Between adaptation and virulence

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Chapter 1

Introduction and scope

Why studying bacterial pathogenesis?

The presence of microorganisms that live in the human body has been acknowledged since the end of the 19th century. Nonetheless, it has been only from the beginning of the 21st century that efforts to expand our knowledge about the microbiota have gained higher priority, a development that has been enabled by technological advances in genome sequencing. The communities of microorganisms inhabiting our bodies, as well as the bodies of other multicellular organisms, comprise a multitude bacterial species, fungi, parasites, and viruses which, importantly, have evolved together with us. Consequently, the resulting commensal and symbiotic relationships have had an influence on the development and functioning of host systems, like the gastro-intestinal tract or the immune system. However, even though most of these microorganisms are commensals, or have mutualistic relationships with the host, some of them can cause disease under certain conditions.

Disease-causing microorganisms are often transmittable and therefore do not only have an impact on one individual but can potentially affect whole communities. Therefore, diminishing the impact and prevalence of such pathogens is of utmost importance. In this regard, understanding the molecular mechanisms that allow microorganisms to adapt to and harm the host would shed light on potential opportunities to develop treatments.

In particular, opportunistic bacterial pathogens like *Staphylococcus aureus* have evolved features that increase their chances of survival and spreading, while being challenged by the host immune system or antibiotic therapies. These bacterial adaptations are not only geared towards the production of molecules that have a negative impact on the host, but also to hiding from our immune defenses, granting access to scarce resources or optimizing their use. Consequently, *S. aureus*

evolved into a pathogen that is renowned for its virulence and high capacity to develop resistance to physical and chemical insults. For this reason, most research on *S. aureus* has been focused on the mechanisms this bacterium employs for infection and antibiotic resistance. In contrast, relatively little is known about its role as a commensal (1). Gaps of knowledge in this area are mainly related to the great flexibility of *S. aureus* to adapt, genetically or metabolically, to the many distinct niches in the human body. Of note, although the adaptations that *S. aureus* displays under limiting conditions have been widely studied *in vitro*, its adaptive behavior *in vivo* received relatively little attention. This relates to the fact that the conditions to which the bacteria need to respond *in vivo* are complex and frequently changing. This makes the pathogen's responses equally complex and dynamic, reflecting the continuous communication between host and pathogen. Understanding and perhaps being able to predict the course of these dynamic interactions is not only an intriguing scientific challenge, but also a necessity for the development of new and more sustainable antimicrobial therapies.

Staphylococcus aureus

S. aureus is a Gram-positive and facultative anaerobic bacterium commonly found as a human commensal in the anterior nares, throat, and on the skin (2). Although this bacterium is part of our microbiota, it is better known for being the causative agent of several diseases ranging from mild skin infections or food poisoning, to life-threatening conditions, like sepsis or necrotizing pneumonia. More importantly, its capability to swiftly develop resistances to antibiotics makes this bacterium a public health threat in the community, as well as in hospitals (3).

The first report on *S. aureus* dates back to 1880, when the surgeon Sir Alexander Ogston observed the presence of spherical microorganisms while studying purulent infections. His illustrations depict cocci that grew in clusters, looking like "roe of fish". This observation led him to subsequently coin the name

Staphylococcus for this microorganism (4). However, it was not until 1884 that Friedrich Julius Rosenbach distinguished *S. aureus* from the closely related *Staphylococcus epidermidis*, based on the golden color of its colonies (hence the term *aureus*). *S. epidermidis*, on the other hand, was temporarily called *albus*, due to the white color of its colonies (5).

The outstanding capability of *S. aureus* to develop resistances against antibiotics was noticed promptly after their introduction as a common treatment of infections. During the first half of the 20th century, in fact, *S. aureus* infections were still considered highly fatal. It was only around 1940 that these infections started to be treated with penicillin, reducing the mortality rates. However, *S. aureus* isolates that developed resistance to this antibiotic were noticed shortly after the discovery of penicillin (6), and within a few years, penicillin resistant lineages had emerged in the clinic (7). Later on, methicillin was introduced as treatment for staphylococcal infection, but resistant strains (all defined as methicillin resistant *Staphylococcus aureus*, or MRSA) emerged already in 1961, only one year after its introduction (8). During the 1970s and 1980s there were several MRSA outbreaks worldwide, but these were limited to hospital settings (9). In response to the increasing incidence of MRSA infections, vancomycin started to become more frequently used, leading to the appearance of strains that were tolerant to this antibiotic in 1997 (10).

Nowadays, as penicillin resistance is commonly found in *S. aureus* strains, methicillin and related β-lactam antibiotics have been used as the recommended treatment for infections caused by this bacterium. Consequently, the worldwide incidence of MRSA has increased over time and many countries have reported a prevalence of methicillin resistance in 50% or more of the clinical *S. aureus* isolates (11, 12). Of note, the high capability to develop resistance to antibiotics is common among *Staphylococcus* species, but none of these is equally aggressive as *S. aureus*,

which makes MRSA a major concern for our health and wellbeing (13). Congruently, infections caused by resistant *S. aureus* lead to higher morbidity and mortality rates (3, 14).

