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CHAPTER 1

Introduction and scope of this thesis

Staphylococcus aureus – 'the Jekyll & Hyde microbe'

The here presented research has its origin in the late 19th century, when Sir Alexander Ogston discovered *Staphylococcus aureus* in pus from surgical abscesses in Scotland. At the moment of discovery no one would have thought that one day, *S. aureus* would become one of the most common causative agents of infectious disease worldwide. Due to the high numbers of *S. aureus* cases associated with antibiotic resistance, especially resistance against methicillin, it has become a renowned public health-associated bacterium in the 21st century. In the United States of America (USA) the mortality rate of severe, invasive methicillin-resistant *S. aureus* (MRSA) infections is approx. 20% and since the numbers of MRSA infections exceed the numbers caused by HIV/AIDS, it is the leading cause of death by a single infectious agents [1]. Moreover, the USA is experiencing the most distinct community-associated MRSA (CA-MRSA) epidemic worldwide, which is almost solely caused by a single strain type (sequence type [ST] 8 with the pulsed-field gel electrophoresis profile [PFGE] USA300) [1,2]. In Europe, the mortality rates differ extensively between different countries, but they are in general higher for MRSA than for methicillin-sensitive *S. aureus* (MSSA) [3]. Moreover, MRSA is estimated to cause approx. 170.000 healthcare-associated infections (HAIs) each year, corresponding to almost half of all HAIs, whereas it has not yet gained predominance in the community [2].

The Gram-positive coccus *S. aureus* is a facultative anaerobe that is frequently encountered in the human microbiota (Figure 1). Generally, it colonizes the skin and mucosae, with its preferred niche in the nasopharynx [4]. Three *S. aureus* nasal carriage patterns have been described: persistent, intermittent and non-carriage. Only about 20% (range 12-30%) of the general population are persistent carriers of *S. aureus*, whereas approx. 30% (range 16-70%) are intermittent and approx. 50% (range 16-69%) are non-carriers [4]. Persistent carriers are mostly colonized by a single type of *S. aureus*, whereas intermittent carriers may carry different types over time. Moreover, persistent carriers have an increased risk of developing staphylococcal infections, which are in 80% of the cases caused by the endogenous *S. aureus* type [4]. In spite of this, persistent carriers have a lower risk of death caused by bacteraemia compared to non-carriers [5]. Although a reclassification of nasal carriage types was introduced in 2009, with a distinction only between persistent carriers and others [6], the first classification is still the most commonly used. Nasal carriage of *S. aureus* is in general asymptomatic, whereas the mechanisms leading to the successful colonization of the human host are multifactorial. Since the nasal colonization remains generally unnoticed by the human host, it suggests that there is a balance between the bacterium and the host that has probably resulted from co-evolution [7]. However, upon disturbance of this balance, either by an increase in bacterial virulence, an improper host immune response, an impaired barrier function or an unknown mechanism of the host and/or bacterium, *S. aureus* can transform into a dangerous invasive pathogen [7]. Especially the disruption of epithelial barriers occurring during surgical procedures, through intravascular catheters or implants or by basic mucosal damage or trauma [8] represent a risk for staphylococcal infections. Accordingly, post-operative wound infections and other HAIs are a major threat for already compromised patients (e.g. elderly, immunocompromised). Unfortunately, the underlying mechanisms causing the switch from commensal to pathogen are still unknown. Once *S. aureus* becomes invasive it can cause an array of infectious diseases that can be grouped into local, toxin-mediated and systemic infections [9]. Local infections caused by *S. aureus* are relatively mild skin infections, including boils, abscesses and impetigo. Toxin-mediated infections have so far only been described for a limited number of toxins, like the toxic shock syndrome toxin-1 (TSST-1) and exfoliative toxins that cause toxic shock syndrome and staphylococcal scalded skin syndrome, respectively [10-12]. Lastly, systemic infections caused by *S. aureus* comprise more life-threatening conditions, such as bacteraemia, pneumonia and endocarditis [13]. However, incidences and outcomes of any disease caused by *S. aureus* strongly depend on a plethora of host factors, in particular the complex immune system. Consequently, there is no predictable correlation between a certain *S. aureus* infection and disease outcome [9]. Moreover, apart from the few toxin-mediated diseases, it remains difficult to predict the onset and course of an *S. aureus* infection in a given patient solely on the basis of the gene repertoire and phenotypic results of the infectious isolate. As an example, *S. aureus* isolates carrying the *tst-1* gene have also been identified

in patients that did not suffer from toxic shock syndrome. Clearly, the vast majority of severe *S. aureus* infections are probably caused by the concerted action of multiple bacterial and host factors. These facts highlight the 'Jekyll & Hyde' character of *S. aureus* that is responsible for the transition between its harmless and extremely dangerous lifestyle.

Figure 1. Clinical isolates of *S. aureus* grown overnight on a blood agar plate.

Staphylococcal virulence – 'the bullets of *S. aureus*'

Virulence refers to the ability of a microorganism to cause disease [14]. The virulence of *S. aureus* is orchestrated by a diverse and extensive repertoire of different factors. The mechanisms by which *S. aureus* can cause disease and exerts its virulent lifestyle are manifold and involve colonization of the host, adhesion to host cells and tissues, invasion of the host, damage caused by toxins, inhibition of host functions and evasion of the host immune system [1]. Importantly, these processes should not be viewed as separate entities but more as a complex network. Many different proteins, whose functions are partially redundant, are involved in these processes and are collectively termed virulence factors [15]. The repertoire of virulence factors may vary significantly between different *S. aureus* lineages due to the presence or absence of the respective genes [16]. The lineage-specificity of certain virulence factors is mainly a consequence of acquisition or loss of virulence genes that are encoded on mobile genetic elements (MGEs) [16,17]. The majority of *S. aureus'* proteinaceous virulence factors that mediate interactions with the host are localized to the cell surface (surfacome) or exported into the extracellular milieu (secretome) (for a schematic overview see Figure 2 [9]) [18-20]. Most of *S. aureus*' virulence factors are regulated by global pleiotropic regulatory loci, such as the accessory gene regulator (*agr*), the staphylococcal accessory element (*sae*) and the staphylococcal accessory regulator (*sar*), which are all part of the interactive network ensuring a coordinated temporal expression [21-23]. Altogether, 16 two-component regulatory systems have been identified so far and are, to different extents, involved in the execution and maintenance of staphylococcal virulence [24].

Surface-exposed proteins can be non-covalently wall-bound proteins, covalently wall-bound proteins, lipoproteins or membrane proteins. These proteins carry out a range of different functions, including adherence to and colonization of host tissues or immune evasion as exemplified by protein A, the immunoglobulin-binding protein (Sbi) and coagulase [25]. The largest class of surface proteins are the 'microbial surface components recognizing adhesive matrix molecules' (MSCRAMMs), which interact with host extracellular ligands, such as collagen, fibrinogen, fibronectin, vitronectin, elastin, prothrombin or von Willebrand factor [26]. The most studied surface-exposed proteins implicated in adhesion are clumping factor A and B (ClfA and ClfB), fibronectin-binding protein A and B (FnbpA and FnbpB), the iron-regulated surface determinants A, B and H (IsdA, IsdB and IsdH), the serine-

aspartate repeat-containing proteins C, D and E (SdrC, SdrD and SdrE), the surface proteins SasB, C, D, F, G, H and K, protein A, the collagen adhesion (Cna) protein, the extracellular adherence protein (Eap), the extracellular matrix-binding protein (Ebh), the elastin-binding protein (EbpS), the immunodominant staphylococcal antigen B (IsaB) and the serin-rich adhesion for platelets (SraP) protein [17]. A few studies investigating the surfacome of the *S. aureus* reference isolates RN6390, Newman, COL and USA300 demonstrated the high surfacome variabilities of these isolates as they differed not only in the presence or absence of proteins, but also in the composition of the identified peptides/proteins [8,25].

The complex secretome of *S. aureus* has been under investigation with a larger number of isolates compared to the surfacome studies, but also only with a limited number of studies so far. It has become clear from the few available studies, that the secretome of *S. aureus* displays an extensive heterogeneity, which has a multifactorial origin. The key factor driving the extensive heterogeneity of virulence factors is the genome plasticity of *S. aureus*, where inter- and more importantly intra-species exchanges of MGEs occur [20,27]. However, also several other factors drive the heterogeneous virulence potential of *S. aureus*, such as the host immune system and environment, gene expression regulation, protein modification processes, single nucleotide polymorphisms (SNPs) in genes or regulatory sequences, differential activities of gene regulatory systems, and posttranscriptional regulation mechanisms [20,28-30]. A study investigating the difference in the secretome between an *S. aureus* nasal carrier isolate and a genetically similar non-carrier isolate suggested that the carrier isolate produces a greater number of proteins related to adhesion, protein transport and immune evasion [31]. Studying the establishment and maintenance of *S. aureus* nasal carriage, a switch from strong expression of wall teichoic acid (WTA) biosynthetic genes at the initiation of the colonization to the production of the adhesive proteins ClfA and IsdA was reported in a different study [29]. Among the most-studied secreted *S. aureus* virulence factors that have implications in immune evasion are coagulase (Coa), the extracellular fibrinogen-binding protein (Ecb), the fibrinogen-binding protein (Efb), the extracellular matrix protein-binding protein (Emp), the virulence factors EsaC, EsxA and EssC, the FPRL1 inhibitory protein (FLIPr), Sbi, the staphylococcal complement inhibitor (SCIN), the chemotaxis inhibitory protein (CHIP), the staphylokinase (SAK), protein A, and the von Willebrand factor-binding protein (VWbp). Several virulence factors such as the covalently cell wall-anchored sortase substrates IsdA, IsdB and others like ClfB, SdrC, SdrD and protein A were not only identified within the surfacome, but also in the extracellular space, showing that surface-bound proteins are regularly parts of the secretome [8]. Studies investigating the proteome of *S. aureus* clearly indicate the need for in-depth DNA sequence analysis to explain the differences observed within the complex proteome [32]. It has been hypothesized that the profoundly heterogeneous expression patterns of virulence factors observed under identical *in vitro* conditions could reflect a very high degree of variability *in vivo* [33]. From the above-discussed studies it is clear that many parts of the complex proteome of *S. aureus* not only under *in vitro* but more importantly *in vivo* conditions still need to be unravelled. Altogether, it can be concluded that *S. aureus* resembles a 'loaded gun' capable of firing different types of 'bullets' (virulence factors) at its host either for offensive or defensive purposes.

