση του σταφυλοκόκκου σε συγκεκριμένο σημείο αποίκισης. Αυτό το σύστημα λέγεται *agr*.

Το σύστημα *agr* ρυθμίζει την έκφραση αυτών των παθογόνων παραγόντων κατά τέτοιο τρόπο ώστε οι παράγοντες που εκκρίνονται να εκφράζονται μόνο όταν τα βακτήρια είναι παρόντα σε μεγάλους αριθμούς. Όταν τα βακτήρια μολύνουν τον οργανισμό μας αρχικά βρίσκονται σε μικρούς αριθμούς. Τότε, παράγουν πρωτεϊνικούς παράγοντες οι οποίοι βρίσκονται κολλημένοι στα βακτήρια. Αυτοί οι παράγοντες βοηθούν τον σταφυλόκοκκο να ξεγελάσει το ανοσοποιητικό μας σύστημα και να αποικίσει κάποιον ιστό ή όργανό μας. Αφού καταφέρει να νικήσει το ανοσοποιητικό μας σύστημα και να επιβιώσει μέσα στον οργανισμό μας τότε αρχίζει να πολλαπλασιάζεται. Όταν ο αριθμός τον βακτηρίων αυξηθεί αρκετά τότε το *agr* σύστημα ενεργοποιεί την έκφραση των εκκρινόμενων παραγόντων.

Στο **δεύτερο κεφάλαιο** αυτής της διατριβής δείξαμε ότι το *agr* σύστημα είναι ρυθμιστής της κινητικότητας του σταφυλοκόκκου. Η κινητικότητα που έχει παρατηρηθεί στον σταφυλόκοκκο ονομάζεται colony spreading δηλαδή διασπορά αποικίας. Σε αυτό το κεφάλαιο δείξαμε ότι ο σταφυλόκοκκος μπορεί να πραγματοποήσει αυτή τη διασπορά αποικίας μόνο όταν το *agr* σύστημα είναι ενεργό. Σε βακτήρια στα οποία το *agr* σύστημα έχει απενεργοποιηθεί, η ικανότητα διασποράς της αποικίας χάνεται.

Στο **τρίτο κεφάλαιο** δείξαμε ότι *agr* σύστημα ρυθμίζει την κινητικότητα του σταφυλοκόκκου μέσω της ρύθμισης της έκκρισης παθογόνων παραγόντων. Αποδείξαμε ότι οι παράγοντες αυτοί είναι τα λεγόμενα Phenol-soluble Modulins (PSM) πεπτίδια. Αυτά τα πεπτίδια έχουν επιφανειοδραστικές ιδιότητες που σημαίνει ότι ελαττώνουν την επιφανειακή τριβή και έτσι επιτρέπουν στα βακτήρια να μετακινηθούν σε διάφορες επιφάνειες. Ο σταφυλόκοκκος παράγει 7 τέτοια πεπτίδια. Τέσσερα από αυτά ονομάζονται PSMα1-4 πεπτίδια, δύο ονομάζονται PSMβ1-2 και το τελευταίο ονομάζεται PSMγ. Για να αποδείξουμε ότι αυτά τα πεπτίδια είναι υπεύθυνα για τον συγκεκριμένο φαινότυπο προχωρήσαμε σε μεταλλαγή των γονιδιακών τόπων που κωδικοποιούν για τα PSM πεπτίδια. Κάνοντας αυτές τις μεταλλαγές σταματήσαμε την έκφραση μόνο των PSMα1-4 πεπτιδίων ή μόνο των PSMβ1-2 πεπτιδίων ή και των έξι. Αυτά τα πειράματα απέδειξαν ότι τα PSMα1-4 πεπτίδια και το PSMγ πεπτίδιο είναι αυτά που ρυθμίζουν κατά μεγάλο ποσοστό την διασπορά αποικίας του σταφυλοκόκκου.

