Breaking the barriers: how Staphylococcus aureus reaches different human host niches

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Chapter 7

Summary, conclusions and future perspectives
Summary

*Staphylococcus aureus* is a coccoid, facultative aerobic, Gram-positive bacterium that is highly capable of colonizing the human body. Consequently, this bacterium is generally regarded as a member of the human microbiome with the skin and mucosa being its major reservoirs. The *S. aureus* carriage and colonization rates may vary in different human populations, where *S. aureus* will persistently colonize about 20% of all individuals (Brown et al. 2014; Mulcahy et al. 2016; Sakr et al. 2018; Raineri et al. 2021). Despite the generally asymptomatic carriage or colonization, *S. aureus* can be the cause of a multitude of infectious diseases in the human host and, as a pathogen, this bacterium can manifest itself with different epidemiology and pathophysiology (Tong et al. 2015; Turner et al. 2019). The efficacy of available antimicrobial treatments varies depending on the multi-drug resistance of the infecting strain and the type of infection. Inadequate eradication of the infecting *S. aureus* strain due to antimicrobial resistance may result in a relapse of the infection. However, such relapses may also relate to the presence of hidden *S. aureus* reservoirs in the human body that harbor persistent bacteria, for instance intracellularly (Horn et al. 2017; Chambers and DeLeo 2009; Tong et al. 2015). In this regard, there are still many uncertainties about the contributions of different niches in the human body to staphylococcal infections and on the roles of phagocytic or non-phagocytic cells as hiding places for asymptomatic *S. aureus* survival (Raineri et al. 2021; van Belkum 2016; Sakr et al. 2018; Thwaites et al. 2011; Krezalek et al. 2018; Horn et al. 2017; Gagnaire et al. 2019). Additionally, little is known about the molecular traits that dictate the epidemiological behavior of community-acquired (CA) or hospital-acquired (HA) isolates, which is especially relevant in the case of methicillin resistant *S. aureus* (MRSA) (Mekonnen et al. 2017; 2018; Mulcahy et al. 2016; Lee et al. 2018; Guimaraes et al. 2019). The research described in this dissertation was aimed at shedding more light on the poorly understood mechanisms used by different *S. aureus* lineages to survive in the human body, and the human host responses towards *S. aureus* in the context of the gut, the bloodstream, the endothelium, and the lung epithelium. This aim was achieved following multiple different approaches, including comparative genomics, transcriptomics, and proteomics, as well as *in vitro* and *in vivo* infection assays.

A general introduction to the state of the field at the start of the present PhD research is presented in chapter 1 of this thesis. This chapter also gives a succinct overview of the molecular features used by *S. aureus* to survive in multiple niches, as well as the possible host cell responses and barriers to infection. In fact, different *S. aureus* isolates employ a wide array of virulence factors, mobile genetic elements (MGEs), mutations and adaptive strategies in response to the challenges imposed on them in different human host niches, which may allow
the bacterium to colonize or infect its host. Importantly, *S. aureus* behavior is counteracted by the host’s immune responses and other host-specific barriers to infection. Chapter 1 also touches upon the diversity of *S. aureus* isolates in terms of epidemiology and the available tools that can be used to characterize *S. aureus* isolates, especially transcriptomics and proteomics. Chapter 2 reviews the different staphylococcal reservoirs in the human body, especially the gut, nasopharynx and oral cavity, where *S. aureus* can thrive and survive, or from where this bacterium can be transmitted to other body sites or other individuals. In particular, this chapter sheds light on the role of the human gut as an endogenous *S. aureus* reservoir of which the contribution to infection is so far poorly investigated and possibly underestimated. In healthy individuals and patients, the frequency of *S. aureus* intestinal carriage is ~20% on average with variations depending on the health condition and age. Additionally, *S. aureus* intestinal carriage may lead to the development of infections both in the community and in hospital environments. However, the diverse mechanisms allowing *S. aureus* to colonize the human gut, or to invade deeper tissues by breaching the human gut barriers, are still poorly understood. The same applies to the relationships between *S. aureus* colonization of endogenous reservoirs in the human body and the onset of invasive disease, which are multifactorial and complex processes. Chapter 2 also reviews recent advances in understanding the dynamic interactions of *S. aureus* with immune cells that lead to survival and dissemination in the human body. In fact, these interactions may enhance the staphylococcal virulence, internalization or colonization by *S. aureus*, or they may promote other cellular activities and inflammation. Additionally, the available literature showed that the dissemination of *S. aureus* within the host can proceed inside different types of leukocytes, including neutrophils.

