Chapter 1

General introduction and scope
Interaction between *Staphylococcus aureus* and the human host

The communities of commensal micro-organisms that live in and on the human body are important for many aspects of human health, especially the digestion of food, the provision of vitamins and the protection against pathogens. In particular, the human microbiota includes bacteria, archaea, fungi and viruses that sustain a network of complex interactions between each other and the host. Research on the human microbiota was essentially started by Antonie van Leeuwenhoek in the 17th century when he discovered the existence of microorganisms with his microscopes. Over the years, the development of increasingly sophisticated research tools furthered the microbiological studies from the simple microscopic description of single cells to advanced investigations of the complexity of entire microbial communities hosted by humans. Today, we know that most microbes are beneficial or at least harmless, while a relatively small group of other microbes can cause a variety of diseases. The research on the interactions between the microbiota and the human body has revealed a plethora of mutual adaptations and a continuous evolution of the complex and dynamic cross-talk between both entities, which is only partly understood. This calls for fundamental research to unravel the complexity of dynamic host-microbiota interactions that may help to develop new therapies for a wide range of infectious and chronic diseases (Fan et al. 2021; Malla et al. 2019; Ursell et al. 2012).

Opportunistic pathogens are microorganisms that, in healthy individuals, display a commensal lifestyle as part of the human microbiome. However, under certain conditions, they will take advantage of the human host and cause infections. A wide range of different bacteria may cause such opportunistic infections. This is most prominently exemplified by the Gram-positive bacterium *Staphylococcus aureus*, which is known to be the causative agent of several diseases. The first identification of *S. aureus* was reported in 1880 when Sir Alexander Ogston isolated this bacterium from surgical abscesses. Since then, it has been demonstrated that this pathogen has evolved a wide range of complex mechanisms to thrive both as a colonizer and as a pathogen, not only in humans but also in livestock. In particular, *S. aureus* developed intricate mechanisms to evade the host immune defenses and, in recent years, this bacterium evolved mechanisms to resist successive generations of newly developed antibiotics. As a consequence, *S. aureus* has become one of the most successful pathogens that managed to disseminate widely in both the community and hospital environments (Chambers et al. 2009; Foster 2017). Despite 140 years of research, the dynamic roles of this bacterium as a commensal and pathogen are still under investigation, and there is still a great need for new preventive and therapeutic strategies to tackle the diverse infections this bacterium causes in
humans. One of the main current knowledge gaps is that we still do not know exactly which mechanisms are employed by S. aureus to hide asymptotically in the multitude of different niches within the human body, or how both bacterial and human factors determine the outcome of S. aureus-host interactions (Horn et al. 2017; Raineri et al. 2021; Sakr et al. 2018; Foster 2017).

Importantly, S. aureus is carried by about one-third of the human population with the nasopharyngeal mucosa and the skin being its best-studied niches. In fact, from the bacterial perspective, these niches are ideal for S. aureus to promote transmission and subsequent invasion of the human body through (in)direct personal contacts, contaminated food products, trauma and surgery. Thus, the opportunist S. aureus can quite readily become an integral part of the human microbiome and, subsequently, adapt to and colonize different host niches. In fact, S. aureus is a common resident of the anterior nares, the oral cavity, and the skin, but it is also known to colonize the upper respiratory tract and the human gut (Figure 1). Judged by nasal carriage, S. aureus is persistently present in ~20% of the human population. However, defining the precise nature of S. aureus carriage is challenging due to the possibility of ineffective sampling, due to the fact that carriage is usually asymptomatic, and due to changes in the carriage over time (Sakr et al. 2018; Mulcahy et al. 2016; Wertheim et al. 2005; Acton et al. 2009; Horn et al. 2017; Gagnaire et al. 2017; van Belkum 2016).

Once S. aureus has crossed the epithelial barrier and escaped the host immune defenses, it may cause serious diseases that range from skin and soft tissue infections to bacteremia, sepsis, endocarditis and necrotizing pneumonia. In recent years, various studies have demonstrated the link between asymptomatic carriage of S. aureus and infection, focusing attention on this bacterium’s endogenous reservoirs that are less harmless than they appear to be. In fact, it was shown that S. aureus bacteremia isolates are often clonally identical to the endogenous nasal colonization isolates of the respective patients (Sakr et al. 2018; Brown et al. 2014; Wertheim et al. 2004; 2005; von Eiff et al. 2001). Among, the different clinical S. aureus manifestations bloodstream infections are very well investigated, because such infections are associated with high patient morbidity and mortality (Tong et al. 2015; Laupland et al. 2013). Also, S. aureus bloodstream infections can lead to additional complications, as exemplified by abscesses, osteomyelitis, endocarditis, or implant-associated infections. Such complications and relapse of infections may relate to antimicrobial resistance of the infecting bacteria and intracellular persistence in different types of phagocytic and non-phagocytic cells (Thwaites et al. 2011; Horn et al. 2017; Chambers et al. 2009). For instance, the so-called small
colony variants of *S. aureus*, which display a reduced metabolic activity and slow growth rates appear to be well adapted to intracellular persistence and show higher antibiotic tolerance (Kahl et al. 2016; Tuchscherr et al. 2010).

