THE IMPACT OF GLYCOPEPTIDE- AND METHICILLIN- RESISTANCE ON STAPHYLOCOCCUS AUREUS AND ITS VIRULENCE IN LOCALIZED AND SYSTEMIC INFECTIONS

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ABSTRACT

Adaptation of bacteria to their environment is a necessity to keep their fitness and survival under limiting conditions. In chapter I of this work we demonstrated that adaptation of glycopeptide-resistant S. aureus (GISA) happens at the cost of resistance loss. An in vitro generated step selected teicoplanin-resistant mutant was phenotypically and genotypically characterized and compared to its glycopeptide-susceptible parent S. aureus strain SA113, prior to and after challenging the host in a foreign body infection model. Slower growth, increased thickness of the bacterial cell wall, increased N-acetylglucosamine incorporation, decreased hemolysis, and downregulation of some virulence-associated genes were determined upon in vitro step selection. In vivo host pressure together with the absence of antibiotics were shown to impair this GISA phenotype and to result in the selection of fitter variants at the cost of resistance loss.

In a further study we compared the virulence and pathogenicity of isolates of two epidemic ST8 and ST5 health-care associated methicillin-resistant S. aureus (MRSA) clones with MSSA isolates of variable STs that are prevalent in French clinical settings and with MSSA of the same ST in a murine sepsis model. Mouse mortality, induced by the different groups of MRSA ST8 and ST5 and MSSA ST8, ST5 and var. ST was related to phenotypic and genotypic features. MRSA isolates of both clones ST8 and ST5 caused higher mortality than MSSA expressing var. ST. However, higher mortality was not related to the presence of SCCmec, since only ST5 but not ST8 isolates showed a higher virulence in the presence of this resistance determinant. Despite the same genetic background individual clonal MRSA isolates had heterogeneous virulence properties. No specific virulence factor determined in vitro, was found related to mortality in mice. In conclusion, in a bacteraemic model, lethality varied with the sequence type, and was modulated by SCCmec. Within the same ST, clonality was not associated with a homogenous outcome in the murine sepsis model.
Whole genome sequencing of clonal lethal and non-lethal isolates will allow identifying virulence factors relevant to the outcome in this model.

In the third part of this thesis we investigated phenotypic properties of the clonal MRSA ST8 isolates that had a variable outcome in the murine sepsis model and measured innate and adaptive immune responses of 7 lethal and 3 non-lethal isolates. Clonality of the 10 MRSA ST8 isolates was certified by MLVA. In vitro assays, simulating steps that occur during the course of infection were performed in order to detect host response discriminating differences between lethal and non-lethal isolates. While chemotaxis, bactericidal activity and apoptosis of PMN were similar for lethal and non-lethal isolates, a preliminary study yielded a different specific immune response induced by a lethal compared to a non-lethal isolate.

The role of the adaptive immunity in the outcome of an infection with lethal and non-lethal isolates will be investigated in further.
AIM OF THE STUDY

Since the introduction of the first β-lactam Penicillin in 1944, the emergence and development of antibiotic resistance in *Staphylococcus aureus* has become a severe problem. Antibiotic resistance of this gram-positive bacterium is a particular threat due to its multiple virulence factors, its immune evasion mechanisms and its great adaptive power to antimicrobial agents. The aim of this thesis was to address the impact of glycopeptide and methicillin resistance on *S. aureus*, and to evaluate the role of resistance and the role of host factors on its virulence during an infection.

Glycopeptide intermediate *S. aureus* (GISA) resistance arises as a result of multiple mutations and alterations in gene expression and is associated with phenotypic alterations, which are a fitness burden for the pathogen. However not all GISA isolates are bearing the same alterations, and the resistance-mechanisms underlying this resistance type are still not well understood. Since stability of the resistance phenotype, fitness of GISA strains, and the impact of the host in the absence of antibiotics is unknown *in vivo*, we aimed studying the resistance phenotype prior to and after an infection in the absence of antibiotics in a well defined murine infection model (Chapter I).