Στο **τέταρτο κεφάλαιο** δείξαμε ότι κάποιοι από τους παράγοντες που βρίσκονται στην επιφάνεια του σταφυλοκόκκου και παίζουν ρόλο στην αποίκιση των ανθρώπινων ιστών και για παράδειγμα της μύτης, παίζουν ρόλο και στην διασπορά αποικίας. Ο ρόλος τους είναι να κρατάνε τα βακτηριακά κύτταρα σε στενή επαφή μεταξύ τους και έτσι δεν μπορούν να μετακινηθούν σε μακρινές αποστάσεις. Όταν όμως αυτοί οι παράγοντες δεν βρίσκονται στην επιφάνεια των βακτηριακών κυττάρων τότε τα κύτταρα του σταφυλοκόκκου μπορούν να

Περίληψη και γενική συζήτηση

διασπαρθούν σε μακρυνές αποστάσεις. Το **πέμπτο κεφάλαιο** αναφέρεται στους παράγοντες της επιφάνειας του σταφυλοκόκκου. Ρίχνει φως στο πώς αυτοί οι παράγοντες φτάνουν στην επιφάνεια και πώς ρυθμίζονται.

Το **έκτο** και τελευταίο **κεφάλαιο** αναφέρεται στους μηχανισμούς κινητικότητας ενός άλλου μη παθογόνου βακτηρίου του βάκιλλου (*Bacillus subtilis*). Το συγκεκριμένο βακτήριο χρησιμοποιείται ευρέως ως οργανισμός μοντέλο για πειραματικές μελέτες. Η ανάλυση μας οδήγησε στην εύρεση καινούργιων παραγόντων που παίζουν ρόλο στην κινητικότητα του συγκεκριμένου βακτηρίου.

Εν κατακλείδι, η συγκεκριμένη διατριβή ανακάλυψε πως τα PSM πεπτίδια τα οποία εκκρίνονται από τον σταφυλόκοκκο είναι υπεύθυνα για τιν κινητικότητα του σταφυλοκόκκου. Πιστεύουμε ότι φάρμακα τα οποία θα στοχεύουν αυτά τα πεπτίδια και τους επιφανειακούς παράγοντες που επηρεάζουν την κινητικότητα του σταφυλοκόκκου, θα βοηθήσουν στην καταπολέμηση λοιμώξεων του σταφυλοκόκκου και στην ρύθμιση της εξάπλωσης του σταφυλοκόκκου.

Appendix 1: Dankwoord

Dankwoord – Ευχαριστίες

And now you have finally reached the most important chapter of the present thesis: the one that comes from my heart and hopes to touch your hearts (maybe make you cry a bit...). I am going to start with my parents of course.

Μπαμπά, μαμά ό,τι και να πώ είναι λίγο. Χωρίς εσάς τίποτα από όλα αυτά δεν θα είχαν συμβεί. Δεν θα κρατούσατε στα χέρια σας αυτό το βιβλίο και δεν θα διαβάζατε τώρα αυτές τις γραμμές. Ένα μεγάλο ευχαριστώ που είστε ΠΑΝΤΑ εδώ για μένα, που κάνατε τα πάντα ώστε εγώ να μπορέσω να έρθω στην Ολλανδία και να ζήσω το όνειρο μου. Ξέρω πόσο πολύ σας στοίχισε το γεγονός ότι ήρθα τόσο μακρυά και για αυτό σας θαυμάζω που παρά τον πόνο σας είσασταν πάντα κοντά μου, με στηρίζατε σε όλες τις δύσκολες στιγμές μου και ποτέ μα ποτέ δεν με με κάνατε να νιώσω τύψεις που έφυγα. Είστε οι καλύτεροι γονείς του κόσμου και νιώθω πολύ τυχερή που είστε οι δικοί μου γονείς. Θράσο, ένα ευχαριστώ και σε σένα που έμεινες πίσω να στηρίζεις τη μαμά κ τον μπαμπά. Μου λείπεις πολύ. Γιαγιάκα μου, η πάντα δυνατή. Σ' αγαπώ.