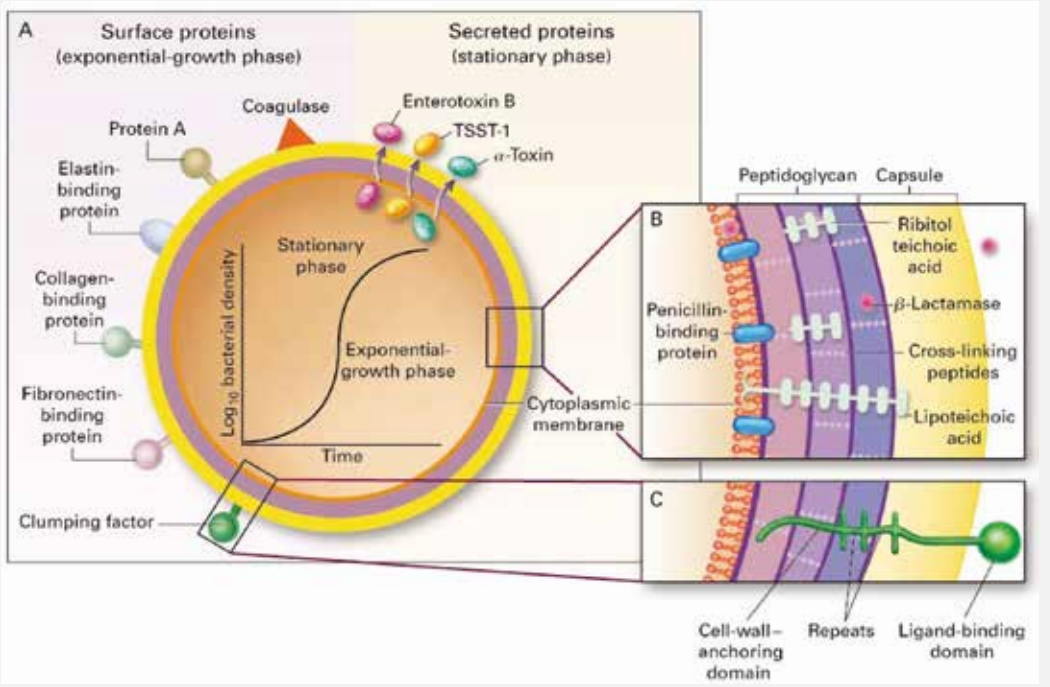


Figure 2. Schematic overview of the surfacome and secretome of *S. aureus*. (A) The synthesis of many surface-associated and secreted proteins is dependent on the growth phase, as shown by the graph, and is controlled by a diverse set of regulatory systems, including *agr*. **(B, C)** Cross-sections of the cell envelope of *S. aureus*. Many of the surface proteins have a structural organization similar to that of clumping factor, including repeated segments of amino acids. Reproduced with permission from [9], Copyright Massachusetts Medical Society.

Host-pathogen interactions – 'the tug of war'

Unveiling the precise mechanistic interaction between *S. aureus* and the human host will be key to understand the onset and progression of staphylococcal diseases or other diseases where an association with *S. aureus* has been suggested. Although *S. aureus* resides within many different hosts, including pigs, cows or companion animals, this section will solely focus on the human host. Before potential associations or interactions can be studied, both the host and bacterial side should be investigated separately. As for *S. aureus* this has been the case since its discovery in the late 19th century on the genomic, transcriptomic and proteomic levels, especially during the last decades. The numerous *in vitro* studies on *S. aureus* have shaped our current understanding of this bacterium and led us to hypothesize which subparts of *S. aureus* might play the most central roles when colonizing or infecting the human host. One disadvantage in today's research is that the majority of investigations still rely on *in vitro* experiments, the results of which may not precisely reflect the *in vivo* situation. Our knowledge about the potential behaviour of *S. aureus in vivo* is very scarce and limited to a few studies using cell cultures or animal models [34,35]. However, even these approaches may not unveil the exact behaviour of *S. aureus* in the living human host during colonization and/or infection. Some researchers have tried to address this issue by investigating colonization of *S. aureus* in human beings and some others used human material (e.g. tissue biopsies or blood) for their investigation [36-38]. The latter approach has become very popular in recent years especially in relation to autoimmune diseases, but limits the outcome of the experiments still due to the low specificity of the applied methods and the working material. The human host is probably the most studied organism worldwide, with an army of researchers trying to understand its complexity. The completion of the human genome project in 2003 was, no doubt, one of the biggest achievements in medical history and sciences and has lead since then to many new discoveries, especially in the treatment of life-threatening diseases, such as

cancer and cardiovascular diseases. Moreover, only very recently the first draft map of the human proteome was published that will certainly drive further advances in the field of medical sciences [39]. Notably, the study of the human complexity, let alone only the human immune system is such a complex undertaking that we can only hope to compile a comprehensive list of clues with the ultimate goal to create a complete profile.

One subsection of the complex immune system that has been under investigations in the cross field of immunology and microbiology is the pathogen-specific immune response. Specifically, the adaptive immune system dealing with anti-staphylococcal antibodies in the blood of both *S. aureus* carriers and non-carriers, *S. aureus*-infected patients and hospital-admitted control individuals and patients with *S. aureus* bacteraemia were investigated focusing on immunoglobulin G, M and A (IgG, IgM and IgA) [18,40-43]. Several different observations have been made in these studies. In brief, persistent carriers had significantly higher levels of antibodies against TSST-1, staphylococcal enterotoxin A (SEA), ClfA and ClfB compared to non-carriers, which could be related to the different outcomes of *S. aureus* infections between these two groups [40]. Studies on patients who suffered from *S. aureus* bacteraemia clearly presented evidence that individual patients develop a unique immune response directed to different staphylococcal proteins with different dynamics over time [41]. Lastly, patients infected with *S. aureus* displayed higher IgG levels directed against several toxins compared to the healthy control subjects [42]. Taken together, these findings indicate a heterogeneous bond between the adaptive immune response and *S. aureus* depending on both the specific type of *S. aureus* and the individual human host. Host-pathogen interactions in both health and disease are of public health concern and importance, and every clue that can be found and explained will be highly relevant. In the following paragraphs, three diseases, namely granulomatosis with polyangiitis, epidermolysis bullosa and buruli ulcer and their associations with *S. aureus* will be briefly reviewed to provide more insight into the respective host-pathogen interactions.

Granulomatosis with polyangiitis (GPA)

GPA, formerly known as Wegener's disease, is a severe, systemic autoimmune disease. It is part of a larger group of anti-neutrophil cytoplasmic antibodies (ANCA)-associated vasculitides (AAV) and characterized by necrotizing inflammation of small- to medium-sized blood vessels. In contrast to other forms of AAV, GPA has been described to start mostly 'localized' in the upper and lower respiratory tract, and progress to a more 'generalized' disease that can include multiple organs, but most frequently affects the kidneys [44-46]. It is therefore a potentially life- and organ-threatening chronic inflammatory autoimmune disease, for which the mortality rate has been drastically reduced in the past decades, due to early diagnosis and more effective immunosuppressive therapy [47]. One hallmark of GPA is the development and presence of ANCAs that are predominantly directed to proteinase 3 (PR3) of neutrophils in GPA patients in Europe, but can also be directed against myeloperoxidase (MPO) of neutrophils, which is mostly occurring in patients in Asia and Australia [48]. The pathogenesis and pathophysiology of GPA has been studied extensively, resulting in the identification of several genetic and environmental factors. Nevertheless, many different hypotheses have been postulated to be the cause or trigger of this autoimmune disease. However, since the aetiology is clearly multifactorial, consisting of a complex network of host and environmental factors, pinpointing factors that might trigger and maintain autoimmunity in GPA will remain an ambiguous task. Overall, there are many observations that suggest a link between GPA and bacterial infections, and although the precise mechanisms by which pathogens trigger the disease are unknown, a number of mechanisms have been proposed. Interestingly, Friedrich Wegener already suggested that GPA could be triggered by an infection in the upper respiratory tract, creating an infectious milieu and leading to a cascade of events resulting in the autoimmune disorder [49]. Supporting this hypothesis, is the fact, that respiratory tract infections frequently precede or accompany initial GPA symptoms [50,51]. Specifically, *S. aureus* has moved into the centre of studies investigating bacterial implications in the onset and progression of GPA in patients bearing PR3-ANCAs. Intriguingly, approximately 60-70% of PR3-GPA patients are chronic *S. aureus* nasal carriers, compared to approx. 30% in the general population, and *S. aureus* nasal

carriage has been associated with an increased risk of relapses [52-54]. Consistent with these findings, anti-bacterial treatment with co-trimoxazole reduces the risk of relapses [55]. Nevertheless, the precise mechanisms by which *S. aureus* could exert a pathophysiological role in PR3-GPA have remained enigmatic. In view of the persistent activation of circulating T cells, staphylococcal superantigens (SAgs) were invoked as chronic stimuli of inducing the aberrant immune response in GPA [56,57]. Indeed, Popa and colleagues showed that PR3-GPA patients carrying *S. aureus* positive for the TSST-1 have an increased risk for disease relapse [58], although earlier studies had not revealed a correlation between the presence of SAg genes and the expansion of specific T cell subsets in peripheral blood [59]. Notably, studies investigating *S. aureus* or other potentially involved microorganisms in AAV possessing MPO-ANCA instead of PR3-ANCA are so far lacking. In conclusion, an association of *S. aureus* with PR3-GPA could be determined, but the precise mechanisms and its exact role still remain to be unravelled.