With the acquisition of the SCC mec element *S. aureus* became one of the most frequent pathogens responsible for hospital-acquired infections. Most MRSA strains belong to six clonal complexes (CC) that are disseminated worldwide. Patient studies aiming at evaluating differences in pathogenicity and virulence between MRSA and MSSA isolates are impaired by multiple confounding factors; they do not allow the analysis of virulence that is associated with methicillin-resistance. Furthermore, in most patient studies genetic analysis of infectious strains is missing. To eliminate host-associated confounding factors we choose to study the virulence of two epidemic MRSA clones predominating in France in a controlled murine sepsis model. Furthermore by precise molecular characterization of the isolates we aimed assessing the role of factors responsible for virulence and for a successful dissemination (Chapter II).
The outcome of an infection is defined by parameters of both the host and the pathogen. We found a variable virulence among MRSA isolates belonging to the same clone, which was not attributable to any of the measured toxins or adhesins. As we postulated that lethal and non-lethal MRSA isolates would elicit a differing immune response, we investigated innate and adaptive host response to the clonal isolates in vitro and ex vivo. This might allow us to conclude back on factors differing between the isolates (Chapter III).

All projects were focused on the consequence of antibiotic resistance upon virulence in murine infection models. The results revealed a) that glycopeptide resistance was unstable in vivo and imposed a fitness burden b) that in a systemic infection SCCmec variably affected virulence of S. aureus depending on the clonal background, and c) that within clonal isolates unknown factors modulate virulence.
GENERAL INTRODUCTION

Staphylococcus aureus

*Staphylococcus aureus* (*S. aureus*) is a 1μm gram-positive bacterium with a circular chromosome, growing in grape-like clusters and belonging to the family of Micrococcaceae. The *S. aureus* genome ranges in the size from 2.8 - 2.9 Mb and is composed of a core genome and an accessory genome. The core genome is making up approximately 75% and is highly conserved among isolates. The accessory genome represents approximately 25% and consists of mobile genetic elements, like bacteriophages, pathogenicity islands, plasmids and transposons, which often carry genes conferring virulence or resistance (33). The multitude of virulence factors combined with the great adaptive power to evolve and acquire resistance mechanisms declares *S. aureus* to one of the major human pathogens, causing both healthcare and community-acquired infections. Infections caused by this pathogen can be localized, e.g. soft tissue infections and abscesses, toxin-related, e.g. food poisoning and toxic shock syndrome, or systemic and life threatening, e.g. endocarditis and sepsis (55). *S. aureus* is colonizing nose and throat of 20% of the human population persistently, 60% are intermittently colonized, while 20% never carry this pathogen (28). Nasal carriers of *S. aureus* bear an increased risk to become septic, once bacteria gain access to the bloodstream due to breaches in the nasopharyngeal or other mucosal colonized niches. Interestingly, mortality rate from sepsis among carriers is significantly lower compared to non-carriers (60, 63, 64). Colonization and adhesion to host cells are not only the critical events before invasion and subsequent infection of the host, but also the driving force for a successful inter-human dissemination. During the exponential growth phase, *S. aureus* is regulating colonization and dissemination by expressing surface molecules, which are important for adhesion, while during the stationary phase secretion of toxins and enzymes enable tissue disruption and thus invasion (34).
The cell wall of \textit{S. aureus} is one of the major targets of antibiotics. Since the thesis focuses on cell active antibiotics and resistance against them the cell wall is described in depth. Fifty percent of its cell wall is composed of peptidoglycan (PGN), a large polymer consisting of alternating \textit{N}-acetylglucosamine (GlcNAc) and \textit{N}-acetylmuramic acid (MurNAc), which form a dissacharide subunit. The MurNAc subunit carries a stem peptide composed of L-Ala–D-Gln–L-Lys–D-Ala–D-Ala. The sugar subunits are linked together by 1,4-β linkages forming glycan strands, which are in turn crosslinked by pentaglycin brigdes between the L-Lys in the 3\textsuperscript{rd} position of the one stem peptide and the D-Ala in the 4\textsuperscript{th} position of the neighboring stem peptide. Most clinical \textit{S. aureus} isolates are encapsulated expressing either capsule type 5 or 8, which have anti-phagocytic functions (32). Several groups were able to demonstrate that the cell wall of \textit{S. aureus} is not only a biochemical structure that is essential to withstand the cytoplasmic turgor pressure. The composition of PGN, teichoic acids (TA), and lipoproteins (LP) are main stimuli of host-response via TLR2 and Nod2 (52, 56).