Τα φιλαράκια μου, οι δικοί μου 'friends' από πού να αρχίσω και πού τα τελειώσω… Αποστόλη, Αραξή, Αργύρη Αφροδίτη, Βίκυ 1, Βίκυ 2, Εύη, Νίκο, Θεοδοσούλα, Λίτσα, Μούλη, Νατάσα, Νίκο, Οβ, Παναγιώτη, Σοφία, Χοσέ, Χριστίνα. Σας ευχαριστώ για τα υπέροχα πράγματα που έχουμε κάνει μαζί τόσα χρόνια. Όπως συνειδητοποιήσατε όταν φτιάχνατε το βιντεάκι για το πάρτυ μου, έχουμε κάνει και περάσει πολλά μαζί. Σας ευχαριστώ που δεν με ξεχάσατε, που δεν με κάνατε να νιώσω ποτέ ότι δεν είμαι μέρος της ζωής σας πλέον. Σας ευχαριστώ για τα ταξίδια που κάναμε μαζί και για την υπέροχη πρτωτοχρονία που μου χαρήσατε. Δεν μπορώ ούτε να ξεκινήσω να λέω πόσο μου λείπετε. Σας αγαπώ πολύ. Σταυρούλα και Κώστα σας ευχαριστώ που με κάνατε κουμπάρα σας και που μου χαρήσατε έναν υπέροχο βαφτισιμιό.

Ερχόμενη όμως εδώ έκανα επίσης πολύ καλούς φίλους. Φίλους που δεθήκαμε σαν οικογένεια. Δημητράκο, Ευάκι, Εβελινάκι, Δεσπουλίνι, Βασίλη, Χρύσα, Μαριέττα σας ευχαριστώ γιατί κάνατε την ζωή μου στην Ολλανδία πιο εύκολη, πιο ευχάριστη, πιο ζεστή. Σας ευχαριστώ για τα υπέροχα και αστεία πράγματα που κάναμε μαζί. Για τα μαζέματα και τραπεζώματα στο σπίτι του Δημήτρη, για τα καφεδάκια μας και τα αλα Εύη Sinterklaas παρτάκια μας. Ευάκι και Δημητράκο μου λείπετε απίστευτα. Το δέσιμο που έχουμε δεν μπορεί να σπάσει όσα χιλιόμετρα κι αν μας χωρίζουν. Χρύσα σ' ευχαριστώ μου με βοήθησες με τα Ολλανδικά, που έμαθες στον Μαρκ την βάση των ελληνικών. Για τα μαζεματά μας στο σπίτι σας, για τα ωραία απογεύματα που περάσαμε παίζοντας παιχνίδια. Ελπίζω να σας έχω στη ζώη μου για πολλά πολλά πολλά ακόμα χρόνια. Σας αγαπώ πολύ.

Τι νόμιζες σε ξέχασα; Όχι βέβαια, αλλά έχεις ξεχωριστή θέση στην καρδιά μου άρα και εδώ. Σ' ευχαριστώ που ήρθες στο Groningen που με έκανες να σε συμπαθήσω και τελικά να σε αγαπήσω σαν αδερφή μου. Όσο είμασταν Αλεξανδρούπολη δεν συμπαθιόμασταν αλλά χαίρομαι πάρα πολύ που αυτό το αλλάξαμε. Σ'ευχαριστώ για τον υπέροχο χρόνο που περάσαμε μαζί στο σπιτάκι μας, που δέχτηκες να έχεις δύο και όχι έναν συγκάτοικο, που ποτέ δεν παραπονέθηκες. Σ' ευχαριστώ που είσαι πάντα εδώ για μένα. Σε θαυμάζω για το πόσο δυνατή είσαι, και για το πόσο δίνεσαι στους ανθρώπους που αγαπάς. Σ' αγαπώ πολύ. Και για όποιους δεν κατάλαβαν, ναι μιλάω για την Δανάη.

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Appendix 1: Dankwoord

leuke avonden. Ook bedankt voor onze nieuwe neefje Max. Ja ja Patrick, ik weet het; Maximus Patricius Paulus Bernhard de Boer☺. Bedankt voor de mooie dagen die ik samen met jullie en Max heb gehad toen hij net geboren was.