Epidermolysis bullosa (EB)

EB refers to a group of inherited connective tissue disorders caused by mutations in various genes that encode essential structural proteins of the skin leading to friction and skin fragility. Patients suffering from EB develop blisters in the skin and mucosal membranes as a result of a defect in the anchor between the epidermis and dermis [60]. Subsequently, the ulceration of the skin leads to the formation of wounds that ultimately become heavily colonized by different bacterial species that are part of the general human microbiota [60]. Specifically, these wounds are predominantly colonized with *S. aureus* that show high genetic diversity within one individual in a spatial and temporal manner [61]. Interestingly, a recent publication unveiled high anti-staphylococcal antibody titres in patients with EB related to long-term colonization with alternating types of *S. aureus* [61]. More specifically van der Kooi-Pol *et al.* reported that the increased anti-staphylococcal antibody titres in EB patients applied to IgGs against nine important virulence factors, including the SAgs SEM, SEN, and SEO [62]. As the underlying disease in the case of EB is a genetic disorder, one might hypothesise that *S. aureus* and other microbes in the wounds of these patients delay wound healing and therefore negatively affect the already compromised health and wellbeing of these patients.

Buruli ulcer (BU)

BU is a neglected tropical disease, manifested as a chronic skin and soft tissue infection, caused by the bacterium *Mycobacterium ulcerans* [63]. After tuberculosis and leprosy, BU is the third most common mycobacteriosis found in humans. One key characteristic in the pathogenesis of BU is the production of mycolactone, which is designated as the major virulence factor. Until today, the natural reservoir of *M. ulcerans* has not been identified unambiguously. The general hypothesis is that once *M. ulcerans* has reached and probably breached the skin, the disease onset results in nodules, papules, plaque or oedema. Without treatment, these lesions break open and an ulcer that can progress to a large necrotic lesion develops. In general, arms and legs are affected, but lesions also occur on other parts of the body with lesser frequency. Regions with tropical, subtropical and temperate climates, such as West Africa, Australia and China, report BU cases annually. In particular, in countries like Ghana and Benin in West Africa, BU is endemic and recognized as a distinct disease that places a major burden on the affected population and health facilities [63]. The majority of cases occur in rural communities and nearly half of the people affected in Africa are children under the age of 15. Early diagnosis and treatment are critical to minimize morbidity and prevent disability. A combination treatment with streptomycin and rifampicin has been successfully used to treat BU since 2004 [64]. However, due to the occurrence of secondary infections that are thought to be causally involved in severe complications in BU, additional antibiotics are often prescribed. No exact information is so far available on the incidence of secondary infections in BU, which bacteria these antibiotics should target and more importantly what the precise antibiotic susceptibility pattern of these bacteria is [64]. Moreover, very limited data is available on the overall resistance of microbes in West African countries, and even less on the potential resistances of microbes that are colonizing BU patients [64]. One study investigating

the rational use of antibiotics for suspected secondary infections in BU patients identified species like *Enterobacteriaceae*, *Pseudomonas aeruginosa*, Group A and Group B or C *Streptococcus* and *S. aureus* [64]. Interestingly, 38% of the *S. aureus* isolates from BU patients were resistant to oxacillin [64]. The author suggested that this high prevalence of MRSA in the wounds of BU patients might complicate treatment. Further studies are therefore urgently needed to describe the population dynamics of *S. aureus* in BU patients, including their antibiotic susceptibility pattern. In addition, the genomic repertoire of relevant *S. aureus* isolates should be analysed to describe potential routes of transmission and implications in the progression of BU.

Taken together, it seems that *S. aureus* shows different adaptive responses to different host environments. As for the evolutionary adaptation to a specific host environment, the livestock-associated MRSA ST398 lineage (MRSA ST398) is an excellent example, since it seems to have lost the genes for the human immune-modulating factors *sak*, *chp*, *scn* in the course of its adaption to the new livestock host [65,66]. Nevertheless, although a balance between the host and *S. aureus* has been suggested that has probably resulted from co-evolution, during the 'tug of war' between these two entities, this balance can shift to either side, which is decisive for either health or disease of the host.

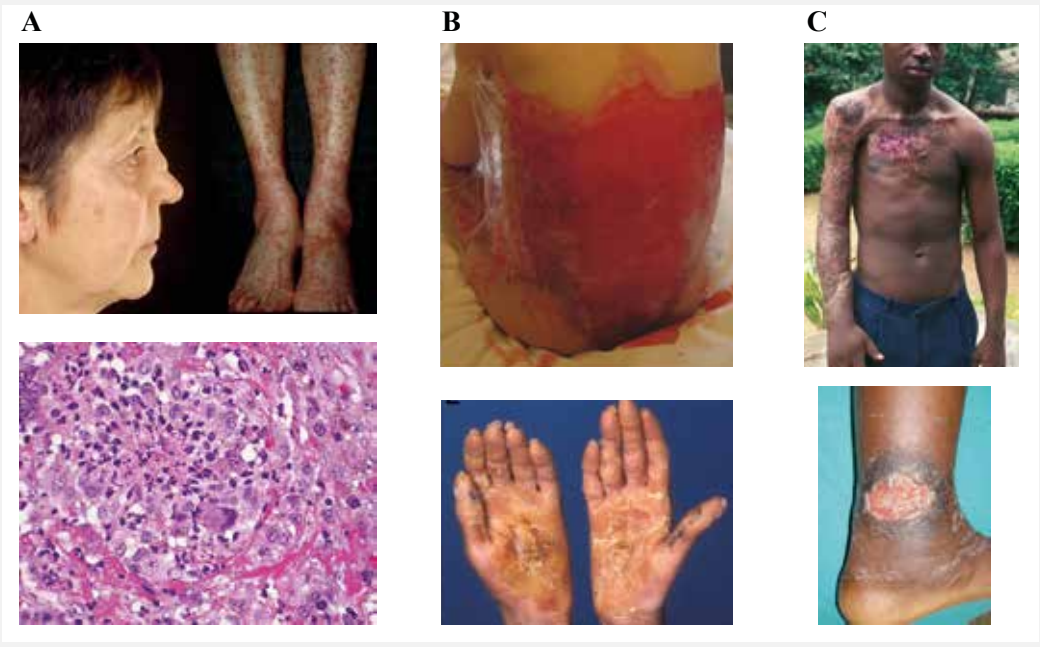


Figure 3. Clinical manifestations of the three diseases. (A) Symptoms of GPA in two patients (upper panel) and a microscopic image of an acute necrotizing lesion in the lung of a GPA patient (lower panel, arrow indicates an adjacent multi-nucleated giant cell next to the microabscess formation) [67]. **(B)** Chronic wounds of patients with EB suffering from severe generalized recessive dystrophic EB (upper panel) and Dowling-Meara type EB simplex (lower panel) [60]. **(C)** Chronic wounds of patients suffering from BU (images were kindly provided by Nana Ama Amissah).

Evolution – 'it was good as long as it lasted'

Evolutionary biology has been very popular in many scientific fields ranging from projects on single cell microbes, to mammals, and complete ecosystems. Charles Darwin, the author of 'The Origin of Species' and many other masterpieces, who described for the first time the mechanisms of 'the survival of the fittest' and 'natural selection' once said: "It is not the strongest or the most intelligent who will survive but those who can best manage change." One very remarkable example of evolution, which is easy to observe in species with a quick generation turnover, is the large-scale experiment started by Prof. Richard Lenski in 1988. Since more than 20 years, 12 independent *Escherichia coli* populations are growing and evolving *in vitro* [68,69]. These have been started from a single ancestor strain and have

already reached more than 50,000 generations, with the accumulation of genetic changes resulting in adaptation to the environment, such as the developed ability to utilize citrate as a nutrient [69].

Evolution of *S. aureus* is also clearly evident, with the emergence of antibiotic resistance as the most prominent example. Accordingly, staphylococcal evolution has been one of many foci of microbiological research in the past century. Especially with the recent developments in whole-genome sequencing (WGS), and therefore the large number of available *S. aureus* genomes through publically available databases, our understanding of the evolution of *S. aureus* has been greatly re-defined. Almost 10 years ago, when only about seven genomes of *S. aureus* were available, comparative genomics already allowed the identification of variations in the genome and helped to understand the mechanisms that led to the high genome plasticity of this versatile bacterium. From these very early comparative genomic studies it became clear that the *S. aureus* genome is made up of two moieties: the conserved core genome and the highly variable accessory genome. The core genome is present in all *S. aureus* isolates and comprises approx. 75% of the genome, whereas the accessory genome is unique for one isolate or can be found in a certain group/lineage of *S. aureus* isolates [27,70].

The core genome contains mainly genes that are essential for growth and survival, such as metabolic genes, and most of all species-related genes that cannot be found in other staphylococcal species. Variations within the core genome can be grouped into three classes: SNPs, sequence divergence within genes and operons and repeat variations [27,71]. Firstly, SNPs can occur across all coding and non-coding regions, but their effect depends on the exact position within the genome and their nature. Many SNPs are phenotypically silent, as they result in synonymous substitutions. Non-synonymous substitutions however, can result in phenotypic changes. This in turn, can be advantageous or disadvantageous, depending on whether they occur in coding or non-coding regions, their exact position, and on the newly encoded amino acid residues. SNPs in gene regulatory systems or other coding regions have been repeatedly associated with the development of antibiotic resistance, especially *in vivo* when patients underwent antibiotic treatment and their microbiota was under selective antibiotic pressure [71-73]. SNPs are also the instrument of the typing method multilocus sequence typing (MLST), where the sequencing of seven housekeeping genes results in a sequence type (ST), and different alleles of these genes will ultimately define a particular ST. Secondly, sequence divergence within genes and operons might reflect some form of genetic exchange and homologous recombination. One example is the *agr* cluster that is encoded in the core genome of *S. aureus*, consisting of *agrABCD*. The activation of the *agr* system takes place through an autoinducing peptide (AIP) that derives from AgrD, and which is processed by AgrB. Interestingly, a total of four groups of the *agr* system have been identified so far, each possessing a different AIP structure. Through sequence comparisons between the different *agr* systems, variable regions in *agrBDC* were identified [21,74]. Thirdly, repeat variation is a widespread phenomenon across bacterial genomes, including the *S. aureus* genome. Many studies have described different types of repeats, such as perfect repeats and tandem repeats [27]. The latter are being targeted in the molecular typing methods multiple-locus variable number tandem repeat (VNTR) analysis/fingerprinting (MLVA/MLVF) and *spa*-typing. Large regions of variable repeats can be found within genes for surface-associated proteins of *S. aureus*, such as the *clfA* gene that is also addressed in the MLVF method. The repeat regions in the ClfA protein involve dipeptide repeats composed predominantly of aspartic acid and serine residues. Determination of the numbers of repeats found within *clfA* revealed repeat sizes between 580 and 1320 bp [27]. These repeat variations, especially in surface-associated proteins, have been hypothesized to have a role in colonization and/or infection of the host.