Chapter 3 describes studies to pinpoint the molecular distinctions between gut-colonizing *S. aureus* isolates and isolates that caused serious bloodstream infections. To this end, such isolates were collected and investigated by whole-genome sequencing. The thus characterized *S. aureus* isolates with sequence types 1 or 5 (ST1, ST5) displayed no distinct genetic signatures that would distinguish *S. aureus* isolates from the gut or the bloodstream. To further characterize these isolates, proteomics and virulence assays were performed. The analysis of the cellular proteome and the exoproteome revealed no distinguishing signatures that would allow a proteomic separation between *S. aureus* isolates from the gut or the bloodstream. However, *in vitro* analyses with gut-colonizing isolates or bacteremia isolates, and the use of an *in vivo* *Galleria mellonella* infection model showed that gut isolates can actually be more virulent than invasive isolates from the bloodstream. Moreover, one of the decisive factors for invasive staphylococcal disease seems to be disruption of the gut epithelial barrier. Gut
epithelial cells with an intact barrier were barely infected by \textit{S. aureus} isolates, irrespective of their site of isolation. Only when the cell-cell junctions were disrupted, significant invasive behavior was observed. These findings showed how, depending on the integrity of the barrier imposed by the epithelial layer, as well as the underlying immune defenses, the outcomes of \textit{S. aureus} carriage can be extremely different.

To further investigate the molecular traits and adaptation mechanisms causing the epidemiological behavior of pathogenic isolates, the studies described in chapter 3 were complemented with the analysis of a collection of MRSA isolates. \textbf{Chapter 4} focuses attention on MRSA isolates of the USA300 lineage, which can cause fulminant infections and are resistant towards most antibiotics. The USA300 collection used for this study was based on closely related CA- and HA-isolates. Through transcriptome analysis, the CA- and HA-USA300 isolates were shown to display distinct profiles in terms of gene expression. In fact, differential expression levels were observed for 460 genes, including genes encoding virulence factors (e.g., leukotoxins and phenol-soluble modulins), metabolic pathways and regulators (e.g., the Agr quorum-sensing system, defenses against oxidative stress, and biosynthetic pathways for purines, pyrimidines, and fatty acids). The HA-isolates displayed a preferred intra-phagocyte survival, which may reflect an adaptive mechanism to survive the frequent antibiotic challenges in the hospital settings. The CA-isolates do not need this protection and accordingly, they show a higher killing activity towards neutrophils and of larvae of \textit{Galleria mellonella} compared to the HA-isolates.

From the bloodstream, \textit{S. aureus} can invade different parts of the human body by crossing the endothelial barrier. The studies presented in \textbf{Chapter 5} were aimed at investigating the fate of MRSA isolates of the USA300 lineage with distinct epidemiological origin upon infection of endothelial cells, involving the use of two \textit{in vitro} infection models that mimic two different conditions of the endothelium. As shown by flow cytometry and fluorescence- or electron microscopy, the presence of an intact barrier with cell-cell junctions sets limits to the invasiveness of \textit{S. aureus}, but such a barrier is more likely to sustain persistent intracellular infection. Clear strain-specific differences were also observed with the HG001 reference strain infecting the highest numbers of HUVEC and displaying the longest intracellular persistence. In contrast, the investigated CA- and HA-MRSA strains reproduced faster intracellularly. This study showed how different \textit{S. aureus} strains can take advantage of endothelial cells to survive intracellularly for a prolonged period of time by remaining confined in membrane-enclosed lysosomal or vacuolar compartments.
As shown in Chapters 2, 3, 4 and 5, *S. aureus* can interact with immune cells in the bloodstream, but this pathogen can also invade the non-phagocytic cells of the endothelium and epithelium. Additionally, *S. aureus* is known to be the cause of recurrent infections in the respiratory tract. Little is known regarding the adaptive mechanisms staged by intracellular *S. aureus* in the lung environment. The aim of the studies documented in chapter 6 was therefore to fill this knowledge gap by analyzing *S. aureus* infecting human bronchial epithelial cells by mass spectrometry and different microscopy techniques over 4 days post-infection. The results show that, over time, the dynamic bacteria-host cell interaction is dependent on the virulence factors produced by *S. aureus*, as well as reciprocal host-bacterial metabolic adaptations. The latter adaptations seem to reflect competition for resources between the internalized pathogen and the host cell. Most likely to optimize its fitness, the internalized *S. aureus* displays a population heterogeneity as evidenced by two subpopulations of *S. aureus* with distinct replication rates and subcellular localization. A replicating *S. aureus* population was localized to vesicles, causing lysis of the host cells. Conversely, a population with a dormant phenotype was shown to survive over time mainly in the host cytosol. As shown by mass spectrometry, upon internalization, the *S. aureus* bacteria modulate the expression of proteins related to growth in nutrient- and oxygen-deprived environments (e.g., fermentation). Additionally, the bacteria optimize energy production from alternative carbon and nitrogen sources (e.g., increase of proteins related to the TCA cycle and amino acid degradation). Over time, also the host displays metabolic adaptations, resulting in changes in the central carbon metabolism and the amino acid metabolism.