**Antibiotic resistance and the emergence of MRSA**

The high capability of *S. aureus* to acquire antimicrobial resistance was recognized shortly after the discovery of penicillin by Sir Alexander Fleming in 1929. This marked the start of several waves of antimicrobial resistance development in *S. aureus* against multiple antibiotics. The mechanisms that drive the development of resistance in *S. aureus* were shown to be horizontal gene transfer from other micro-organisms and chromosomal mutation, following selective antibiotic pressure. In fact, upon the clinical introduction of penicillin, there was an increased incidence of strains producing the plasmid-encoded penicillinase BlaZ that hydrolyses the beta-lactam ring of penicillin and thereby nullifies its antimicrobial activity. This instigated the development of a new antibiotic called methicillin. However, the introduction of this antibiotic in the clinic was soon followed by the emergence of the first methicillin-resistant *S. aureus* (MRSA) strains in 1961. Two decades later, it was discovered that these resistant strains had acquired the *mecA* gene, which encodes the low-affinity penicillin-binding protein, PBP2a, that can complement for the methicillin-impaired activity of PBP2. Subsequent research showed that the *mecA* gene is located within a mobile cassette element called ‘SCCmec’ (staphylococcal cassette chromosome mec). Since the identification of the first SCCmec at least eight SCCmec allotypes have been described ("International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements [IWG-SCC]" 2009). Here, it is interesting to note that methicillin resistance itself evolved actually long before the clinical introduction of this antibiotic, as evidence by the co-evolution of mecC-expressing MRSA with a β-lactam-producing dermatophyte in hedgehogs (Larsen et al. 2022). To treat MRSA infections, the antibiotic vancomycin was introduced for use in hospital settings in the late 1980s (Foster 2017; Chambers et al. 2009; Lowy 2003). However, vancomycin-intermediate *S. aureus* (VISA) strains and vancomycin-resistant *S. aureus* (VRSA) strains emerged, respectively, in the 1990s and in 2002 (McGuinness et al. 2017). This resistance was caused by the *vanA* gene, which was encoded by a plasmid-borne transposon that was acquired from *Enterococcus faecalis* (McGuinness et al. 2017). In recent years, other last-resort antibiotics, such as daptomycin and linezolid, were approved for the treatment of infections caused by MRSA. The emergence of daptomycin and linezolid resistance in *S. aureus* was, however, shortly thereafter demonstrated (Baltz 2009; Dortet et al. 2013; Yoo et al. 2020; Besier et al. 2008).
When MRSA emerged, it was initially considered as a healthcare-associated pathogen mainly causing bloodstream infections. However, in the 1990s, it was discovered that MRSA was also spreading within the community of healthy individuals. These community-acquired (CA-)MRSA lineages were causing skin and soft tissues infections as well as more fatal infections, especially necrotizing pneumonia, purpura fulminans, and post-viral toxic shock syndrome (Kong et al. 2016; Chambers et al. 2009). The CA strains harbored a new SCCmec called type IV and a variety of toxins, including the Panton-Valentine leukocidin (PVL) encoded by the lukS and lukF genes, which were not evidently present in the earlier hospital-associated (HA-)MRSA strains. Nowadays, HA-MRSA strains generally harbor the SCCmec I, II or III, while the CA-MRSA strains harbor SCCmec IV or V (Peng et al. 2018; Turner et al. 2019).