The accessory genome of *S. aureus* is more complex and highly heterogeneous between different *S. aureus* isolates and is made up of various MGEs that determine a large part of the heterogeneity. The presence or absence of MGEs, but also their numbers and compositions contribute to the overall variation within the accessory genome. So far six different types of MGEs have been identified and described in many different *S. aureus* isolates, namely: transposons, bacteriophages, *S. aureus* pathogenicity islands (SaPIs), staphylococcal cassette chromosomes (SCC), plasmids and genomic islands and islets. Interestingly, the composition of the different MGEs seems to be restricted since

virulence genes tend to be found on bacteriophages and SaPIs, whereas resistance genes rely on SCC, plasmids and transposons, probably for easier transfer between cells [27]. Three mechanisms for horizontal gene transfer (HGT) are present within the bacterial kingdom, and all three seem to be active in *S. aureus*. However, transformation and the conjugative transfer of gene elements does not appear to be common in *S. aureus* [75]. Instead, genetic exchange in *S. aureus* seems to depend mostly on transduction by bacteriophages. The independent evolution of the different *S. aureus* lineages and their very specific genetic repertoire is sustained by effective restriction modification (RM) systems, one of the natural barriers for HGT [76]. *S. aureus* possesses two RM system, where one prevents solely the uptake of DNA from different bacterial species while the other also prevents the uptake of DNA from different *S. aureus* lineages [27,76]. The transfer of MGEs between *S. aureus* cells of the same lineage on the other hand might occur at high frequencies and there is increasing evidence that HGT is the main driver of adaptation, evolution and the overall success of *S. aureus*. The diversity between different *S. aureus* isolates and lineages due to different MGEs, bacteriophages and transposons will be briefly introduced in the following. Bacteriophages have been shown to carry important *S. aureus* virulence genes, including the genes for Panton-Valentine leukocidin (PVL), enterotoxin A and exfoliative toxin A, as well as the *sak*, *chp* and *scn* genes that make up the immune evasion cluster (IEC). Bacteriophages are acknowledged as the most common MGEs in *S. aureus*, with most of the investigated isolates carrying between one and three bacteriophages [27,77]. The classification of *S. aureus* bacteriophages is based on the integrase gene (*int*) sequence and the integration site, whereas they all belong to the Siphoviridae family and can be grouped into eight families (φ1- φ 8) [78-80]. A recent investigation revealed a high variability but lineage-specificity of the eight known bacteriophage groups within a large *S. aureus* collection [78]. Although most bacteriophages genes were dispensable and extensive mosaicism could be observed, certain virulence genes were tightly associated with specific phage families. In contrast to phages, transposons (Tn's) often carry resistance genes. For example, the Tn554 encodes resistance to erythromycin, Tn552 encodes β-lactamase resistance, Tn1546 confers resistance to vancomycin and the Tn916 encodes tetracycline resistance [27,81,82]. Interestingly, transposons are often associated with other MGEs, such as SCC or plasmids, for successful HGT that usually occurs via a 'hitchhiking' type of process. All transposons feature a transposase gene that is essential for excision, replication and integration of the transposon. Via this mechanism and the ease of HGT, transposons are widely distributed among *S. aureus* isolates. Thus, the transfer of antibiotic resistances is a realistic danger that can cause tremendous clinical problems and needs to be controlled. Clearly, from the staphylococcal perspective genome evolution is crucial as the conditions in the host can be subject to critical changes, either due to innate and adaptive immune responses or to antibiotic therapy. Thus, a staphylococcal genome is only 'good as long as it lasted' in the continuous battle with the host.

Antibiotic resistance – 'the rise of the superbugs'

One crucial characteristic of *S. aureus*, which complicates effective control measures and treatment of infections, is its high ability to escape natural defences and therapeutic interventions. Resistant lineages of *S. aureus* have been in the centre of numerous studies worldwide in the past decades as the medical significance of antibiotic resistance is clearly of public interest. In general, *S. aureus* is intrinsically susceptible to all discovered and developed antibiotics. Resistance to antibiotics by *S. aureus* is generally acquired via two pathways: (1) HGT of antibiotic resistance genes from other microorganisms and (2) genomic mutations that occurred spontaneously or upon antibiotic pressure. A brief look into the history of antibiotic resistance clearly shows that, in the 21st century, the vast majority of antibiotics are no longer effective against *S. aureus* (Figure 4, adapted from [83]). Antibiotics have clearly improved life and longevity and, more importantly, saved countless millions of lives, since the ground-breaking discovery of penicillin by Sir Alexander Fleming in 1928. However, antibiotics have also come to a level of saturation in every corner of human life and the environment. In the pre-antibiotic era the mortality of patients suffering from *S. aureus* bacteraemia went beyond 80% and over 70% developed metastatic infections [9]. Twelve years after its discovery, penicillin was introduced in the clinics and drastically improved the disease outcome of patients with staphylococcal and other infections [9]. However, in

1942, only one year after this drug was introduced, the first penicillin-resistant *S. aureus* isolates were identified, initially in hospitals and subsequently in the community [9]. By the 1950s more than half of all *S. aureus* isolates in large hospitals were resistant to penicillin [9,82]. In addition, *S. aureus* was able to develop resistance to the other available antibiotics such as erythromycin, streptomycin, and tetracycline [82]. The flow of resistant microbes first identified in hospitals with the subsequent spread to the community is nowadays a well-established pattern that occurs with every new antibiotic that has been developed and introduced so far. A new ground-breaking development in the antibiotic era was the introduction of methicillin, a semisynthetic penicillinase-resistant penicillin, in 1959 [9,82]. Although many scientists thought to have found a solution to their penicillin resistance problem, history repeated itself, and only two years after its clinical introduction, the first methicillin-resistant *S. aureus* isolates were reported [82]. Since its first occurrence, *S. aureus* resistant to methicillin dominated infectious diseases worldwide, presenting itself in a changing epidemiology [84]. The first cases of MRSA were solely associated with hospitalized patients, leading to the term hospital-associated MRSA (HA-MRSA; some scientists prefer to use the term hospital-acquired MRSA). Since the late 1990s, MRSA has also started to be present and spread among healthy individuals resulting in the term CA-MRSA [84]. It has to be noted that the separation of these two types of MRSA isolates has become quite challenging and different definitions for them are currently being used all over the world. One definition takes into account the time of stay at the hospital until the first isolation of *S. aureus*, but this requires active screening of all patients when entering a hospital, which is currently not mandatory in Europe or other parts of the world. Additionally, in the early 2000s a new lineage designated livestock-associated MRSA (LA-MRSA), with ST398 being the predominant lineage of LA-MRSA has been identified [85]. This lineage is nowadays a large problem, especially in countries like Denmark and the Netherlands, as it has widely spread among farm animals, mainly pigs, and farmers and their direct contacts [86-88]. In connection with this new lineage that is highly associated with farm animals, the clear over-usage of antibiotics on farms all over Europe dramatically increased the numbers of resistant lineages that in turn now threaten public health. As exemplified with the two antibiotics penicillin and methicillin, the introduction of new antibiotic therapies is usually rapidly followed by the emergence of resistant strains [9,89]. Thus, antibiotic therapy seems to be the central driver of the emergence of new antibiotic-resistant lineages in accordance with the 'survival of the fittest' principle.

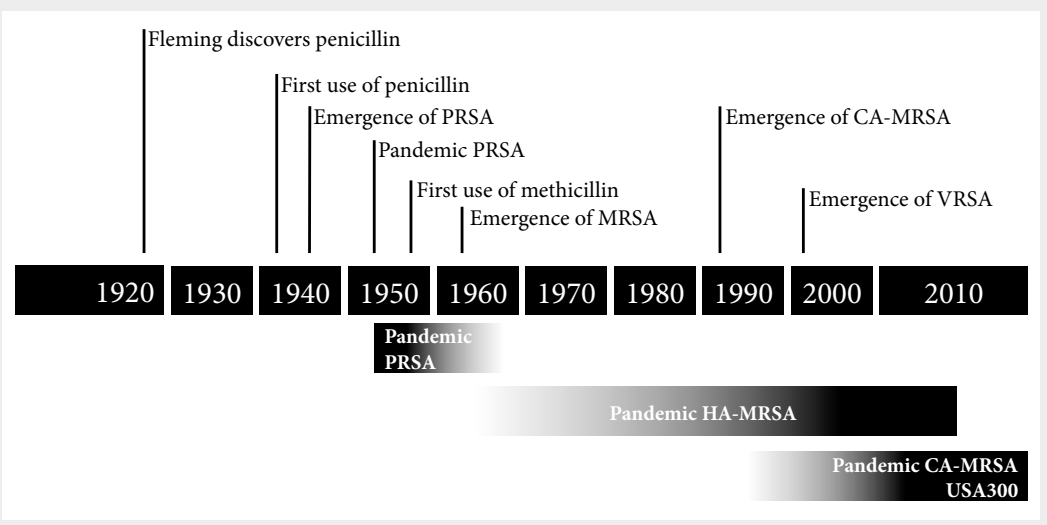


Figure 4. Emergence of antibiotic-resistant *S. aureus* and epidemic waves in the United States. Timeline indicates the year in which an event occurred or was reported. Arrows indicate approximate length of time for each pandemic/epidemic (adapted from [83]). Abbreviations: PRSA = penicillin-resistant *S. aureus*, MRSA = methicillin-resistant *S. aureus*, CA-MRSA = community-acquired/associated MRSA, VRSA = vancomycin-resistant *S. aureus*.