**Antibiotic resistance in \textit{S. aureus}**

\textit{S. aureus} has a great adaptive power to antimicrobial agents and this represents a severe problem in infections since there exist meanwhile resistances to all antibiotics, which are available in clinical practice (61). Target pathways of antibiotics are cell wall biosynthesis, transcription, DNA replication, protein synthesis, and biosynthesis of essential-small molecules. Resistance mechanisms are based on one of three strategies:

a) Inactivating the antibiotic.

b) Preventing the antibiotic from reaching the target.

c) Altering the target to render it insensitive to the drug.

These survival strategies are mediated in general either by mutation of a gene or by acquisition of genetic material carrying resistance determinants. A spontaneous mutation frequency of $10^{-8}$ enables the emergence of resistant mutants within the host, even before the first confrontation with the drug takes
place. Thus, once starting treatment with antibiotics, those mutators have a certain advantage to cope with antibiotic pressure (7). The genetic changes may often impair original functions of genes or gene subsets resulting in a fitness cost, which is described as reduced replication rate, decreased survival, reduced transmission rate and reduced virulence (4). However, since natural and clinical environments select for fitter variants, bacteria respond either with reversion of the mutation resulting in the loss of resistance or with a compensation by secondary mutations allowing maintenance of the resistance and with that the fitness (42).

Further factors contributing to resistance against antibiotics are e.g. the formation of biofilm or small colony variants (SCV), mechanisms usually used to protect bacteria from the host defense. The resistance of bacteria within a biofilm is not due to genetic mechanisms as known for their planktonic counterparts, but due to several characteristics of the biofilm itself: e.g. i) the extracellular matrix, which is limiting the diffusion of certain drugs; ii) the waste accumulation and alteration of the microenvironment due to high bacterial density compromising the antimicrobial action; iii) the upregulation of certain destroying enzymes like beta-lactamases; iv) the alteration of growth behavior and v) the development of persister cells, which are not affected by antibiotics, since these drugs act only on growing cells (14, 53). Bacteria further escape of the host response mechanisms by residing within host cells thereby forming often SCVs. They are characterized by a low membrane potential, which makes them resistant to all antibiotics (5).

The development of antibiotic resistance is promoted by the fact that bacteria occur in high numbers, possess a short generation time, and efficient mechanisms for gene exchange, which is additionally favored by the widespread and sometimes inappropriate use of antimicrobial agents (7, 54).

Whenever a new class of antibiotics was introduced onto the market sooner or later resistance against the present drug emerged. Since the introduction of the first antibiotic penicillin more than 60 years ago, there is competition between drug development and the emergence of new resistance mechanisms.
Meanwhile, 12 classes of antibiotics composed of several subgroups were introduced, each acting on a different target of the bacterial cell, trying to combat with resistant strains, each being successful for a certain time but, nevertheless being overcome by new resistance mechanisms acquired or developed by the pathogen. Resistances emerged against all of them: beta-lactams and glycopeptides acting on the cell wall biosynthesis; fluoroquinolones targeting the DNA replication and DNA repair mechanisms; aminoglycosides, tetracyclines, chloramphenicol, macrolides-lincosamides-streptogramin B (MLS), fusidic acid, sulfonamides, trimethoprim, and mupirocin inhibiting protein synthesis.

In the history of antibiotics, the most useful agents treating staphylococcal infections have been the beta-lactam antibiotics with penicillin as prototype. The mode of action of penicillin is explained by the structural resemblance between the drug and the natural substrate of the transpeptidases. Binding of the beta-lactam moiety instead of the PGN precursor to the transpeptidases weakens the crosslinking of the cell wall and results in cell death. The occurrence of uniformly beta-lactam resistant strains, i.e. strains producing beta-lactamases and thus destroying the beta-lactam ring, led to the development of further semi-synthetic beta-lactams and other classes of antibiotics. However the irresistible resistance development awarded glycopeptide antibiotics the prominent role of being the last choice of an effective agent.