Spreading of the CA-MRSA occurred very rapidly and a great diversity of clones was identified in many countries around the globe (Chambers et al. 2009; Turner et al. 2019). Notably, the original clinical definition to distinguish CA- and HA-MRSA is essentially based on the timing of isolation of MRSA in relation to the respective patient’s admission to the hospital. An MRSA isolate is considered to be CA if it is isolated from individuals in the community who were generally healthy and who were hospitalized not more than 48 h before isolation of the MRSA. Also, these individuals had not been hospitalized in the 2 years preceding the identification of their MRSA carriage and were not receiving outpatient care. While this definition makes perfect sense from a clinical epidemiological perspective, it does not take into account the aforementioned distinctive molecular features of CA- and the HA-MRSA (Turner et al. 2019). On the other hand, these features are subject to continuous evolution and therefore less robust over time. This view is nowadays underscored by the fact that CA-MRSA can also cause infections in hospitals and that HA-MRSA circulates in the community, especially among adults (Wang et al. 2015; Biber et al. 2012). Additionally, colonization with both MRSA and methicillin-sensitive S. aureus (MSSA) can persist asymptomatically for prolonged periods of time, which makes the distinction of CA- and HA-isolates even more complicated (Kluytmans-VandenBergh et al. 2006; Sanford et al. 1994).

**Bacterial and human determinants for colonization or infection**

The many years of research on the epidemiology of S. aureus have shown that S. aureus-host interactions are governed by a multitude of factors that are decisive for asymptomatic carriage, colonization or infection. To interact with host cells, S. aureus can employ a wide range of virulence factors, encoded by the core genome or acquired mobile genetic elements (MGEs), as well as genetic or regulatory metabolic adaptations to different host niches depending on
the availability of nutrients and stresses imposed by the host cells (Sibbald et al. 2006; Ziebandt et al. 2010; Mäder et al. 2016; Nagel et al. 2018). Consequently, it is the cross-talk with the host that is critical for the ultimate outcome in terms of \textit{S. aureus} elimination, colonization of the host niches, superficial infection or invasive disease (Palma Medina et al. 2019). The acquisition of MGEs is a strategy that is frequently used by \textit{S. aureus} to facilitate the adaptation to selective pressures imposed by the host immune system or ‘environmental’ conditions such as the presence of antibiotics (Haaber et al. 2017). The known MGEs include the aforementioned SCC elements, as well as insertion sequences, transposons, bacteriophages and prophages, staphylococcal pathogenicity islands, and plasmids (Novick et al. 2010). These MGEs carry a wide range of genes with different functions in virulence (e.g. PVL), immune evasion, resistance to antibiotics and metabolism (e.g. the ‘arginine catabolic mobile element’ ACME). The horizontal transfer of MGEs in \textit{S. aureus} takes place via conjugation and transduction (Malachowa et al. 2010; Lindsay 2014), but possibly also via genetic competence (Morikawa et al. 2012). Additionally, adaptations can be consolidated by SNPs and other mutations in the bacterial genome that contribute to the virulence (Messina et al. 2016; Acker et al. 2019).

\textit{S. aureus} virulence factors come in different flavors (Sibbald et al. 2006; Dreisbach et al. 2020). They may be proteinaceous or non-proteinaceous molecules, small or large, and surface-associated or secreted (Cheung et al. 2021; Dreisbach et al. 2020). The main groups of cell wall-associated proteins are the ‘microbial surface components recognizing adhesive matrix molecules’ (MSCRAMMs), near iron transporter NEAT motif family proteins, three-helical bundle proteins, proteins of the G5-E repeat family, and proteins of which the structure is still uncharacterized (Foster et al. 2014). Other \textit{S. aureus} virulence factors are secreted, serving roles in the evasion of host responses and causing damage to host cells. In the latter group, we can find diverse exotoxins and extracellular enzymes that cause pore formation in host cells, cause host cell lysis or inactivate particular functions in the host’s immune defenses (Tam et al. 2019). Additionally, to fine-tune the production of virulence factors and relevant metabolic pathways in response to changing host environments, \textit{S. aureus} can employ a vast array of transcriptional regulators (Cheung et al. 2004; Jenul et al. 2018; Priest et al. 2012). There are several major groups of regulators, including two-component regulatory systems and proteins of the SarA family (e.g., SarA, Rot and MgrA) (Cheung et al. 2004; Jenul et al. 2018; Priest et al. 2012). The two-component regulatory systems are composed of a sensor component that senses particular external or intracellular stresses and that, upon activation, transfer this signal to a response regulator by phosphorylation (e.g. the \textit{agr} quorum-sensing system, SaeRS,
SsrAB, ArlRS) (Cheung et al. 2004). Moreover, the virulence of *S. aureus* can also be modulated through two alternative sigma factors (SigB and SigH) (Jenul et al. 2018; Horswill 2018). Particular regulators have also central roles in responding to the availability of carbon and nitrogen sources or oxygen (e.g. CcpA, CodY, and Rex) (Reed et al. 2018; Waters et al. 2016; Somerville et al. 2009). Altogether, these regulators are responsible for orchestrating metabolic adaptations to different host niches and the balanced expression of virulence factors, which is fundamental for the survival of *S. aureus* at different body sites, such as the human nasopharynx and the gastrointestinal tract, or inside phagocytic and non-phagocytic host cells (Raineri et al. 2021).