S. aureus is capable of acquiring resistance to antibiotics through several processes, which include mutations in the core genome, as well as the acquisition of exogenous resistance genes carried by plasmids and other mobile genetic elements. These mechanisms started to become better understood after publication of the first whole genome sequences of two isolates of this bacterium in 2001 (15). Specifically, the latter study was carried out using two MRSA isolates, N315 and Mu50, thereby unveiling the genetic background of staphylococcal resistance, as well as the major role of horizontal gene transfer in spreading resistance between staphylococci. Importantly, this study also represents the first report on the presence of multiple virulence genes in this bacterium.

Although *S. aureus* is generally acknowledged for its role as a pathogen, carriage of this bacterium is generally asymptomatic. The 'harmless' commensal state of *S. aureus* was acknowledged in the mid-1940's, when the nose was identified as its most common niche (16). In fact, approximately 20% of the human population can be classified as recurrent carriers, while another 30% are regarded as temporal carriers of *S. aureus* in the anterior nares (17, 18). The carriers were shown to be more prone to develop nosocomial infections caused by *S. aureus*, and it has actually been reported that 80% of bacteremia cases are caused by the endogenous strain of the patient (19, 20). Of note, carriers not only have a higher risk of *S. aureus* infection, but they also transmit *S. aureus* to other individuals in the population (21). In the last 20 years, MRSA strains have adapted to spread among the community, causing infections in healthy individuals and displaying a more virulent behavior. Since *S. aureus* represents a general and serious threat for the human population, efforts have been undertaken to develop vaccines against this

pathogen but, unfortunately, none of the tested candidates has so far passed the stage of clinical trials (22, 23).

Epidemiology

The aforementioned capability of *S. aureus* to develop resistance to antibiotics has enabled this bacterium to prevail in the population and to spread around the world in the 'antibiotic era', turning this bacterium into one of the major public-health threats. One of the first documented drug-resistant lineages that have rapidly spread to different countries was the strain 80/81. The pandemic caused by this clone lasted from 1954 to 1957, starting in Australia and expanding to several countries, including the USA and the UK, where this clone was responsible for several outbreaks (24). At that time, the incidence of *S. aureus*-caused infections was 3 per 100.000 person-years but increased to approximately 20 per 100.000 person-years during the next 30 years. This rise has been attributed to nosocomial infections, acquired after invasive medical interventions. Nowadays, the rate of *S. aureus* infections appears to be stable, but the actual incidence varies over time and is dependent on geographical location. Of note, less affluent regions of the world exhibit higher rates of the infections caused by *S. aureus* than the wealthier regions (25).

Today, MRSA is one of the most commonly identified pathogens around the globe including America, Europe, North Africa, and the Middle east (24). Infections caused by MRSA strains are no longer restricted to hospital settings and even healthy individuals are at risk of developing an infection. Of note, some individuals within the human population are particularly susceptible to develop *S. aureus* bacteremia, including babies within the first year of life, adults over 70 years old, HIV- infected individuals, intravenous drug users, and hemodialysis patients (25).

Especially since the emergence of community-associated (CA) MRSA strains, the burden of *S. aureus* infections has risen in many countries (21, 26). The first report on CA *S. aureus* came from Australia in the early 1990's, concerning patients from a remote population who had limited access to large hospitals (27). By the end of the same decade, the first CA strain from the US was isolated from healthy children that did not exhibit the commonly known risk factors (28). Subsequently, CA-MRSA has been reported to spread in very diverse communities including native Americans, islanders in the Pacific, athletes, prisoners, military personnel and individuals in day care centers (21).

Since their very first discovery, CA-MRSA isolates displayed a more virulent behavior than the HA-MRSA isolates (29, 30). In particular, CA *S. aureus* has a high capacity to produce sepsis and fatal infections like necrotizing pneumonia, purpura fulminans and post-viral Toxic Shock Syndrome (TSS). HA-MRSA is more commonly associated with respiratory infections, while CA-MRSA, on the other hand, is more often associated with skin and soft tissue infections. Enhanced virulence has been linked to the capacity of CA-MRSA lineages to kill neutrophils more rapidly and, therefore, neutralize this first line of immune defense (31–33). This also explains the more pronounced inflammation and tissue damage that are usually associated with infections caused by CA strains (34).

Identification of the virulence factors that are most representative for each group and the differences in their virulence potential is still a matter of debate. It has been suggested, however, that toxins like the Phenol-soluble Modulins (PSMs) or LukSF (produced by the Panton-Valentine leukocidin [PVL] genes) are responsible for the increased virulence of CA-MRSA (31, 35, 36). One of the strongest arguments for this hypothesis is the higher prevalence of the PVL genes in most of the CA isolates compared to HA isolates (29, 37). Nevertheless, conflicting results regarding the real contribution of PVL to either the fitness or virulence of CA-

MRSA isolates have led to the hypothesis that additional factors may contribute to the CA phenotype.