**Resistance to beta-lactam antibiotics**

Staphylococcal resistance against beta-lactam antibiotics is based either on the production of penicillinases or on the production of a novel penicillin-binding protein 2a (PBP2a), which are both key-players in the cell wall synthesis in the presence of beta-lactam antibiotics.

Penicillin resistance
Only two years after the introduction of penicillin in the early 1940s, the first penicillin-resistant strains were already detected. Today more than 90% of clinical isolates of *S. aureus* are resistant. Resistance to penicillin is provided by
the penicillinase beta-lactamase. This predominantly extracellular enzyme acts on penicillins by disrupting the beta-lactam ring, which is a common structural feature of this antibiotic class. The transcription of beta-lactamase is tightly regulated by *blaZ* and its adjacent regulatory genes *blaR1-blaI*, which are all located on a large plasmid. Upon exposure of staphylococci to beta-lactam antibiotics the transmembrane beta-lactam sensing protein BlaR1 is autocatalytically cleaved. The cleaved BlaR1 in turn cleaves BlaI the repressor protein of *blaZ* and *blaR1-blaI*. The inactivation of BlaI and its release from the operator region allows the transcription of *blaZ* and its adjacent regulatory genes, resulting in the production of beta-lactamase (27). (Fig.1A)

Methicillin resistance
Methicillin was the first penicillinase-resistant semi-synthetic penicillin introduced into the market in 1960 to treat penicillinase-producing *S. aureus*. Already one year later, the first methicillin-resistant *S. aureus* (MRSA) was isolated. Meanwhile, MRSA is responsible for outbreaks of nosocomial *S. aureus* infections and causes also community-acquired staphylococcal infections mainly occurring in USA. Resistance to this drug results from the acquisition of the staphylococcal chromosome cassette (SCCmec), a chromosomally integrated mobile genetic element composed of two essential gene complexes; the *mec* gene complex harboring *mecA*, a 2 kb gene encoding for the additional 78 kDa PBP2a, which has a low affinity to beta-lactams, and the *ccr* gene complex, harboring *ccrA* and *ccrB* encoding recombinases, which are responsible for the site-specific integration and excision of the entire SCCmec. The integration occurs at the bacterial chromosomal attachment site (attBSCC), which is downstream of *orfX*, an open reading frame of unknown function near the origin of replication. *orfX* is well conserved among clinical strains, being present in both MRSA and MSSA (26). The origin of the mec-element is not clear, but since there is more than 80% homology to a mec-like gene present in *S. sciuri*, an evolutionary relation is likely. However, the native *S. sciuri mecA* gene does not confer methicillin resistance (11).
Five different types of this cassette (SCCmec I-V) have been defined by the specific combination of the *ccr* and the *mec* gene complex. The remaining part of the SCCmec complex is designated Junkyard region (J-region), which carries nonessential components of SCCmec. Based on differences in the J-region SCCmec IV is divided in SCCmec IVa, IVb and IVc. While SCCmec type I, IV and V do not carry any further resistance genes to antibiotics except *mecA*, SCCmec type II and type III MRSA do carry additional resistance determinants and therefore are also known as multi-resistant-methicillin-resistant *S. aureus*. Multi-resistant strains represent a huge problem in terms of infections to treat (23).

PBPs are grouped in class A and class B PBPs that are responsible for the polymerization of the GlcNAc-MurNAc chains (transglycosylation) and the crosslinking of the stem peptides (transpeptidation). Class A PBPs are bifunctional and catalyze both transglycosylation and transpeptidation in cell wall biosynthesis, whereas class B PBPs are monofunctional and have only transpeptidase activity (22). In the presence of antibiotics, the four native staphylococcal PBPs get acylated and inactivated in their transpeptidation function. Due to its low affinity to beta-lactam antibiotics PBP2a takes over the function of the other 4 PBPs in the presence of beta-lactams. However, as belonging to class B PBPs thus having only transpeptidase activity, PBP2a uses the still active transglycosylation site of the native PBP2, a class A PBP, and enables cell wall synthesis (46).