The latest larger class of antibiotics that has been introduced into the clinic are carbapenems, a class of β-lactam antibiotics with a broad spectrum of antibacterial activity. Due to their structure, carbapenems are highly resistant to most β-lactamases and are antibiotics designated as last resort for treatment of many bacterial infections. To date, the vast majority of *S. aureus* isolates are still sensitive to carbapenems and, to prevent the emergence of resistance, carbapenems are not used to treat staphylococcal infections. Instead carbapenems (e.g. imipenem, meropenem, ertapenem, doripenem) are used for the treatment of life-threatening infections caused mainly by *E. coli* and *Klebsiella pneumonia* [90]. Both *E. coli* and *K. pneumonia* belong to the family of *Enterobacteriaceae*, a large family of Gram-negative bacteria that are frequently encountered pathogens in humans, causing both community- and hospital-acquired infections. One key property of this bacterial family is its ability to transmit easily between humans but also from contaminated food or water to humans [90,91]. Their ubiquity and frequent acquisition of MGEs means that their human hosts are regularly exposed to new strains with novel genetic repertoires – including antibiotic resistance – through food and water, or from other animate and inanimate sources in the community, hospitals and during travel. Over the last three decades, *Enterobacteriaceae* have become increasingly resistant against first-line and second-line antibiotics, which include extended β-lactam antibiotics, such as third generation cephalosporins (caused by the production of so-called extended spectrum β-lactamases or ESBLs) but also against fluoroquinolones and aminoglycosides. This is mainly due to adaptive prescription of antibiotics, which favours carbapenems (third-line antibiotics) as an early and empirical treatment option in almost all European regions where ESBL-producing bacteria have become frequent among patients [92,93]. With the emergence of *Enterobacteriaceae* capable of producing carbapenem-hydrolysing enzymes in many parts of the world, including Europe, the Indian subcontinent and the US, the current situation has become extremely volatile [91,94,95]. In general, carbapenem-resistance in *Enterobacteriaceae* can arise from two main mechanisms: (1) acquisition of a carbapenemase gene that encodes for an enzyme capable of degrading carbapenems or (2) a combination of phenotypic changes such as a deficiency in porin production and an overproduction of β-lactamases that possess weak affinity for carbapenems [90]. The most important carbapenemases are categorized in three types of enzymes: (1) the *K. pneumoniae* carbapenemase (KPC) type enzymes first described in the USA but now found worldwide; (2) the Verona integron-encoded metallo-β-lactamases (VIM), the IMP-type metallo-β-lactamases (IMP), and the New Delhi metallo-β-lactamases (NDM); and (3) the OXA-48 type enzymes [96-98]. Acknowledging the ineffectiveness of almost all alternative antibiotics and resistance even to novel compounds, which are still under development, there is a growing awareness that carbapenemase-producing *Enterobacteriaceae* (CPE) may thwart the ability to cure life-threatening infections in the future even more than multi-drug resistant MRSA lineages. This would create a tipping point from where antibiotic resistance becomes again a significant cause of mortality. Thus, the not even a century-long trend of disappearance of major bacterial infectious diseases from Europe would be reversed and public health would be threatened by the decay of antibiotic effectiveness. The sole examples of resistance to penicillin, methicillin and carbapenems clearly underscore the notion that the problem of microbial antibiotic resistances in the 21st century has been caused by antibiotic overuse that, in turn, has led to 'the rise of the superbugs'.

Typing, typing, typing – 'the rogues' gallery'

Humans have a compulsion to group and categorize both the living and non-living world and this is particularly true for scientists. Intriguingly, humans themselves can be grouped by their genotype and phenotype, which could be characteristics such as eye or hair colour, gender or shoe size, but also features such as nationality, job or the brand of the smartphone owned. Moreover, all human individuals have their personal fingerprints and genetic features. One particular focus of scientists is the grouping of microbes, in particular bacteria. This so-called typing has led to the identification of different types of bacteria within a certain species [99]In the beginning of the typing era, methods such as serotyping, biotyping, phage-typing or the antibiogram have been used for the classification of bacteria. However, methods that type and therefore determine the relatedness of bacteria on the

molecular level have clearly revolutionized our understanding of evolution, transmission and, more importantly, the diversity of bacteria. In the context of public health, molecular typing of *S. aureus* is important to decide on effective surveillance and control strategies as well as to understand the spread, the complex population biology and the infectious status of this rapidly evolving opportunistic pathogen. To monitor the emergence of new *S. aureus* types, various typing techniques have been developed, including PFGE, MLST, MLVA, *spa*-typing and MLVF (for an overview see Figure 5), which will be introduced in the following.

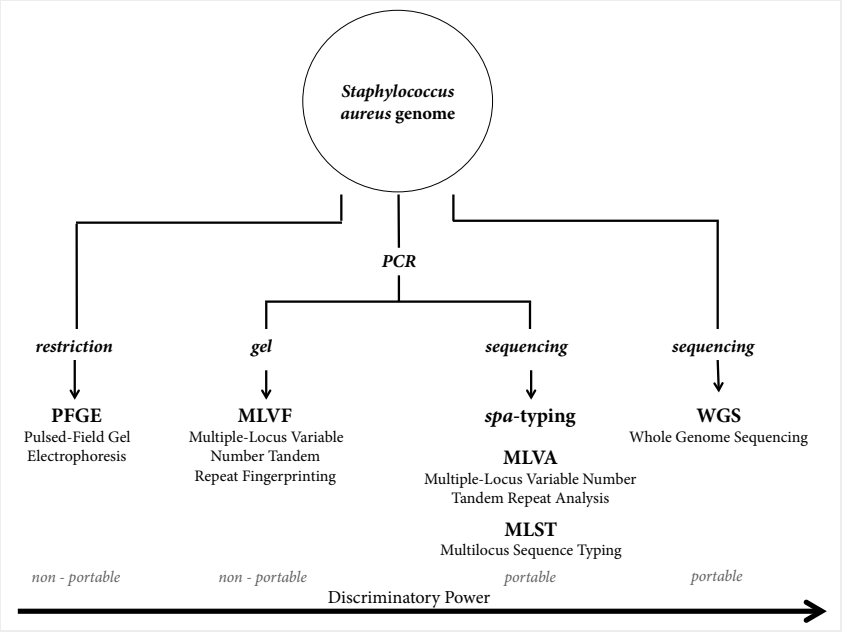


Figure 5. Overview of available and here discussed molecular typing methods for the classification and identification of *S. aureus*. Typing methods are ordered by their application (PCR, sequencing, restriction and gel electrophoresis) and the portability and discriminatory power is displayed as well.

PFGE has been considered and acknowledged the 'gold standard' among molecular typing methods since its first publication and application in the early 1990s (Figure 6) [99]Surprisingly, it is still the most frequently used approach to characterise bacterial isolates in outbreak situations, as exemplified with the successful implementation of Pulsenet.org in the USA (http://www.cdc.gov/pulsenet/) [99] Although it has a high discriminatory power and high epidemiological concordance, PFGE is relatively labour-intensive and inter-laboratory comparison of data produced by PFGE is challenging [100]L. As a revolutionary step in the advancement of molecular typing, several studies have shown that related *S. aureus* types with indistinguishable PFGE profiles can be further differentiated with PCR- and sequence-based typing methods [101-105].

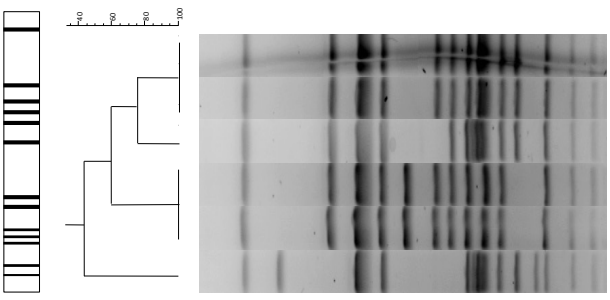
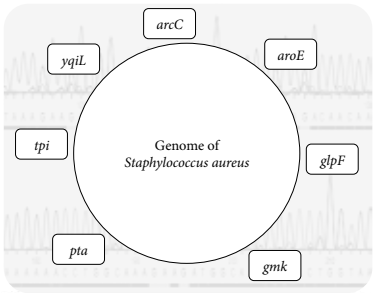


Figure 6. Schematic representation of the experimental output of PFGE. In brief, genomic DNA is cleaved with a restriction endonuclease with the subsequent separation on an agarose gel by pulsed-field electrophoresis. For more information refer to [99,106].

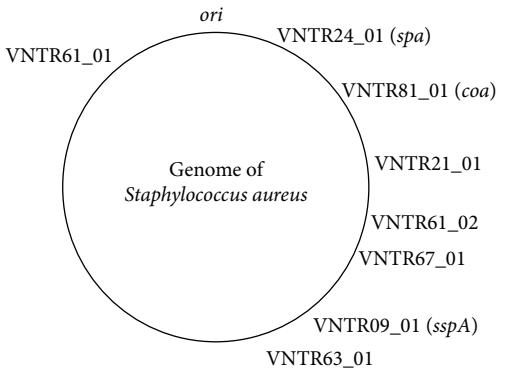
Sequence-based typing methods can be focused on a single locus (e.g. *spa*-typing) or on multiple loci (e.g. MLVA and MLST). Since its development in 1998 for *Neisseria meningitides*, MLST became a popular tool for the determination of the clonal evolution and moreover for global epidemiological studies on *S. aureus*, but also for other microbes such as *K. pneumonia* (Figure 7) [107-109]. The great advantage of MLST is that all data produced by this method is totally unambiguous due to an internationally standardized and acknowledged nomenclature, and is therefore highly reproducible. Moreover, the allele sequences and ST profiles are collected and are publicly and easily available in large central databases (http://www.pubmlst.org and http://www.mlst.net). These databases also provide software (eBURST) for the determination of the genetic relatedness between different *S. aureus* types as well as maps to locate and track isolates of a certain ST across the world and time [110]. The great disadvantage of MLST is its high cost, as it involves the sequencing of seven loci and moreover, it is labour-intensive and time-consuming.