The discrimination between HA- and CA-MRSA infections has conventionally been based on the time span between admission of a patient into a healthcare institution and the detection of a MRSA-positive culture. In general, a pathogen is considered CA if it is detected within the first 48 h after a patient's admission to a healthcare institution and if this patient has not been hospitalized within the two years prior to the detection of this patient's MRSA carriage. If it is detected after 48 h of hospitalization, the pathogen is labeled as HA. In special cases, where a patient presents particular risk factors associated to health care centers, the causative infectious agent is considered HA community onset, even if the infection is detected during the first 48 h after admission. Nonetheless, molecular characterization of HA- and CA-MRSA isolates has shown that the two groups are no longer restricted to their original locations, but rather they are migrating from the hospital into the community and *vice versa*. In consequence, the preferred characterization of clinical isolates should not only take into account clinical data, but also a molecular characterization (38).

Molecular typing

In order to grasp the genetic variability of *S. aureus*, different methods for classification of strains have been implemented over time. These methodologies have expanded our understanding of the evolution of *S. aureus*, the spreading of this bacterium worldwide and the association of different pathologies with different genetic backgrounds. There are several classical methods to classify the different strains of *S. aureus*: two gel-based methods: Pulsed-Field Gel Electrophoresis (PFGE) and Multiple-Locus Variable Number Tandem Repeat Fingerprinting (MLVF), and three sequence-based: *spa* typing, Multilocus Sequence Typing (MLST), and multiple-locus variable-number tandem-repeat

analysis (MLVA; Sabat et al., 2012). Another method, SCC*mec* typing, is utilized only for classification of MRSA strains (12, 40). However, advancements in sequencing technologies have increased their availability in hospital settings, and currently whole-genome sequencing emerges as an efficient method for epidemiological identification of *S. aureus* isolates (41, 42).

PFGE has been one of the most frequently used methods for characterization of isolates during outbreaks. This gel-based typing method compares isolates based on their banding pattern after digestion of genomic DNA with the restriction enzyme SmaI and separation of the resulting fragments on agarose gels with alternating current directions (43). Although commonly used, this method is labor-intensive and achieving good reproducibility between laboratories is challenging (44). Strains classified by this method are clustered by 80% of similarity and the resulting clusters are designated "USA" according to the Centers for Disease Control and Prevention guidelines (45). The second gel-based typing method, MLVF, is particularly useful in a local setting but, like PFGE, it doesn't produce data that are portable among laboratories. The MLVF technique is based on the gel banding pattern of PCR fragments derived from five staphylococcal variable number tandem repeat loci (*sdrCDE, clfA, clfB, sspA,* and *spa*). Unfortunately, it is not possible to relate the respective DNA banding patterns to the number of repeats per locus (46).

MLST is a sequence-based technique where single nucleotide variations in seven housekeeping genes (*pta, tpi, yqiL, acrC, aroE, glpF, gmk*) are examined. The allelic variation in each locus is used to designate a sequence type (ST) to the investigated strains (47). Although this method is relatively expensive and labor-intensive, its main advantage lies in its good reproducibility and the portability of data between laboratories using an online database for comparisons (48). Up until very recently, MLST has been the most commonly used method to assess

evolutionary relationships between *S. aureus* linages. In particular, isolates that show identity in 5 out of the 7 sequenced genes are clustered in so-called clonal complexes (49). MLST classification is often paired with SCC*mec* typing for a more refined identification of MRSA isolates (48). *Spa* typing is also based on sequence analysis of variable number tandem repeats, but in this case only the *spa* gene is targeted. *Spa* typing is widely used due to its low cost and higher discriminatory power compared to MLST (48). Moreover, this typing technique does not only take into account the number of repeat variations, but also point mutations found within the *spa* gene (50). MLVA was developed to overcome limitations of PFGE, MLST, and *spa* typing. It is as discriminatory as PFGE and produces portable data that can be readily interpreted, similar to data obtained by MLST and *spa* typing.

SCC*mec* typing is a technique used to differentiate MRSA isolates based on the Staphylococcal Cassette Chromosome *mec*, which carries the *mecA* gene, its regulator genes, and the so-called *ccr* recombinase genes. Importantly, the *mecA* gene is responsible for the methicillin resistance by encoding an additional penicillin-binding protein (PBP2A) that does not bind β-lactam antibiotics (Katayama et al., 2000). Currently, at least 7 types of SCC*mec* (I to VII) can be differentiated by the respective combinations of *mec* and *ccr* genes and additional resistance genes (24, 51).

All the afore-mentioned typing methods have proven to be useful for the identification and distinction of *S. aureus* isolates. However, most clinically relevant isolates of *S. aureus* belong to a limited number of lineages, and often the classical typing methods are not sufficient for detailed differentiation (52). Moreover, these techniques are laborious and time-consuming. Consequently, whole-genome sequencing is becoming increasingly popular for rapid identification and characterization of pathogens in clinical settings. Although sequencing of 16S rRNA genes has been widely used for the assignment of

bacterial isolates to pathogen species, it does not provide further details on the specific nature of the investigated microorganism. Therefore, the characterization of clinical isolates of *S. aureus* nowadays involves whole-genome sequencing. In recent years, the availability of this method in clinical microbiology laboratories has rapidly increased. Nonetheless, so far there is no standard methodology for sample preparation and sequence data analysis, which poses a great challenge for the implementation of this method in routine clinical diagnostics (53, 54).