**Conclusions and future perspectives**

Altogether, the studies presented in this thesis provide important clues that shed light on the complex network of interactions between the human body and *S. aureus*. In particular, the epidemic behavior of *S. aureus* depends on multi-factorial traits governed by the bacteria. However, the bacterial pathogenic potential is contained by the physiological state of the specific human body site. In fact, bacterial molecular traits, as well as metabolism and fitness, are important determinants of virulence. This was exemplified in different chapters of this dissertation, where the characterization of various groups of *S. aureus* isolates obtained from different body sites and causing different clinical symptoms, were described. Additionally, every human host niche is shown to present various ‘barriers’ that help to contain the invasive behavior of *S. aureus* and to adapt to bacterial survival. However, changes in the homeostasis of the body reservoirs and the silent *S. aureus* intracellular trafficking may represent stimuli that lead to the transition from the harmless colonizer to the deadly pathogen. This view is supported
by the present findings, which show that a particular isolate can display a more invasive behavior when there is a weakening of ‘body’ barriers, or when this isolate reaches different host niches. Additionally, the implementation of a combination of genomics, transcriptomics, and proteomics combined with *in vitro* and *in vivo* infection experiments helped to shed more light on the complex network of interactions between the human body defenses and *S. aureus*.

Further studies should consider infection models with more variables, which mimic the complexity of the human host. In this regard approaches based on *ex vivo* models, such as organoids, organs-on-a-chip or tissue implants, could be used in combination with *in vitro* analyses of single cells or co-cultures of cells grown in two-dimensional (2D) or three-dimensional (3D) settings. In fact, the combination of 3D structure, immune response, and flow conditions could bring us a step closer to the physiological conditions of endogenous *S. aureus* reservoirs. Another important variable that should be addressed in future research is the interaction of *S. aureus* with other bacteria residing in the human body. The complexity of the microbiota of endogenous reservoirs could be limiting or potentiating *S. aureus* behavior (García-Pérez et al. 2018). For example, beneficial microbes in a certain human niche can occupy the adhesion sites and limit the invasiveness of opportunistic pathogens. Additionally, it should be noted that the complexity of the microbiota can undergo changes over time, for instance after antibiotic exposure or changes in diet. The clearance of *S. aureus* from the human body reservoirs may also provoke changes in the microbiome that lead to more or less invasive behavior of other facultative pathogens. Another important variable to take into consideration in future studies is the media composition used for culturing the bacteria, which should be adapted to mimic different human reservoirs as closely as possible. Here it is conceivable that essential components are still missing. For example, future research on gut-colonizing *S. aureus* isolates should be performed under many different conditions and the consequences for the cellular composition should be analyzed by transcriptomics and proteomics. This would lead to the design of *in vitro* conditions that enable us to align the bacterial gene expression and proteomic signatures to be studied as closely as possible with the *in vivo* conditions. The different host adaptations following the interaction with *S. aureus* should also be further investigated to address possible changes in terms of metabolic adaptation and competition for resources. For example, little is known about *S. aureus* adaptation to the gut environment and the subsequent responses of host cells interacting with *S. aureus*. This could be complemented by *in vivo* ‘omics’ approaches targeting infected tissues in order to consider the bacterial and host interactions directly *in vivo*. 
From a clinical perspective, it would be highly relevant to perform more longitudinal comparative studies based on asymptotically carried *S. aureus* isolates obtained from different endogenous reservoirs of particular individuals, and on those isolates that have caused infection in the respective colonized individuals. Furthermore, there is a need to obtain a deeper understanding of the immune imprint of *S. aureus* during asymptomatic colonization, of the dynamic cross-talk between bacteria in the different endogenous staphylococcal reservoirs, and of the possible migration of bacteria to different sites in the human body over time. This will require the design of novel diagnostic and therapeutic modalities to visualize and eradicate *S. aureus* reservoirs that are currently perhaps under-appreciated or at least difficult to detect, as exemplified by the intracellular bacterial persistence and the asymptomatic colonization of the human gut. In fact, research on the clinical relevance of endogenous *S. aureus* reservoirs and on new therapeutic approaches will be beneficial for many people who are at risk of contracting staphylococcal infections, especially the very young, immunocompromised patients or the elderly. The same applies to patients with compromised barrier functions, most evidently due to trauma or surgery, but probably also due to the actions of other microorganisms, or due to inflammatory diseases or genetic predisposition. In essence, this calls for the development of more personalized diagnostic approaches and therapeutic interventions that take into account the multiple faces of *S. aureus* interactions with the human host. Further research into achievable and affordable diagnostic and therapeutic approaches that are tailored to the specific needs of the individual patient will be invaluable for providing the best possible clinical outcome, not only for the patient but also for the healthcare system at large.

In conclusion, the main future challenge for fully appreciating all clinically relevant aspects of *S. aureus*-host interactions resides in the characterization of all the different staphylococcal reservoirs in the human body and in understanding the mechanisms employed by the bacteria to reach the respective niches. In this context, the concerted barrier function of tissues, the immune system, and other microorganisms deserves further in-depth investigations, because the breaking of these barriers is central in the switch of *S. aureus* from a harmless colonizer to an invasive pathogen. A deeper understanding of the underlying principles would almost certainly lead to more refined guidelines for the prevention of *S. aureus* infections, and to the development of innovative personalized therapeutic interventions that support or reinforce the critical barriers that protect us from infection.
Chapter 7- Summary, conclusions, and future perspectives

References