The resistance regulation is similar to that of *blaZ*. The transmembrane protein MecR1 functions as a beta-lactam sensor and is autocatalytically cleaved upon exposure to beta-lactams. Subsequently, the inhibitor Mecl gets inactivated, which in turn enables the transcription of *mecA* and thus the synthesis of PBP2a. Due to a sequence homology of *mecl-mecR1* with *blaZ-blaR1*, *mecA* is under dual control. The repressors Mecl and Blal are interchangeable, so that Blal can also repress *mecA*. (Fig. 1B)
Figure 1: Resistance regulation to beta-lactam antibiotics
Exposure to beta-lactam antibiotics leads to the autocatalytic cleavage of the transmembrane sensor protein BlaR1/MecR1 and to the subsequent inactivation of DNA binding protein BlaI/MecI. This results either in induction of beta-lactamase synthesis (A) or induction of PBP2a synthesis (B).
**Resistance to glycopeptide antibiotics**

Vancomycin, the first glycopeptide antibiotic, was introduced into clinical practice in the 1950s, after it was discovered as a second metabolite of the soil bacterium *Streptomyces orientalis*. Despite its efficacy in gram-positive infections, its use remained limited because of the low purity of the first lots of vancomycin, and the introduction of second and third generation beta-lactams like methicillin. However, with the increasing emergence of multiresistant MRSA glycopeptide antibiotics became the last resort for treatment of infections.

Teicoplanin, another important member of the glycopeptide family, extracted from *Actinoplanes teichomyceticus* came onto the market 30 years after the introduction of vancomycin and is still available for clinical use in Europe only. Even with the development of semi-synthetic derivatives, vancomycin and teicoplanin are still the most commonly used drugs for treatment of serious infections caused by MRSA.

Glycopeptide antibiotics act by inhibiting the extracellular steps of the peptidoglycan (PGN) biosynthesis of the cell wall. During the cell wall formation PGN precursor molecules are translocated from the cytoplasm to the extracellular face of the cytoplasmic membrane. At the outer face they are immediately polymerized by transglycosylases to build long glycan strands and in further steps crosslinked by transpeptidases to build a dense network. Glycopeptides inhibit the cell wall reticulation by building a non-covalent complex via five hydrogen bonds with the free D-Ala-D-Ala dipeptides of the uncross-linked PGN pentapeptides. This complex formation prohibits further transpeptidation and transglycosylation steps by steric hindrance, where the responsible enzymes cannot interact appropriately with their substrates. The result is a loose and weakened cell wall leading to the death of the bacterium (47).