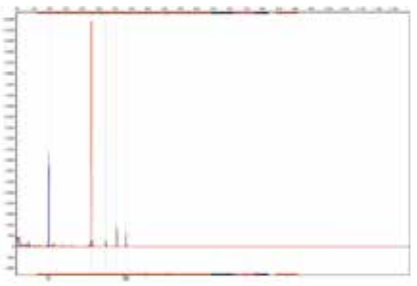


	arcC	*aroE*	*glpF*	*gmk*	*pta*	*tpi*	*yqiL*
ST59	19	23	15	2	19	20	15
ST30	2	2	2	2	6	3	2
ST239	2	3	1	1	4	4	3

Figure 7. Schematic representation of the experimental input and output of MLST. In brief, seven coding regions in the genome of *S. aureus* are PCR-amplified and sequenced. Depending on the identified allele, a number is assigned to each locus, and based on the combination of the identified alleles an ST can be determined (lower part). For more information refer to [107].

Whereas MLST is solely based on the exact sequence of the seven loci, *spa*-typing and MLVA are based on regions with VNTRs. MLVA determines the number of VNTRs at eight different loci, mostly non-coding regions, within the *S. aureus* genome [104]. The exact number of repeat units in each MLVA locus is determined by the use of capillary electrophoresis on an automatic DNA sequencer and the labeling of primers with different fluorescently coloured dyes (Figure 8) [104]. Once the number of repeats in each of the eight loci has been assessed, allele numbers corresponding to the number of repeat units at each MLVA locus result in an allelic profile (e.g. 16-1-1-5-1-11-8-6), which can be easily compared to a publically available reference database on the Internet (http://www.mlva.net). MLVA has been developed to overcome the inherent limitations of PFGE, MLST, and *spa*-typing and it was shown that MLVA was at least as discriminatory as PFGE and at the same time produced portable data with ease of interpretation comparable to that of MLST and *spa*-typing. Moreover, significant congruence of the results produced by MLVA, PFGE, MLST, and *spa*-typing was observed [104,111]. Nevertheless, it is as labour-intensive and expensive as MLST.





MLVA type	MLVA Complex	V09_01	V61_01	V61_02	V67_01	V21_01	V24_01	V63_01	V81_01
7	7	16	1	1	5	1	11	8	6
621	621	16	2	2	2	99	8	2	6
1035	621	16	2	2	2	99	8	1	6

Figure 8. Schematic representation of the experimental input and output of MLVA. In brief, the exact number of repeat units in each locus are determined with the use of sequencing and the labeling of primers with different fluorescently labelledcoloured dyes. Based on the amplicon size, the repeat number for each locus is calculated. Together, the repeat numbers represent the final allelic profile and, based on the combination of the eight alleles, a MLVA type can be assigned (lower part). For more information refer to [104].

As to a single locus sequence-based molecular typing method, *spa*-typing has become a highly popular and widely utilized typing approach, in particular for surveillance and epidemiological studies at the international level. For *spa*-typing, the *spa* gene is PCR-amplified and sequenced. Based on the detected VNTRs and their composition, a specific *spa*-type is assigned to the investigated *S. aureus* isolate (Figure 9) [112-114]. The key advantages of this approach are the transportable data production and the accessibility of the *spa* server through the internet (http://www.spaserver.ridom.de). Additionally, *spa*-typing can be performed in a high-throughput manner, is highly reproducible, and cheap compared to MLST and MLVA as it only involves sequencing of a single locus. The latter is however also a limiting factor that has been shown to make *spa*-typing less discriminatory than PFGE, MLVA and MLVF [115,116].

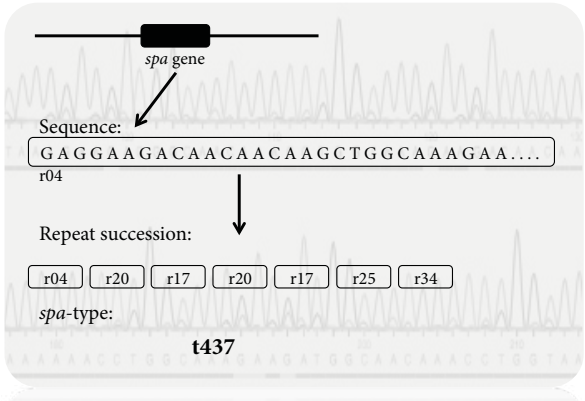


Figure 9. Schematic representation of the experimental input and output of *spa*-typing. In brief, the polymorphic X region, of the *spa* gene, containing VNTRs, is sequenced. Each identified repeat is associated to a repeat number and the order and nature of the repeats result in the assignment of a *spa*-type. For more information refer to [114].

MLVF is an attractive high-throughput alternative for PFGE that is complementary to *spa*-typing [105,111,117]. This approach is solely based on PCR amplification of five staphylococcal VNTR loci (*sdrCDE*, *clfA*, *clfB*, *sspA*, and *spa*). The multiplex PCR products can then be investigated by standard agarose gel electrophoresis, or for improved reproducibility and more stable conditions with a microfluidic chip that is based on microcapillary electrophoresis (Figure 10) [105,111]. As the precise number of repeats per locus cannot be calculated, it is referred to as a fingerprinting method. MLVF is the cheapest, fastest, and easiest to perform compared to all other typing methods. Moreover, it has the highest discriminatory power among the different PCR-based molecular typing methods. Its only disadvantage is that it does not produce portable data, and is therefore suggested to be used only at the local level (e.g. a single hospital) for the rapid and highly discriminatory clustering of clinical *S. aureus* isolates [105,111].

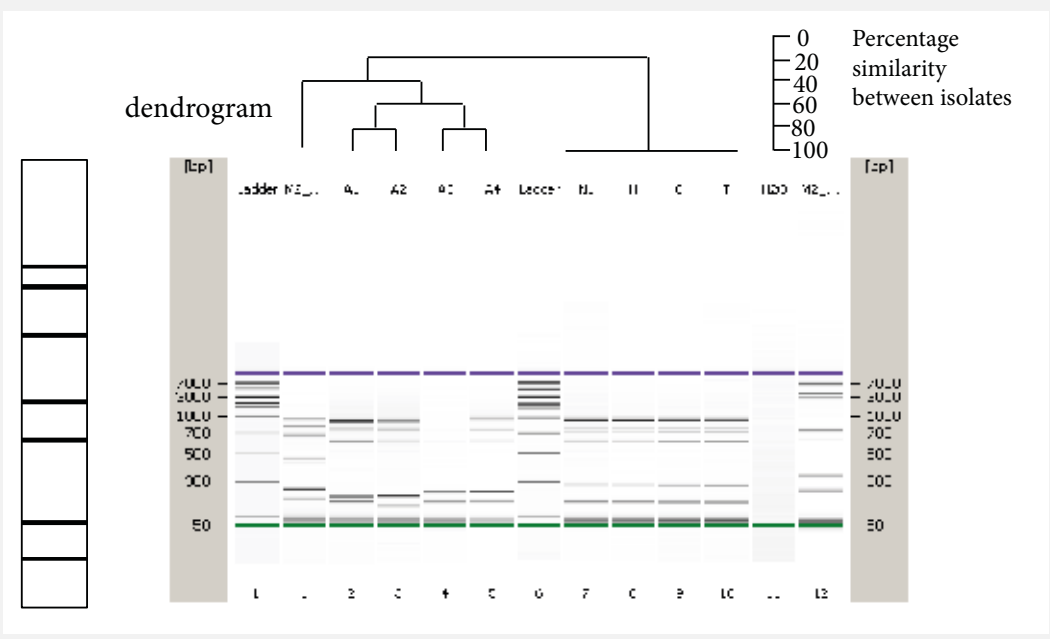


Figure 10. Schematic representation of the experimental output of MLVF. In brief, five loci, containing VNTRs, are PCR-amplified and subsequently separated with a fully automated microfluidic chip-based system, resulting in a better resolution compared to standard agarose gels. The internal markers and simplified analyses with an automated software is exemplified with the illustrated dendrogram. For more information refer to [111,118].

In recent years, a whole new chapter in the field of bacterial molecular typing has arisen with the application of WGS, which has changed our understanding of bacterial evolution, transmission and adaptation and more importantly, it has started to uncover the diversity of the microbial world, including *S. aureus*. This new WGS era has transformed microbiological molecular investigations by providing an in-depth view into many genomes, and disclosing enormous amounts of new genetic information that has, thus far, not been addressed in the commonly used molecular typing methods. The clear advantage of WGS compared to the traditional Sanger sequencing, is the generation of millions of genomic reads in single runs at comparatively low costs, with prices currently around 50 USD per *S. aureus* genome on an Illumina HiSeq (San Diego, USA) machine including sample preparation, library quality control (quantification and size assessment) and sequencing (Figure 11) [99]Major drawbacks in this new era of WGS are the non-existence of an internationally accepted proficiency standard and the presence of numerous different pipelines for the analysis of the massive amounts of data. There are currently a plethora of different bioinformatic tools and infinite different ways on how to analyse WGS data. This is a potential pitfall as it slows down the fast developing process of WGS and leads to the accumulation of insufficiently used WGS data on numerous servers in the world. On the positive side, WGS also made two

fields, namely microbiology and bioinformatics merge, as microbiologist have no deep understanding of the possibilities to analyse this vast amount of data. Nevertheless, in the near future, WGS will become a highly powerful tool for outbreak investigations, transmission studies and more importantly surveillance schemes in routine clinical practice. However, this will require proficiency standards and internationally accepted standard operating procedures. WGS, by revealing genetic relatedness of *S. aureus* isolates in detail, has become the new gold standard in studies of evolution, transmission and strain relatedness. As the number of available and implemented typing methods for *S. aureus* exceeds the ones presented in this section and, more crucially, as more and more genomic information will be forthcoming through WGS, endlessly diverse fingerprints of *S. aureus* will be at hand to generate the 'rogues gallery'.