Once again I have to switch language. First of all, I would like to thank Jan Maarten. Thank you, for accepting me as an exchange student in 2007. Thank you for giving me the opportunity to do real research and making me fall in love with bacteriology. Moreover, thank you for offering me my PhD position. When they told me that you wanted to talk to me, I was terrified that I had disappointed you with my lack of experimental knowledge. Instead of that, you offered me a PhD position. I really want to thank you for this great opportunity. It changed my scientific life but also my personal life. Thank you for all our talks and for making me a better scientist. I really enjoyed going through my papers with you, correcting them and making them better. I really learned a lot from you. Speaking of learning, Emma comes to my mind. You are a great scientist and a great person. You taught me so many things. Thank you for helping me feel more self-confident, for helping me to be a real PhD student, for replying to my numerous silly questions, for our discussions, for helping me through my frustrations, for the amazing time I had working with you in the lab. But above all, thank you for being my friend. A special thanks to Annette as well, for her support and help through my PhD, for teaching me new techniques, for always being there when I needed her, for answering all my questions. You are a great scientist and a great person. I am glad I meet you and worked with you. Monika, thank you first of all for being there for me from the beginning. You and Girbe were pretty much the only persons I was talking to during my first research project. Thank you for all our talks, for the help in the lab, for the nice dinners. Thank you for being my zusje. Girbe, thank you for all the discussions we had. Sjouke, Ruben, Ewoud thank you for the amazing conversation during our dinners at Emma's place. Vivianne, thank you for coming to our group and making it more lively. Thank you and Geoff for coming to our wedding and for inviting us to yours. We had an amazing time in South Africa and we came back home with a nice souvenir☺. Thank you for our Pilates evenings as well. Of course, a big thanks to all MolBac members (old and new), Mark, Thijs, Rene, May, Jessica, Corina, Henrik, Vahid, Federico, Magda, Gosia, Dennis, Rense, Carmine, Lakshmi, May, Francisco, Marcus, Jolanda, Jetta, Hermie, for these amazing four years. I would also like to thank my students, Jolien and Gijs, for their help, amazing work and support. I also want to thank you for making me a better supervisor.

I would also like to thank the reading committee, Michael Hecker, Wim Quax, and Henk Busscher, for reading and approving my thesis and for their critical comments.

Wow, you reach the end of this book. And you think you had enough of it. I am sorry to disappoint you, but this is not the end. I still have to talk about the love of my life, my husband, Mark. I could go on and on and on talking about him but I am not going to do it. First of all, Mark, thank you for supervising me when I was a student. Thank you for introducing me to your project and *S. aureus,* for letting me do the colony spreading which ended up being the main topic of my PhD thesis. Thank you for asking me to join you to a reggae concert (because I was the only one you knew that liked reggae music☺), which was the beginning of a great story, our story. Thank you for all the support during these years. Thank you for answering my questions, helping me out, and teaching me how to work with *S. aureus.* Thank you so much for helping me preparing this thesis: it would have never been this way without your help. But most of all, thank you for supporting me psychologically and for making my life bearable while being away from my family and friends. Thank you for making me want to spend the rest of my life with you and to love you as no one else. You are an amazing person. Ζουζουνάκι μου, σ' αγαπώ πολύ για πάντα.

Ελένη

List of Publications

1. Sibbald, M.J.J.B., Winter, T., van der Kooi-Pol, M.M., Buist, G., **Tsompanidou, E.**, Bosma, T., Schäfer, T., Ohlsen, K., Hecker, M., Antelmann, H., Engelmann, S., van Dijl, J.M. (2010) Synthetic effects of *secG* and *secY2* mutations on exoproteome biogenesis in *Staphylococcus aureus*. *J. Bacteriol.* **192**, 3788-3800.