Importantly, upon invasion of the human body, *S. aureus* has to withstand both the innate and adaptive immune responses. Firstly, this involves the evasion or inhibition of the classical and alternative pathways for complement activation and bacterial opsonization (Buchan et al. 2019). Further, innate immune responses will be activated with the help of pattern recognition pathways that detect non-specific markers of bacterial infection. This leads to the subsequent activation of neutrophils and macrophages that may then phagocytose and kill the bacteria (Rosman et al. 2021). At later stages, once the release of cytokines occurs and with the presentation of bacterial antigens by antigen-presenting cells, the adaptive immune responses are elicited. Here, B-cells will produce specific antibodies that opsonize the bacteria and activated T-cells will amplify the immune response and actively kill the bacteria (Medzhitov 2007). In this context, the immune history of an individual, as well as the immune imprint by the bacteria during an asymptomatic period of colonization, are likely to direct the *S. aureus* host-interactions either towards bacterial clearing, asymptomatic colonization or infection. In fact, the association of specific host traits and asymptomatic *S. aureus* carriage was evidenced in different previous studies (Mulcahy et al. 2016; Nouwen et al. 2004; Brown et al. 2014), and this was also shown for the association between asymptomatic carriage and infection (Wertheim et al. 2004; von Eiff et al. 2001; Brown et al. 2014). In addition to the innate and adaptive immune responses, other host factors play critical roles in the outcome of *S. aureus*-host interactions. In particular, it was shown that physical barriers represented by the skin and the mucosa are formidable defense lines against infection and that changes in their homeostasis can entice the invasive behavior of *S. aureus* (Abdallah et al. 2017; Raineri et al. 2020; Palma Medina et al. 2020; Fukuda et al. 2020).

Despite all the accumulated knowledge, there it is still a major challenge to treat severe infections by multi-drug resistant MRSA lineages and there is still no clinically approved vaccine
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to prevent such infections (Bekeredjian-Ding 2020; Daniels et al. 2016; Veloso et al. 2015; Giersing et al. 2016). Consequently, further investigations into both the bacterial and human determinants are required to deepen our understanding of the avenues leading to S. aureus infection and how such infections can best be prevented.

**Molecular techniques and omics approaches for strain characterization**

To investigate S. aureus epidemiology and the genetic variability of this pathogen, different molecular typing methods were developed (Sabat et al. 2013). Using these methods, it became possible to group the different isolates into lineages which allowed a better understanding of the evolution of S. aureus. One of the first techniques used at a global scale was pulsed-field gel electrophoresis (PFGE). This technique is based on the digestion of S. aureus genomic DNA with the restriction endonuclease SmaI, and the subsequent separation and comparison of the generated DNA fragments by pulsed-field electrophoresis on agarose gels. With the advance of DNA sequencing techniques, the multi-locus sequence typing (MLST) was introduced, which allows the distinction of sequence types (STs) based on nucleotide sequence differences in seven housekeeping genes of the core genome of S. aureus. The more simple spa-typing is a relatively recently developed method based on analyzing and comparing the sequences of the variable number of tandem repeats within the spa gene encoding the staphylococcal protein A (Chambers et al. 2009; Fitzgerald et al. 2016). However, these molecular typing approaches do not always guarantee sufficient resolution to unequivocally establish lines of S. aureus transmission, or for the prediction of disease phenotypes. With the introduction of whole-genome sequencing techniques, these limitations can now be overcome and much deeper insights into the genetic relationships between different staphylococcal isolates can be obtained. For example, S. aureus pangenome analyses allow the division of the bacterial genome into a core genome that is represented in all bacteria belonging to this species and an accessory genome that is typical for particular lineages or isolates (Bosi et al. 2016). Whole-genome sequencing analyses can also be used to approximate the pathogenicity of S. aureus isolates by charting their virulence and antibiotic resistance genes (Gordon et al. 2014.; Fitzgerald et al. 2016; Köser et al. 2014).