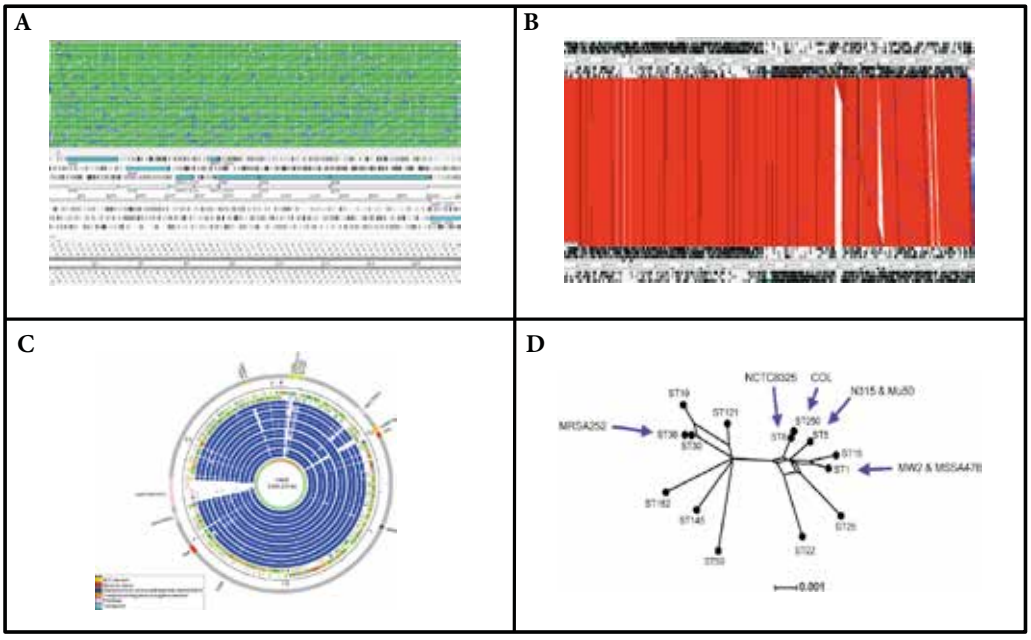


Figure 11. Schematic representations of possible outputs of WGS. Please see the text for details. **(A)** Burrows-wheeler alignment of sequence reads aligned to a reference genome and displayed in Artemis [119]. **(B)** Comparative genomics performed with BLASTN between two *S. aureus* isolates and displayed in ACT [120]. **(C)** Schematic circular diagram of the *S. aureus* TW20 genome taken from [121]. **(D)** Example of the phylogenetic diversity of *S. aureus* strains displayed in a split decomposition tree constructed by using Splitstree (taken from [122]).

Epidemiology, public health and emerging threats – 'the bad, the worse and the ugly'

The epidemiology of *S. aureus* has changed drastically in the late 1990s, as MRSA isolates were until then largely confined to hospitals and long-term facilities and have then started to emerge in the community [84]. To determine the distribution of different *S. aureus* clones and clonal lineages on the local, national and international levels, the application of molecular typing tools is required. With the implementation of these methods, several important observations regarding the evolution, epidemiology and spread of clones with particular public health importance have been made. In the past decade, the surveillance of antimicrobial resistant MSSA and MRSA clones was agreed to be of major importance, as certain clones have disseminated over wide geographical regions, especially in Europe. Surveillance of *S. aureus*, but also other bacteria with relevance for public health, is also crucial in terms of combating antimicrobial resistance. For this purpose, the European Antimicrobial Resistance Surveillance Network (EARS-Net, formerly known as EARSS) has been established in 1998 by the Dutch National Institute for Public Health and the Environment (RIVM, the Netherlands) to monitor variations of antimicrobial resistances in seven species over time across Europe. Currently, 30 countries being part of Europe or the European economic area (EU/EEA) participate in this

network and continuously collect invasive isolates of eight species and determine their antimicrobial susceptibility patterns. A report summarizing the results between 2009 and 2012 documents that for MRSA the population-weighted EU/EAA mean decreased significantly over the last four years, but that it remains a public health priority with percentages above 25% in seven out of the 30 participating countries [123]. Unfortunately, molecular typing is not part of the EARS-Net and also not of other currently on-going surveillance projects in the world. Nevertheless, numerous studies and projects have been performed and published in the past decade to capture the national and international epidemiology of *S. aureus* in Europe and other parts of the world with focus on the molecular level.

One of these projects on the European level is the network of staphylococcal reference laboratories (SRLs), which is partly overlapping with EARS-Net that performed two structured surveys on invasive *S. aureus* isolates [124, Grundmann *et al*., manuscript submitted]. These structured surveys took place in 2006/2007 and 2011, lasted six months in total and captured the first ten successive MSSA and MRSA bloodstream infection isolates per hospital with the number of included hospitals being proportional to the size of the respective country. During both structured surveys the molecular typing method *spa*-typing was the instrument for the determination of the geographic distribution of *S. aureus* clones [124, Grundmann *et al*., manuscript submitted]. The major finding of the first structured survey from 2006/2007 was that MRSA *spa*-types have a predominantly regional distribution in Europe, whereas MSSA *spa*-types are more widely distributed across Europe [124, Grundmann *et al*., manuscript submitted]. This indicates that a limited number of clones stay within the boundaries of the health care networks, suggesting that infection control efforts that prevent the spread and transmission of MRSA within and between health care institutions would ultimately dampen the numbers of MRSA cases in the hospital and subsequently in the community [124, Grundmann *et al*., manuscript submitted].

For over a decade, it has been the remit of the EARS-Net to monitor the occurrence of bacterial pathogens with epidemiological and clinical relevant antibiotic resistances causing invasive infections in the participating countries across Europe. One drawback of EARS-Net is inherent to its sampling frame. By restricting the surveillance to blood culture isolates (which undoubtedly improves compliance and comparability) the sensitivity is diminished, which probably leads to a considerable under-appreciation of other resistance threats, such as carbapenems resistance. Thus, during the annual EARS-Net (then still named EARSS) meeting in Athens 2009, the urgent need for a European-wide consultation about increasing CPE was recognised. Subsequently, a workshop for experts in the surveillance of antibiotic resistance in *Enterobacteriaceae* from 31 European countries was organised at the RIVM in April 2010. During a two-day meeting, gaps in diagnostic and response capacity were identified, and participants established a novel five-level staging system for the magnitude of CPE and provided elements of a strategy to combat this complex public health issue in a concerted manner [125]. Subsequently, a structured questionnaire survey in the fall of 2010 provided valuable insights into the prevalence of different CPE-associated resistance determinants and the degree of spread of CPE in all EU member states and 3 European Free Trade Association (EFTA) countries. The European Survey on Carbapenemase-Producing *Enterobacteriaceae* (EuSCAPE), a novel initiative of the European Centre for Disease Prevention and Control aiming to improve the understanding of the occurrence of CPE in Europe with a resolution currently not achieved by EARS-Net, is a direct continuation of these activities and a fulfilment of the expert recommendations published in 2010 [125]. Importantly, this initiative has the endorsement of all national experts who strive to improve active surveillance of CPE in their country, based on another consultation among the 31 European countries [May 2012, unpublished]. Another European survey performed in 2010, focussing on NDM-1 producing *Enterobacteriaceae* and the appropriate public health responses also came to the conclusion that active surveillance of CPE must be enhanced in Europe and effective control measures identified and implemented [126]. Recently, a review published by Levy Hara *et al.* reports recommendations from an international working group including experts from different continents, focussing on detection, treatment and prevention of CPE [127]. The two European reports and the latest inter-continental report clearly indicate that a more systematic and structured approach for the surveillance of CPE is urgently needed. Thus, the collected information during the course of the EuSCAPE project

shall inform consensus guidelines for the identification and confirmation of CPE and the curricular contents of capacity building workshops that shall improve current practice paving the way towards enhanced surveillance at the local, regional, national and European levels. In view of public health threatening pathogens that are in the focus of this section, it will probably never be possible to say with complete certainty which are 'the bad, the worse or the ugly', as any of them could meet one, two or all three of these qualifications. Nevertheless, at present it seems that MRSA qualifies for the 'bad' while the different CPEs represent 'the worse and the ugly'.

SCOPE OF THIS THESIS

The overarching aims of the research described in this thesis were to capture the diversity of staphylococcal fingerprints across Europe, and to give a preview of the next wave of resistant bacteria that started to cast a shadow over this continent and the rest of the world. The basic background information on 'the case' is introduced in **Part I** of this thesis. The prime suspect in **Part II** of this thesis is the 'Jekyll & Hyde microbe' *S. aureus*, followed in **Part III** by the CPE as 'accomplices'. While *S. aureus* is 'bad', the CPEs can be described as 'the worse and the ugly'. Although the scientific focus of the present work lies on the opportunistic pathogen *S. aureus*, experts across the globe predict that the group of CPEs introduced in **Part I** and investigated in **Part III** will take the upper hand in the field of infectious diseases and public health in the near future.

The aims of this thesis were primarily reached through the implementation of a diverse set of DNA typing tools, introduced in **Chapter 1**. Secondarily, information on antibiotic resistance, metadata of the patients/carriers as well as phenotypic and molecular data of the respective bacterial isolates were also utilized to complete the bacterial fingerprint picture. Withal, **Chapter 5** explored the more advanced technology of WGS in combination with proteomics and in **Chapters 6 and 7** the human host with focus on the anti-staphylococcal antibody response was investigated. Thus, the compilation of the presented bacterial fingerprints across Europe in this thesis was accomplished by the combined analysis of six parameters: (1) particular selected *S. aureus* lineages; (2) selected *S. aureus* carrier groups and patients; (3) different geographical locations; (4) different time points; (5) experimental methods and, last but not least, (6) other relevant bacterial species.