2. **Tsompanidou, E.**, Sibbald, M.J.J.B., Chlebowicz, M.A., Dreisbach, A., Back, J.W., van Dijl, J.M., Buist, G., Denham, E.L. (2011) Requirement of the *agr* locus for colony spreading of *Staphylococcus aureus*. *J. Bacteriol.* **193**, 1267-1272.

3. Sibbald, M.J.J.B., Yang, X.-M., **Tsompanidou, E.**, Qu, D., Hecker, M., Becher, D., Buist, G., van Dijl, J.M. Partially overlapping substrate specificities of staphylococcal group A sortases. Submitted for publication in *Proteomics*.

4. **Tsompanidou, E.**, Denham, E.L., Sibbald, M.J.J.B., Yang, X.-M., Seinen, J., Friedrich, A.W., Buist, G., van Dijl, J.M. The sortase A substrates FnbpA, FnbpB, ClfA and ClfB antagonize colony spreading of *Staphylococcus aureus*. Submitted for publication in *PLoS One*.

5. **Tsompanidou, E.**, Denham, E.L., Becher, D., de Jong, A., Buist, G, van Oosten, M., Manson, W.L., Back, J.W., van Dijl, J.M., Dreisbach, A. Distinct roles of phenol-soluble modulins in spreading of *Staphylococcus aureus* on wet surfaces. Submitted for publication in *Journal of Biological Chemistry*.

6. **Tsompanidou, E.**, Tanaka, K., Noirot, P., Shyns, G., van Dijl, J.M., Denham, E.L. What is required for motility and pellicle formation in *Bacillus subtilis*. *In preparation*.

Background information on the cover

Staphylococcous aureus, the Gram-positive bacterium described in the present thesis, is one of the major causes of hospital-acquired infections. Because of its ability to transmit within the hospital settings and due to the difficulties that medical doctors are facing in treating patients with staphylococcal infections, these infections are causing 'nightmares' both to medical staff and to patients. *S. aureus* is also called a 'Superbug' and I therefore took the freedom to compare it with Gods form the Greek mythology. On the front cover *S. aureus* is compared with Morfeas (Μορφέας). Morfeas was one of the brothers called the Oneiroi (Dreams), which were the Gods of the dreams. These Gods were sending dreams to humans. Morfeas could take different human shapes (**morphologies**) and this is how he was appearing in dreams. The word morphology that is so often used in science thus has its roots in Morfeas' name. Morfeas was pictured with strong wings that could help him travel to the end of the earth. So like Morfeas, *S. aureus* is sending 'dreams' to humans and can take monstrous appearances. The phenol-soluble modulin peptides (PSMs), described in the present thesis, could be imagined then as the wings that help *S. aureus* translocate over wet-surfaces.

On the back cover, *S. aureus* is compared with Proteus (Πρωτεύς). Proteus was a sea-God that could take up any morphology he wanted. Proteas could transform himself into any kind of animal, plant, bird, or even fire and water. From this feature of Proteus comes the adjective protean, with the general meaning of 'versatile', 'mutable', 'capable of assuming many forms'. 'Protean' has positive connotations of flexibility, versatility and adaptability as does *S. aureus*. Like Proteus, *S. aureus* can take different morphologies on the soft agar plates used to study the motility of this bacterium. *S. aureus* can be seen as a flower or tree, as a bird or butterfly and finally as an animal. Not only can *S. aureus* take up different morphologies on plates, but this bacterium is also highly adaptable to environmental changes and to host-specific microenvironments. It can acquire numerous antibiotic resistances and it can mutate itself (e.g. the *agr* locus). Thus, it seems to me (as a Greek with great imagination ☺), that if the ancient Greeks had encountered *S. aureus*, they would have for sure proclaimed this bacterium a God. In fact, the name of *S. aureus* has a Greek root, it is often encountered in the Greek hospitals (more than 50% prevalence of MRSA in positive blood cultures). It so much resembles the Greek Gods that it has been studied by Greek people (e.g. myself). I therefore dare to say, yes, we Greeks are proud to proclaim *S. aureus* a semi-God among the bacteria.

The cover was designed by Jan Kluitenberg and Eleni Tsompanidou.

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