The 'toolbox' of *S. aureus* for infection

Virulence factors

The virulence potential of *S. aureus* is mostly determined by its capability to produce proteinaceous and non-proteinaceous molecules that help this pathogen to colonize and invade host tissues, get access to the host's resources, or hide from the immune response of the host. These virulence factors disturb the normal function of host cells and may severely affect the host organism. Additionally, some virulence factors are responsible for triggering specific and sometimes excessive immune responses. In general, virulence factors can be distinguished by their subcellular or extracellular localization. Proteins that are linked to the surface of the pathogen are mostly involved in adhesion to extracellular matrixes or binding to other host molecules, while secreted proteins target the host's primary barriers, disrupt cell membranes and neutralize, modulate or allow to evade immune cells (55, 56).

Surface virulence factors

Proteins that are anchored to the cell wall of *S. aureus* can be classified into 4 classes of proteins based on their structure, namely: i, the microbial surface components recognizing adhesive matrix molecules (MSCRAMMs); ii, the near iron transporter (NEAT) motif family; iii, the G5-E bundle family; and iv, the

three-helical bundle family. Regardless of their classification, all these proteins have a secretory signal peptide at the N-terminus and a sorting signal peptide at their C-terminus (57). These proteins can be regarded as genuine virulence factors, since mutant strains defective in any of these cell wall-associated proteins were shown to be attenuated in infection models, or cause decreased bacterial loads (58–61). Alternatively, they were shown to promote nasal colonization (62–64).

The MSCRAMMs form the largest group of surface proteins in *S. aureus.* They are characterized by the presence of two IgG-fold domains close to their N-terminus. In general, these proteins help the bacteria to colonize host tissues by attaching them to molecules from the host's extracellular matrix, like collagen, fibronectin, or fibrinogen (65). The MSCRAMMs thus include fibronectin-binding proteins A and B (FnbpAB), clumping factors A and B (ClfAB), a collagen-binding protein (Cna), and the Serine-aspartate repeat-containing proteins C, D, and E (SdrC, SdrD and SdrE). Moreover, some MSCRAMMs have secondary functions as exemplified by Fnbps which supports biofilm formation in some *S. aureus* strains (McCourt et al., 2014), or ClfA and Cna which can bind to complement factors thereby interfering with the complement system (66, 67). Importantly, the Fnbps are key for the internalization process into non-professional phagocytic cells, as they bind to the fibronectin located on the host cell membranes and trigger the signal for endocytic uptake (68).

The iron-regulated surface determinant proteins (IsdA, IsdB, IsdC, and IsdH) are part of the NEAT motif family. These proteins are renowned for their role in iron acquisition. Similar to other surface-associated proteins, they also bind to fibronectin and fibrinogen and, therefore, they participate in the internalization process by non-professional phagocytes (69). Nevertheless, being key components of the iron uptake pathway, they play a pivotal role in intracellular survival (70,

71). Lastly, the IsdABCH proteins are involved in immune evasion, but the molecular mechanisms to achieve this are still unclear (63, 72).

In *S. aureus*, the G5-E repeat 'family' consists only of the surface protein G (SasG), which is present only in a subset of *S. aureus* isolates. This protein plays a main role in adhesion to the nasal epithelium and has been associated with biofilm formation. Nevertheless, neither the respective molecular mechanisms, nor the ligands of this protein have been identified so far (57, 73).

Lastly, the Staphylococcal protein A (Spa) has three helical bundles in its N-terminal region that have affinity for the Fc part of immunoglobulins, especially IgG. Thus, Spa protects *S. aureus* from opsonization and, consequently, limits phagocytosis by cells of the host's immune system (74, 75). In addition to its role in immune evasion, Spa also shows affinity to the tumor necrosis factor receptor 1 (TNFR1). This leads to increased production and release of cytokines and recruitment of polymorphonuclear leukocytes (PMNs). Therefore, Spa also acts as a pro-inflammatory molecule that increases tissue damage during the infection process (76, 77).

In addition to the proteins that are anchored to the surface of the bacterium the capsule of *S. aureus* is also a potent virulence factor, due to its role as a bacterial defense mechanism against the host immune responses. In particular, the capsule inhibits recognition of the bacteria by phagocytic cells and therefore prevents phagocytosis and killing by the immune system. The gene clusters involved in production of capsule polysaccharides are prevalent in 90% of the *S. aureus* isolates with types 5 and 8 being present in most of the clinical isolates (78).

Secreted virulence factors

S. aureus produces several virulence factors that are released into the extracellular milieu. In general, these factors are involved in the evasion of and interference

with the host immune system or cause direct damage to host cells and tissues. Secreted proteins that play a role in adhesion or immune evasion apply similar modes of action as the afore-mentioned surface proteins. In particular, the fibrinogen-binding protein (Efb) and staphylococcal coagulase (Coa) bind to fibrinogen to protect the bacteria from phagocytosis and to start the coagulation cascade, respectively (79). Other secreted *S. aureus* proteins like the immunoglobulin G (IgG)-binding protein (Sbi), the staphylococcal complement inhibitor (SCIN), the chemotaxis inhibitory protein of staphylococci (CHIPS), and a variety of superantigens (SAg) modulate immune responses. Sbi is a protein that works similar to Spa and, accordingly, it prevents opsonization and has a proinflammatory effect (80). The SCIN and CHIPS proteins are encoded by the same gene cluster and both have a major impact on the activity of the complement system, which is part of the host's innate immune system. While SCIN binds to the C3 convertase and prevents activation of the complement system, CHIPS binds to the neutrophil receptors that are related to chemotaxis (81–83).