The overview presented in **Chapter 1** forms the foundation for the research presented in this thesis by introducing the double-sided and under certain circumstances 'deadly' potential of *S. aureus*, followed by a brief description of three potential host-pathogen interactions fighting the 'tug of war'. As evolutionary processes give rise to diversity, they are central to this thesis, showing that the genome of a bacterium at a given time point was 'only good, as long as it lasted'. One great example of evolution is 'the rise of the superbugs', describing the history of antibiotic resistances that was recently reported for the first time by the World Health Organization as the most serious worldwide threat to public health. The final piece of the **Chapter 1** foundation is represented by the European epidemiology of bacterial pathogens that threaten public health and can be classified as 'the bad, the worse and the ugly'.

The first experimental research is described in **Chapter 2** in **Part II** 'Rapid and high-resolution distinction of community-acquired and nosocomial *Staphylococcus aureus* isolates with identical PFGE patterns and *spa*-types'. The aim of this project was to overcome a previously reported pitfall for the correct identification of a high-risk CA-MRSA clone with the PFGE profile USA300 and ultimately to pillory the limitations of this 'gold standard' DNA typing method. The employed tool to overcome these limitations was MLVF, an easy to use and relatively cheap multiplex PCR method that was applied to a large number of *S. aureus* USA300 samples from Denmark, possessing two different but related *spa*-types. For control purposes isolates from Germany with a different PFGE profile, but the same *spa*-types were also included. This approach both tested and evaluated the discriminatory power of a DNA typing 'underdog' tool for the identification and possible genomic sub-clustering of potentially dangerous *S. aureus* clones.

The results presented in **Chapter 3** 'High-resolution typing by MLVF unveils extensive heterogeneity of European livestock-associated methicillin-resistant *Staphylococcus aureus* isolates with the sequence type 398' confirm and extend the demonstrated discriminatory power of MLVF reported in the previous chapter. An extremely diverse *S. aureus* collection from seven different European countries that were selected based on the sole criterion to be ST398, were typed with the complementary methods MLVF and *spa*-typing. This *S. aureus* ST398 lineage has emerged in livestock worldwide, especially in areas with high densities of livestock such as the Netherlands and Denmark. Therefore the correct, rapid and discriminatory identification of this particular lineage is of utmost importance. With the anew application of these two complementary DNA typing tools, their usefulness and discriminatory power was validated.

The research reported in **Chapter 4 '***Staphylococcus aureus spa*-type t437: identification of a community-associated clone from Asia across Europe' reports on the methodical continuation of the previous two chapters, examining the applicability of DNA typing methods on another specifically selected *S. aureus* collection. Prior, as *S. aureus* isolate from a patient at the University Medical Center Groningen (the Netherlands) with the *spa*-type t437 and ST338 (belonging to CC59) was identified. This was the incentive for a literature search about this particular *S. aureus* type, showing that *S. aureus* with the *spa*-type t437 in association with MLST CC59 is the most dominant CA-MRSA in Asia. Notably, although this specific lineage has so far only been reported in low numbers among large epidemiological studies in Europe, the identified numbers in some Northern European countries have increased during the past decade. To determine whether the *S. aureus* t437 clone is present in other European countries, and to assess its genetic diversity across Europe, a large sample of *S. aureus* t437 isolates from 11 European countries was analysed. This was again achieved by the implementation of DNA typing tools. Next to the previously proven successful MLVF method, and the usage of its 'sister method' MLVA, the more widely applied and acknowledged MLST was added for a better genomic resolution and clonal comparability.

Chapter 5 'A combined genomic and proteomic portrait of sequential *Staphylococcus aureus* isolates from a patient and his partners', reports on the MRSA-t437 isolate from a patient in our hospital. Since *S. aureus* t437 was demonstrated to represent a tight genomic cluster across Europe (Chapter 4), this particular *S. aureus* type was examined within one patient and his partners. As the new era of WGS is revolutionizing our understanding of bacterial evolution and pathogenesis, and the combination of WGS with proteomics for research or diagnostic purposes has so far been lacking, this combinatorial approach was chosen. Hence, it was applied to examine retrospectively selected sequential *S. aureus* isolates, both MRSA and MSSA, collected over a five-year period from the same hosts. Experimental data was replenished with information on the travel destinations of the patient and, more importantly, the antibiotics the patient was exposed to during this time.

Since the first three experimental chapters (Chapters 2, 3 and 4) dealt entirely with the exploration and evaluation of different DNA typing methods on describing diverse bacterial fingerprints from *S. aureus*, Chapter 5 forms the bridge to the following chapters that take the human host into account. Thus, **Chapter 6** 'High anti-staphylococcal antibody titers in patients with epidermolysis bullosa relate to long-term colonization with alternating types of *Staphylococcus aureus*' is the first chapter dealing with a particular patient group and relating bacterial fingerprints with the respective hosts and their immune system. EB is an inherited skin disease with the clinical picture of mostly chronic blisters and wounds. Moreover, colonization of these wounds with the skin commensal *S. aureus* occurs frequently as an unwanted side effect. To what extent these patients are colonized with specific *S. aureus* types and the potential impact on their immune system is described in this chapter. The temporal and spatial diversity of *S. aureus* isolates from a large Dutch EB cohort was examined with the DNA typing methods MLVA and *spa*-typing. The anti-staphylococcal immune response of EB patients was investigated by determining their IgG levels against 43 different *S. aureus* antigens, including various virulence factors.

In **Chapter 7 '**Low anti-staphylococcal IgG responses in granulomatosis with polyangiitis patients despite long-term *Staphylococcus aureus* exposure' a different patient group compared to the previous

chapter was in the focus of investigation. In contrast to EB, GPA is an autoimmune disease and microbial involvement in the onset/progression of several autoimmune diseases has been hypothesized and debated. In the case of PR3-GPA, it was shown that chronic nasal *S. aureus* carriage occurs at a higher frequency compared to healthy individuals and is a risk factor for disease relapse. Based on these observations, the humoral immune responses against a large set of *S. aureus* antigens in relation to the genetic diversity of their nasal *S. aureus* isolates were assessed. The latter was once more investigated with the complementary application of MLVF and *spa*-typing.

The 'Molecular fingerprints of *Staphylococcus aureus* from ANCA-positive vasculitis patients in the Netherlands' described in **Chapter 8**, expands the research from the previous chapter on *S. aureus* collected from GPA patients. This study was aimed at determining a comprehensive picture of the molecular fingerprints of *S. aureus* isolates collected from PR3-ANCA positive patients suffering from GPA and from MPO-ANCA positive patients suffering from any kind of AAV. *S. aureus* isolates of PR3-ANCA positive patients have been under investigation in the past, while information on *S. aureus* isolates colonizing MPO-ANCA positive patients was so far lacking. In addition to the complementary application of MLVF and *spa*-typing, the overall gene repertoire was assessed with a DNA microarray containing 336 DNA probes coding amongst others for virulence factors, antibiotic resistance and adhesion factors. These results are novel in both the microbiology and immunology field addressing the *S. aureus* population of AAV patients trying to identify disease-associated genetic determinants.

Chapter 9 'Genetic diversity of *Staphylococcus aureus* in buruli ulcer patients' reports on *S. aureus* in relation to a third disease. BU is a neglected necrotizing skin disease caused by *M. ulcerans*. This infectious disease occurs mainly in West African countries, such as Benin, Côte d'Ivoire and Ghana. Wounds of these patients are, in addition to *M. ulcerans*, colonized with *S. aureus* and other bacteria. Although the incidence of secondary infections in BU is unknown, antibiotics are prescribed on a regular basis. No molecular or phenotypic information is so far available on microorganisms colonizing the wounds of BU patients, including their antibiotic susceptibility. The study presented in this chapter was therefore aimed at describing the genetic diversity of a large number of *S. aureus* samples from BU patients from Benin with the two complementary DNA typing methods MLVF and *spa*-typing. In addition, antibiotic resistance profiles and the microbial topography of their wounds were examined.

The studies described within Chapters 2-9 of this thesis were mostly focused on determining the genetic diversity of *S. aureus* with respect to lineage/type or patient/carrier group. The research described in **Chapter 10** 'The dynamic changes of dominant clones of *Staphylococcus aureus* causing bloodstream infections in the European region: results of a second structured survey' followed a different approach by investigating both the spatial and temporal changes of *S. aureus* causing bacteraemia in Europe over a five-year interval. Two structured surveys performed in 2006/2007 and 2011 collected MSSA and MRSA bloodstream infection isolates and explored the possibility of integrating *spa*-typing with epidemiological and clinical information at the European level. Sentinel laboratories in participating countries across Europe were selected in a geo-demographic manner and collected the first ten successive MRSA and MSSA isolates during a six-month period. In addition, metadata to the performance of pathogen-based typing was collected.

S. aureus is one of the most-studied opportunistic pathogens threatening public health worldwide and obviously the prime suspect in all preceding chapters of this thesis. Numerous studies and many initiatives worldwide focused on *S. aureus*, most often solely addressing its multidrug resistant form. Meanwhile a new group of highly resistant bacteria has appeared on the horizon. In **Part III** of this thesis**,** *Enterobacteriaeceae*, a large family of Gram-negative bacteria are addressed. The hallmark of the new generation of these pathogens, which represents a serious threat for human health, is its acquired resistance against β-lactam antibiotics, in particular carbapenems. The latter is a group of antibiotics regarded as 'antibiotics of last resort'. With focus on CPE, **Chapter 11** 'Carbapenemase-producing *Enterobacteriaceae* in Europe: a survey among national experts from 39 countries, February 2013' reports on a new project that has the ultimate goal to reveal the current epidemiology of CPEs across Europe, covering a total number of 39 participating countries. The spread of CPEs is a threat to healthcare delivery, although its extent differs substantially from country to country.

Therefore, in February 2013, national experts from across Europe were invited to self-assess the current epidemiological situation of CPE and provide insight into national management of CPE in their country.

At last, the scientific research presented in this thesis is summarized in **Chapter 12** in **Part IV** 'Summary and future perspectives'. This chapter highlights the overall conclusions with focus on future perspectives for further research in the line of capturing bacterial fingerprints across Europe.

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PART 2

‘I always channel my emotions into my work. That way, I don’t hurt anyone but myself.’
Katniss Everdeen (Hunger Games)