In addition to whole-genome sequencing approaches, other ‘omics’ technologies gained traction for studies on S. aureus-host interactions. These include diverse transcriptomics and proteomics techniques that elucidate genome-wide gene expression and the concomitant production of proteins. Such approaches are nowadays used both *in vitro* and *in vivo* to obtain a deeper understanding of host-pathogen interactions of S. aureus in its natural niches.
(Chaves-Moreno et al. 2016; Burian et al. 2010; Otto et al. 2014; Hecker et al. 2018; Holtfreter et al. 2016; Zhao et al. 2019). Thus, different multi-omics technologies are presently available to close our current knowledge gaps concerning *S. aureus*-host interactions with the final objective to better prevent or treat staphylococcal infections.

**Figure 1.** *S. aureus* colonization and infection of different human host niches. The opportunistic pathogen *S. aureus* thrives and survives in the nasopharyngeal cavities, respiratory tract, gut, wounds and blood. Studying the dynamic interactions of *S. aureus* with the barriers imposed by the human body, especially the epithelial and endothelial barriers, and human immune cells is fundamental to develop new approaches needed to contain *S. aureus* invasiveness and to uncover the many factors that govern *S. aureus* infections.
Scope and outline of the thesis

This dissertation documents studies aimed at uncovering the molecular mechanisms and survival strategies employed by S. aureus to adapt to different human host niches. The molecular characteristics of different S. aureus isolates were dissected at different levels, which included comparative genetic and proteome investigations. Additionally, an important aim of the here documented research was to understand how S. aureus breaks the different barriers for infection to invade different niches in the human body.

Chapter 1 of this thesis presents a short overview of the outcomes of 142 years of S. aureus research on this pathogen’s journey from sites of contamination via the colonization of different reservoirs to the invasion of particular host niches. In particular, this chapter touches upon the mechanisms for virulence, drug resistance and immune evasion employed by S. aureus to pass important barriers and to thrive and survive in the human body. Also, the diversity of S. aureus lineages and the available tools to characterize S. aureus isolates are introduced. Chapter 2 provides an extensive overview of S. aureus niches and the specific mechanisms employed by the bacterium to reach these niches. In addition to addressing the extensively investigated niches, such as the nasopharynx and the oral cavity, the attention is drawn to the human gut as a reservoir of S. aureus. Lastly, the possible determinants that govern the S. aureus switch from colonizer to infectious agent, and the roles of blood cells as vehicles for bacterial dissemination are discussed. Chapter 3 documents investigations on the molecular distinction of gut-colonizing S. aureus isolates and isolates that caused serious bloodstream infections. The different study isolates were characterized by whole-genome sequencing, proteomics, and infection experiments. Interestingly, the different S. aureus gut-colonizing and invasive isolates showed no distinct signatures allowing their separation based on genomic or proteomic traits. Instead, the results imply that an intact gut epithelial layer, rather than the pathogenic potential of an S. aureus strain, is the decisive determinant for transition from gut colonizer to invasive pathogen. On the other hand, Chapter 4 describes a clear molecular distinction at the transcriptional level of closely related CA- and HA-MRSA isolates of the USA300 lineage. Importantly, the investigated CA-isolates showed the highest killing activity towards neutrophils and larvae of the wax moth Galleria mellonella as compared to the HA-isolates. However, the HA-isolates displayed a better intra-phagocyte survival, which is possibly advantageous as an adaptive mechanism to survive the antibiotic pressure of the hospital environment. Chapter 5 reports on the fate of MRSA isolates of the USA300 lineage with distinct epidemiological origins when infecting endothelial cells. In this case, two infection models were applied that mimic two
different conditions of the endothelium. Similar to the results presented for gut epithelial cells described in Chapter 3, the results show that an intact endothelial barrier with cell-cell junctions sets limits to the invasiveness of *S. aureus*. However, the intact endothelial barrier is more likely to sustain persistent intracellular infection. Furthermore, all internalized bacteria remained confined in membrane-enclosed lysosomal or vacuolar compartments over a prolonged period of time. **Chapter 6** presents a time-resolved analysis of *S. aureus* infecting human bronchial epithelial cells by mass spectrometry and different microscopic techniques. Over time, the dynamic bacteria-host cell interaction is shown to depend on the virulence factors produced by *S. aureus* as well as metabolic adaptations of both the bacteria and the host in response to changes in available resources. Lastly, **Chapter 7** summarizes the main findings and conclusions presented in this dissertation. In particular, this chapter reflects on how the different findings can be used to achieve a deeper understanding of the cross-talk between *S. aureus* and its human host during colonization and infection.
References


Chapter 1- General introduction and scope


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