Contrary to the secreted proteins that serve to evade or attenuate the host's immune system, the main function of SAgs is to trigger stronger immune reactions. These proteins, including the toxic shock syndrome toxin-1 (TSST-1), activate and stimulate T-cells leading to their proliferation, excessive cytokine production and ultimately to host cell death (84). The SAgs were initially mostly linked to food poisoning (85), but subsequent research demonstrated their role in the development of infectious endocarditis, dermatitis, necrotizing pneumonia and kidney abscesses (86–88).

Besides proteins that manipulate the immune system, other secreted proteins from *S. aureus* induce lysis of host cells, enable the destruction of tissue and spreading of the bacteria to other tissues. Many of these secreted toxins are known as pore-forming toxins.

α-Hemolysin (Hla) is one of the major virulence factors of *S. aureus*. This pore-forming and pro-inflammatory protein is mainly regulated by the accessory gene regulator (Agr) quorum-sensing system, and contributes to abscess formation during skin infection in animal models (31, 89). Hla interacts with A Disintegrin and Metalloprotease 10 (ADAM10), which is indispensable for the structural modifications of Hla that lead to the formation of a cytolytic pore in the host cell's membrane (90). In addition to this mechanism of host cell lysis, Hla also potentiates the natural cleavage function of ADAM10 towards membrane proteins like e-cadherin, thereby promoting the disruption of tight junctions (89).

Other relevant staphylococcal pore forming proteins are the bicomponent leukotoxins, namely gamma hemolysin (Hlg), leukocidins (Luk)AB (or LukGH), LukED, and the afore-mentioned leukocidin PVL (LukSF). The lysis process caused by these toxins is host-receptor dependent, specifically involving integrin, C5aR1 or CD45 receptors (91–93). Studies have shown that LukAB and LukED play a major role in murine sepsis and renal abscess models (94, 95). Importantly, LukAB contributes to the lysis of PMNs after the pathogen has been phagocytized (91). LukSF, on the other hand, has been proposed to influence other host processes, like mitochondrial functions, leading to the induction of apoptosis (96). Nonetheless, the precise roles of this protein remain to be clarified.

Several studies that compared the impact of PVL proteins, using wild-type *S. aureus* strains and isogenic mutants, showed no significant differences in virulence, neutrophil survival or cytotoxicity in human epithelial cells (Summarized by David and Daum, 2010). Nevertheless, recent studies have highlighted some variables that have not been considered previously and that could explain the mixed results obtained while studying the impact of PVL on virulence. Although all *lukSF* genes are very similar, there is a distinctive substitution at the nucleotide 527 that distinguishes two variants of the PVL

protein termed R and H. These two variants show different geographical distribution and are distinctively associated to CA- or HA-MRSA (97–99). Moreover, the effects of PVL vary in a host-specific manner, and they have different affinity for particular targets depending on the infected organ. In this regard, it is noteworthy that PVL displays high specificity to human and rabbit neutrophils, while their affinity for the respective receptors in murine and monkey neutrophils is low. Therefore, mice are not an appropriate model to measure the impact of PVL on virulence (100). In this regard, there may actually be more variables to consider, such as the diversity in expression rates in different staphylococcal isolates and host systems (101).

The PSM proteins represent another class of cytolytic toxins produced by bacteria of the *Staphyloccocus* genus, including *S. aureus* and *S. epidermidis* (102). Although these proteins are not exclusive to these species, only strains capable of colonizing epithelial surfaces produce them, indicating an evolutionary connection to the colonization site. In particular, *S. aureus* produces seven PSMs: four PSMα, two PSMβ and one PSMδ (delta hemolysin). All these peptides have a similar structure and amphiphilic nature, but not all are as effective as virulence factors. In this respect, virulent strains commonly produce higher amounts of PSMα peptides and lower amounts of PSMβ peptides (36). Of note, PSMs have potent surfactant properties and, accordingly, they have been implicated in staphylococcal spreading over wet surfaces (103).