Some glycopeptides, e.g. vancomycin, enhance their affinity and with that their bactericidal activity to PGN components by forming homodimers. Others, like teicoplanin, in turn enhance their bactericidal activity by anchoring into the membrane via a fatty acid moiety, which facilitates capturing the precursor
molecule right at the site of reticulation (6). Glycopeptides are large and need direct access to their target. Therefore, glycopeptides act specifically on gram-positive bacteria that have a surface exposed and thus easily accessible PGN. In contrast, gram-negative bacteria have PGN covered with an outer lipopolysaccharide layer, that renders them insensitive to these antibiotics. The first resistance against vancomycin was described in Enterococcus faecium in 1986, wherefrom the term vancomycin-resistant Enterococcus (VRE) (31) is derived. The resistance results from an alteration of the drug target, where the D-Ala-D-Ala dipeptides are replaced by D-Ala-D-Lac or D-Ala-D-Ser (21). As described before, the complex formation between drug and the PGN precursor molecule is formed via five hydrogen bonds. Since one of the hydrogen bond donors is the amide hydrogen in the D-Ala-D-Ala dipeptide, the replacement of the terminal D-Ala with D-Lac leads to a 1000-fold decrease in binding affinity. The replacement with D-Ser does not suppress hydrogen bonds but induces conformational changes, thereby reducing the affinity of the drug slightly (8, 21). The so-called van genes, which are located on the transposable element Tn1546, are responsible for the alterations in the PGN structure. Six phenotypes (vanA-vanG) are known, each consisting of a group of genes contributing to resistance. The vanA gene cluster was the first being described and consists of a) vanR and vanS, encoding a two-component regulatory system (3), b) vanH encoding an alpha-keto acid reductase that generates the D-isomer of lactate (2, 8), c) vanA encoding an altered D-Ala-D-Ala ligase, which generates the desipeptide D-Ala-D-Lact (8), d) vanX encoding a metallodipeptidase that degrades D-Ala-D-Ala (48), e) vanY encoding a D,D-carboxypeptidase that removes D-Ala from growing PGN precursor molecules (67), and f) vanZ encoding a protein that seems to play a role in teicoplanin resistance (1) (Fig. 2A + B). The expression of the resistance genes is tightly regulated by the two-component regulatory system VanR/VanS. In the presence of glycopeptides, the membrane bound sensor His-kinase VanS and the response regulator VanR, which are located upstream from vanH, upregulate the expression of the vanHAX and vanYZ genes. The autophosphorylation of VanS leads to the phosphoryl
transfer to VanR, which in turn binds to the promoter region of vanH and induces the transcription of the vanHAX genes. In the absence of glycopeptides, VanS dephosphorylates VanR and thus controls gene expression (3). Resistance in S. aureus towards glycopeptides emerged later compared to Enterococci. 1992 Noble et al. demonstrated for the first time that van resistance from Enterococcus faecalis was transferable to S. aureus by conjugation in vitro (43). While the first teicoplanin-resistant Enterococcus strain was already described in 1990, the first S. aureus strain, which showed an elevated level towards vancomycin, was isolated in Japan from a patient with a MRSA infection treated with vancomycin in 1996. But this first resistance was not due to the van resistance genes, as it was known from Enterococci and thus was classified as glycopeptide intermediate S. aureus (GISA). In 2002 the first highly glycopeptide-resistant S. aureus (GRSA) with a van-resistance mechanism acquired from Enterococci outside the laboratories, was isolated from a patient, who had a co-infection with MRSA and E. faecalis (9). Since S. aureus is a more virulent pathogen than E. faecalis, the resistance in S. aureus is much more threatening than the resistance in E. faecalis.

Depending on the Clinical and Laboratory Standards Institute (former NCCLS) the MIC towards vancomycin for glycopeptide susceptible S. aureus (GSSA) is ≤ 4 μg/ml, for GISA 4-16 μg/ml, and for GRSA ≥ 32 μg/ml. In addition to GISA and GRSA, there are also hetero GISA (hGISA) strains, which are primarily susceptible to vancomycin, but contain subpopulations having intermediate MIC levels (24).

The molecular resistance mechanisms, which underlie GISA, are still not well understood. However, certain phenotypic characteristics, like cell wall thickening, increased PGN synthesis with decreased PGN crosslinking, increased incorporation of GlcNAc, altered PBP expression, longer doubling times, increased adherence to artificial surfaces, and reduced autolytic activities are shared by most of the GISA strains (12, 13, 37, 66). The increased production of PGN precursors and the decreased crosslinking leads to an abundance of free D-Ala-D-Ala termini, which can be bound by glycopeptide antibiotics. The excess
of free binding sites leads to unproductive binding effects, i.e. the glycopeptides are all trapped in the outer layers of the cell wall and are thus prevented from reaching the cell membrane, where they can directly block the reticulation steps of cell wall formation. The fact that during the normal crosslinking process in S. aureus, only every 2\textsuperscript{nd} bond is cleaved, leading to a certain number of free D-Ala-D-Ala binding sites in a highly crosslinked cell wall, indicates that the cell wall constitution of S. aureus per se, is promoting resistance (20).