The importance of PSMs in virulence is linked to their different roles in the host-pathogen interaction. First, they are capable of disrupting host cells by membrane perturbation in a receptor-independent manner. This independence of host receptor proteins makes them relevant in a broad spectrum of diseases (104). Secondly, they trigger inflammatory responses like chemotaxis and priming of neutrophils, and they induce cytokine expression by interaction with the formyl

peptide receptor 2 (FPR2; Kretschmer et al., 2010). Interestingly, this interaction is inhibited by the *S. aureus*-protein FPR2/ALS-inhibitory protein (FLIPr), thereby modulating the host's immune response (106). Thirdly, PSMs take part in shaping the biofilm structure and bacterial detachment from the biofilm, resulting in dissemination and bacterial spreading during infection. Due to their amphiphilic nature, these proteins are capable of opening channels, which allows the diffusion of nutrients into deeper layers of the biofilm. Since excess production of PSMs leads to detachment of the biofilm structure, their production is tightly regulated and varies within a biofilm (107, 108). Lastly, the production of PSMα has been recently linked to excretion of cytoplasmic proteins by inducing damage of the bacterial membrane. This disruption event is not specific for trafficking of proteins, but also prompts the release of other molecules as lipids and ATP into the extracellular milieu (109). Importantly, secretion of cytoplasmic proteins has been proposed as a potential virulence mechanism employed by numerous bacterial species, including *S. aureus* (110, 111).

In summary, *S. aureus* produces a wide range of proteins that are actively involved in the interactions between the pathogen and its host, and that are required for growth, propagation and survival of the pathogen during infection. These different virulence factors that are located at the cell surface or are secreted, modulate the environmental cues in an infection setting to favor different outcomes. Since these proteins affect directly the responses of the host, they are tightly regulated by the pathogen.

Regulators

The adaptability of *S. aureus* to different environments is not only a consequence of its genetic variability and capability to acquire exogenous DNA, but its ability to respond appropriately to chemical and physical stimuli is also highly relevant

for the management of available resources. When it comes to the adaptation to different niches, the bacteria must optimize the consumption of the available nutrients, balancing the production of proteins that are required for survival and fitness (112). During an infection setting, these adaptations will include the production of virulence factors to access the limiting amounts of nutrients, but also activation of pathways to optimally use them. These changes in gene expression are coordinated by a network of regulators that is tightly interconnected (113). Some of the respective regulators directly control the production of virulence factors, while others are indirectly related to virulence by controlling central metabolic pathways.

One of the key challenges that *S. aureus* has to face during infection or colonization is the variable abundance of resources, like carbon and oxygen, which affects the central carbon metabolism and therefore the provision of energy and metabolic precursors (114). The main regulators that are involved in adaptation to conditions with restrictive resources are the catabolite control protein A (CcpA), catabolite control protein E (CcpE) and the GTP-sensing pleiotropic repressor CodY. All of these regulators control the transcription of genes that encode proteins to optimize energy production from alternative sources. In resource-rich environments, these regulators will prevent excessive energy consumption for unnecessary tasks (112).

The activity of CcpA is modulated by the availability of diverse compounds including simple sugars, disaccharides or alcoholic derivatives of them (115). CcpA binds to DNA sequences denominated catalytic responsive elements (CRE), which are located upstream of its target genes. Although it has been determined that CcpA has a low intrinsic affinity to CRE, its affinity is enhanced by the corepressor histidine-containing protein (HPr) in the phosphorylated state (116, 117). This corepressor is an indicator of the availability of carbon sources, because its phosphorylation state depends on the abundance of glycolytic intermediates

(115). Besides its regulatory role in the expression of genes involved in the catabolism of alternative carbon sources, CcpA also has a direct effect on virulence by controlling the expression of Hla, Spa, and TSST-1 (118, 119). Another direct regulator of central carbon metabolism is CcpE, whose activity is modulated by the presence of citrate. CcpE is a positive regulator of the expression of genes encoding proteins involved in the TCA cycle, and it also has been shown that its inactivation enhances bacterial virulence (120, 121).

A third metabolic regulator, CodY, is mainly required for the activation of the TCA cycle, but it also regulates the expression of genes for proteins involved in amino acid biosynthesis (112). The cofactors of this regulator are guanosine-5'-triphosphate (GTP) and the branched-chain amino acids (BCAAs), which increase the affinity of CodY for its target sequences. A low abundance of GTP signals stress conditions or nutrient deprivation (112, 122), while the abundance of BCAAs in the extracellular milieu serves as an indicator for the availability of carbon, nitrogen and sulfur. Diminished quantities of these cofactors trigger the derepression of CodY-regulated genes involved in alternative energy-generating pathways (123, 124). Interestingly, CodY mutants do not only increase the production of enzymes related to biosynthesis and transport of amino acids and other nutrients, but also lead to increased production of virulence factors or proteins used to adequately respond to stress conditions. In this regard, some genes encoding virulence factors, like *hla* or *capA*, have been found to be directly regulated by CodY, but this regulator indirectly affects the expression of more virulence factors by repressing Agr (125, 126).

The afore-mentioned regulators mainly respond to the availability of carbon or nitrogen sources. However, basic cellular functions are also affected by the availability of oxygen. This molecule is, in fact, the main electron acceptor during aerobic respiration, and without it, *S. aureus* is forced to use alternative metabolic

pathways like fermentation. One of the first effects of a lack of oxygen is a disbalance in the redox state of the cell which, in turn, triggers the major regulator redox-dependent transcriptional repressor (Rex) (112). This protein senses the NADH/NAD+ ratio by binding either of these two molecules and regulating the expression of several proteins that play a role in maintaining the redox state. Rex-NADH derepresses the synthesis of proteins from the fermentative pathway, electron transport chain, anaerobic metabolism and regulators of nitrogen metabolism, including the two-component regulatory system SrrAB (127). Additional to the regulation by Rex, the SrrAB system also responds to the levels of oxygen and nitric oxide in the environment, sensing the reduction of menaquinone (128). The genes regulated by this system are related to cytochrome assembly, anaerobic metabolism, iron-sulfur cluster repair and NO detoxification (129).