\textbf{Figure 2: Van-resistance regulation to glycopeptide antibiotics}
A) Arrangement of \textit{van} genes located on \textit{Tn1546} responsible for resistance
B) \textit{van} gene dependent resistance regulation. Ddl = D-Ala-D-Ala ligase, adapted and modified from (21)
Typing, evolution, and clonality of MRSA

The dissemination of MRSA in Europe is characterized by a north-south gradient, with being rare in Scandinavian countries and highly prevalent in Southern Europe (51). To study the epidemiology and the clonal spread of MRSA, different molecular typing techniques are used, among them pulse-field gel electrophoresis (PFGE), typing of the variable tandem repeat region of protein A (spa typing), multilocus sequence typing (MLST), SCCmec typing or ‘multilocus variable number of tandem repeat analysis’ (MLVA). PFGE is based on Smal digestion of bacterial DNA and separation of the DNA fragments according to their size by changing the orientation of the electric field periodically (39, 40). Spa-typing is based on the sequence typing of a region, which consists out of 24 bp repeats and is known as the polymorphic X region of protein A and has proved its discriminatory use in S. aureus outbreaks and global population studies (19, 29). MLST was originally developed to identify hypervirulent lineages of Neisseria meningitidis. It was first applied to S. aureus in 2000 and is based on the sequence analysis of around 450-bp internal fragments of seven housekeeping genes, which are essential in a given species. For each gene fragment different sequences are assigned to distinct alleles (e.g. 3-3-1-1-4-4-16), which can be compared with already known alleles at the MLST website (http://www.mlst.net). Thus, the allelic profile of each isolate is characteristic for its sequence type (ST). Since there are seven loci with an average of 42 alleles present per locus, $42^7$ (more than 200 billion) different allelic profiles and with that as much as different strains are possible, making it highly improbable that two unrelated genotypes have the same ST (15, 35). Depending on their allelic profile, isolates are subdivided in clonal complexes (CC). When at least five of seven housekeeping gene alleles are in common with one other member, they belong to the same CC and when they have the exact same allelic profile, they belong to the same ST, which demonstrates that MLST has an enormous discriminatory value (16). SCCmec typing is either based on PCR, amplifying parts of the mec gene complex and the ccr genes or based on multiplex PCR detecting mecA and either different loci or a single locus on SCCmec (25, 45,
MLVA is based on the amplification of variable number of tandem repeats (VNTR)-containing genes that are found at single genetic loci within the genome. This typing method has high discriminatory power within clonal species. VNTRs are rapidly evolving short nucleotide sequences (in our assay VNTR length varies from 9-500 nucleotides) that are repeated (multiple times) and often vary in their copy numbers between strains from various origins. Variation of repeat number relies on aberrations during chromosome replication, which is relatively frequent in these regions. They have a high mutation rate that leads to the creation of length polymorphisms of alleles at the same locus. By designing PCR primers in conserved VNTR-flanking regions, these differences are detectable by PCR, yielding to different size fragments. The mutation rate of VNTRs is important to determine genetic relatedness of a collection of isolates (18, 58). The first MLVA methodology was developed in 1997 based on *Haemophilus influenzae* genome sequence and intrinsic repeat variabilities (57). The technique was first described for typing MRSA in 2003 and was optimized in 2005 in terms of cost- and labor-saving and discriminatory abilities (18, 50).

There are two theories regarding the evolution of MRSA strains. One is supporting the aspect that all MRSA clones are descendants of one *S. aureus*, which acquired SCCmec on one occasion, and the other theory is supporting the aspect that SCCmec was introduced several times into several lineages of *S. aureus* (30, 41). With the above-mentioned molecular typing methods, it was possible to reveal the existence of MRSA lineages, which are not related to the very first detected MRSA (ST8), thus proving the latter theory. It is also evident by the presence of different SCCmec types in isolates with the same ST, that MSSA has become MRSA on more than one occasion. Indeed, MLST studies revealed that all epidemic hospital MRSA isolates belong to six major lineages or clonal complexes (CC8, CC5, CC45, CC30, CC22, CC1) (10). The fact that these major MRSA lineages have developed from successful MSSA strains by the horizontal transfer of SCCmec and the fact that within the very same successful MRSA lineages already some became less responsive to glycopeptides is alarming, since glycopeptides are the antibiotics of last resort.
Evasion of *S. aureus* from host response: an overview