As described above, the metabolic changes caused by nutrient scarcity lead to changes in the expression of virulence factors. This is mainly connected to the regulation of the Agr system, which has been by far the most studied regulatory system involved in staphylococcal virulence (112, 125). This quorum-sensing system is simultaneously regulated by at least 15 other proteins, among them the afore-mentioned CcpA, CodY and SrrAB regulators (126). The Agr system comprises two loci that are transcribed from two divergent promoters. The P2 promoter is located upstream of the *agrA, agrB, agrC* and *agrD* genes encoding the Agr system. AgrA is a transcriptional regulator that binds to cognate promotor regions and modulates their activity. This binding is dependent on AgrA phosphorylation, which is carried out by AgrC, a membrane protein stimulated by the abundance of the auto-inducing peptide (AIP) in the extracellular environment. AgrD is the precursor of the AIP, which is processed by the membrane protein AgrB. On the other hand, the P3 promotor regulates the expression of Hld, a virulence factor, and RNAIII, a small regulatory RNA

molecule that serves as one of the major regulators of *S. aureus* (130, 131). Currently, more than 174 genes are known to be regulated by Agr, and expression of at least 60 of them is modulated by RNAIII. Upregulation of the Agr system leads to high-level production of toxins like PSMs, Hla, LukSF and LukDE, and reductions in the level of surface proteins like Spa (128). Since Agr regulates most of the known virulence factor genes, it is essential for virulence. This has been corroborated by the generation of mutants of this system and verified in different infection models (132–136). Through the regulation of Agr by metabolic regulators like CcpA, CodY and SrrAB, the production of virulence factors is connected to the environment of *S. aureus*. On top of this, Agr expression is also modulated by additional transcriptional regulatory systems, such as the staphylococcal accessory regulator SarA and alternative sigma factor SigB.

SarA belongs to the SarA family of regulatory proteins, comprising 11 proteins that, notably, are also related to the regulation of the Agr system. In particular, the SarA protein plays an important role in its activation during early stages of bacterial growth (137). Nonetheless, the connection of SarA to the production of virulence factors is not only due to its positive effect on Agr, but it also serves to activate the expression of fibronectin-binding proteins, all hemolysins, and TSST-1 (128, 138–140). Interestingly, SarA also negatively influences the levels of other proteins related to virulence and fitness, like Spa, IsaB, proteases, and the superoxide dismutases (141–143). Thus, SarA is also a principal regulator of virulence, as was demonstrated through the use of mutants in infection models (144–146). The *sarA* gene is controlled by three promotors that are recognized by the sigma factors SigA and SigB (139), and negatively regulated by SarA and SarR, another protein belonging to the same family (147, 148).

Sigma factors are the initiation factors that define the specific binding of RNA polymerase to particular promoters. SigB is an alternative sigma factor which

responds to environmental cues. Its relevance for virulence is underpinned by its influence on the Agr and SarA regulatory systems. Further, the *sigB* gene is part of an operon that also encodes several Rsb proteins responsible for the regulation of SigB activity (Rsb, regulation of sigma B). In brief, the RsbW protein binds SigB under non-stress conditions, thereby preventing its association with target promoters. At the same time RsbW is a kinase that phosphorylates and thereby inactivates its antagonist protein RsbV. Under appropriate stress conditions, the phosphatase RsbU dephosphorylates RsbV, which then binds RsbW, concomitantly releasing SigB from its inhibition by RsbW. The liberated SigB is then able to bind to RNA polymerase and stimulate transcription of SigB-dependent genes (149). Stressful conditions that activate this regulator in shake flask cultures *in vitro* are heat shock and alkaline stress, but also the transition of *S. aureus* into the stationary phase. SigB influences expression of around 200 genes that encode proteins involved in membrane transport, biofilm formation, cell internalization, persistence and virulence (150). Importantly, SigB is also required for intracellular replication and the intracellular persistence of so-called small colony variants of *S. aureus* (SCVs; Pförtner et al., 2014; Tuchscherr et al., 2015).

So far, the complex regulatory network of *S. aureus* has mostly been studied in parts, elucidating the target genes of each known regulator and the influence of different environmental conditions on their expression. Many genes are not regulated by a single regulator but, instead, they are influenced by several of them. Importantly, the specific combination of conditions encountered during the infectious process could promote different interactions between the different nodes of the regulatory network, giving rise to specific *S. aureus* phenotypes that are advantageous to this bacterium. Therefore, it is imperative to study the expression level of bacterial proteins during colonization or infection in a comprehensive manner, taking into account the different regulatory events, in

order to expand our understanding of the contribution of these systems to human health and disease.

S. aureus and the host epithelium

During the course of an infection, *S. aureus* encounters several challenges posed by the host's primary defenses. This network of pathways, cells, and effector molecules may protect the host from reinfection by invasive pathogens and prevent the development of diseases. One of the first lines in the host defense consists of a tight membrane of cells that acts as a physical barrier, preventing the invasion of the pathogen into deeper-seated tissues, and triggering the recruitment of components of the second line of defense, the innate immune system. Although the innate immune system comprises several types of cells, each with a specific function, altogether they adopt a few key strategies to deal with an invasive pathogen. These strategies include phagocytosis, production of antimicrobial peptides, and production or induction of the complement system. The interactions between the pathogen and different cells of the human body are dependent on the nature of the respective host cells and they are highly dynamic. In fact, the continuous communication between a host cell and an infective agent will determine the outcome of an infection.

One immune evasion mechanism of *S. aureus* consists of using the interior compartments of host cells as temporary hideouts that will prevent direct encounters with immune system effectors. Initially, it was thought that *S. aureus* was only capable of internalizing into phagocytic cells, like neutrophils (153). Nonetheless, recent evidence shows that *S. aureus* can be internalized also by non-professional phagocytic cells, where it can persist for long periods of time (152, 154, 155). After *S. aureus* engages non-phagocytic cells through the binding of Fnbp to fibronectin on the host cell surface, the pathogen will activate, through

different mechanisms, the phagocytic pathway. Initially the bacterium will reside inside an endosome, which needs to fuse with a lysosome to kill and degrade the internalized bacterium thanks to the presence of antimicrobial peptides, acidification of the compartment, production of reactive oxygen species, and the presence of highly potent degradative enzymes. Nonetheless, *S. aureus* is capable of manipulating this process, thereby avoiding clearance. In this regard, this pathogen can initiate the autophagocytic pathway, in which it will be encapsulated by a secondary membrane, or it may escape into the cytosol. Regardless of these optional escape routes, *S. aureus* will aim for growth and survival either by consuming the host's resources intracellularly, or by inducing host cell lysis to spread to and propagate in other tissues of the host (156).

Scope and outline of the thesis

The goal of the PhD research described in this dissertation was to expand the understanding of the adaptive mechanisms employed by *S. aureus* to adjust to and survive the diverse conditions encountered in infection-relevant niches of the human body. Particular adaptations of *S. aureus* may consist of changes in the production of virulence factors that allow the bacteria to colonize and spread into host tissues, as well as metabolic adaptations that determine the fitness of the pathogen. Such adaptations are mostly studied separately in the invading bacteria and the respective host cell system. By contrast, this thesis describes a series of investigations where a combined approach was followed. In particular, adaptive responses in infecting bacteria and their host cells were monitored through proteomics, and subsequently the correlations of these responses and their physiological consequences were evaluated. The first two experimental chapters of this thesis focus on the adaptations of *S. aureus* upon a successful infection of bronchial epithelial cells, while the two subsequent experimental chapters describe the differences in the exo- and cytosolic proteomes of closely related MRSA

isolates with a different epidemiological history, linking the outcomes to their predominant modes of infection.

C**hapter 1** of this thesis presents a general introduction on the genetic and epidemiological diversity of *S. aureus*, emphasizing this pathogen's virulence factors and regulators that define the infection process. In addition, a general overview on the molecular interactions of *S. aureus* with cells of the human host is provided. C**hapter 2** covers the interaction between *S. aureus* and bronchial epithelial cells over four days post-infection. This extended period of observation allowed a comparative investigation of the metabolic pathways employed by *S. aureus* in the two subpopulations that it presents during infection and the occupation of different intracellular compartments. Importantly, the detected changes in the expression of metabolic pathways were simultaneously studied and correlated to the changes in the host cell proteome, offering a comprehensive picture of the dynamic interactions that occur between host and pathogen during infection. In this respect it should be noticed that pulmonary infections by *S. aureus* are highly prominent in patients with lung-related illnesses causing lesions in the epithelial lining. Therefore, in the studies described in **chapter 3**, proteomics was employed to study the adaptations of *S. aureus* during internalization within epithelial cell layers displaying two subsequent regenerative stages that occur after injury. In particular, this chapter is focused on those changes in the bacterial cytosolic proteome that occur during the first hours after internalization.

Taking into account the diversity of *S. aureus* lineages and strains, the lung epithelial model of infection employed for the studies described in chapter 2 was used to study interactions with clinical isolates of MRSA. The differences in the proteome of certain closely related *S. aureus* strains isolated in Denmark were, therefore, evaluated in experiments described in **chapters 4 and 5**. These studies include twelve isolates from two distinctive epidemiological groups, namely

community- (CA) and hospital-associated (HA) MRSA isolates. Specifically, **chapter 4** reports on the exoproteomes of these isolates and correlates the outcomes with their behavior upon infection of lung epithelial cells. Since the behavior of the investigated MRSA strains upon infection is also determined by their fitness, the cytosolic proteomes of a selection of these isolates were also investigated as described in **chapter 5**.

Altogether, the studies described in this thesis emphasize critical metabolic adaptations as drivers of the course of infection. The specific results of the present PhD research that have led to this important conclusion are summarized and discussed in **chapter 6**.

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