The first line of defense against bacteria invading the host is provided by the innate immune system, with polymorphonuclear leukocytes (PMN) being the key players. PMN are terminally differentiated cells, descending from pluripotent haematopoetic stem cells containing multi-lobed nuclei and a granular cytoplasm. PMN have a short half-life of about 12 hours and usually circulate in the blood unless they are recruited to sites of infection by chemoattractants to phagocytose and kill bacteria. Intracellular killing by PMN occurs due to reactive oxygen species (ROS), proteolytic enzymes, and antimicrobial proteins. Extracellular killing is caused by degranulation of PMN and the release of antimicrobial peptides and enzymes into the extracellular medium (36). Factors of the complement system function as chemoattractants and promote the recruitment of phagocytic cells to the sites of infection. Furthermore, complement C3b and Fc fragments of Ig opsonize bacteria and allow their phagocytosis by PMN and macrophages (17). In fact, cells of the monocytic lineage, i.e. monocytes and macrophages also play a very important role, since they are the main source of pro-inflammatory cytokines during the initial stage of infection. Macrophages and Dendritic cells (DCs) are professional antigen presenting cells (APCs), which take up bacteria or bacterial components in the tissue. DCs transport them to lymphoid organs and present them there to lymphocytes via a MHCII-antigen complex. Recognition of the bacterium leading to the induction of an inflammation occurs via pattern recognition receptors (PRRs) present on APCs and PMN, and pathogen associated molecular patterns (PAMPs) present on bacterial cells. B and T lymphocytes are the key players of the adaptive immune response. While naïve CD4 T cells, also known as T helper cells, need to be primed by DCs before proliferation and subsequent activation of B cells or macrophages, naïve B cells don't need DCs necessarily to be primed for proliferation. They either get activated by macrophages presenting antigens or take up and process pathogens themselves. In the course of infection with extracellular bacteria, the protective immunity is clearly centered on the B cell...
response, i.e. the secretion of specific antibodies (induced by T helper cells), which favor opsonization and phagocytosis of bacteria. In the early phase of an infection, when pathogen-specific antibodies are not yet produced, ‘natural occurring’ antibodies that can cross-react with many antigens provide the first support of humoral defense.

*S. aureus* has evolved a multitude of immune evasion mechanisms to escape host defense, wherever it is possible. Thus it becomes clear, why *S. aureus* has such a high pathogenicity, once it invades the host.

This will be demonstrated in the following scenario (Fig. 4):

Once *S. aureus* is entering the blood stream, a great proportion is transported by the blood flow to lymphoid organs, especially to the spleen, where they come across lymphocytes. Some of them encounter monocytes and PMN, which are circulating in the blood and which upon contact or upon phagocytosis of bacteria secrete pro-inflammatory cytokines like IL-1, IL-6, and TNFα, and chemokines like MIP-2 and KC. The secretion of bacteria-derived (e.g. N-formyl peptides) and host-derived (chemokines, complement facors) molecules results in the recruitment of further host cells. The expression of so-called adhesins, like fibrinogen-binding (ClfA/ClfB), fibronectin-binding proteins (FnBPA/FnBPB) and collagen binding protein (Cna) on the bacterial surface and the expression of extracellular matrix components on endothelial cells enable the adhesion and subsequent endocytosis of bacteria (44). These incidents induce the further secretion of pro-inflammatory cytokines, chemokines, and the expression of cell adhesion molecules like VCAMs and ICAMs and result finally in micro-environmental changes including vasodilatation. Vasodilatation or active disruption of endothelial cell membranes by proteolytic enzymes, enable bacteria to penetrate through and enter the parenchyma. There, taken up by macrophages, they induce the release of chemoattractants and with that the further recruitment of PMN and macrophages into sub-endothelial tissues. After two days post infection, the adaptive immune response participates in this scenario. DCs present pathogen-derived antigens to naïve T cells and prime them for the activation by further APCs. Primed T cells proliferate and in turn
activate primed B cells for the secretion of antibodies and macrophages to enhance their killing machinery. Priming of B cells usually happens when they encounter bacteria in lymphoid organs. There, pathogen-derived peptides are presented either by resident APCs, which have taken up and processed bacteria, or by APCs, which have taken up bacteria in the periphery and brought them to lymphoid organs.

To evade defensins, secreted by PMN and epithelial cells, *S. aureus* releases certain molecules, which inactivate the functions of these antimicrobial molecules by cleaving them either directly e.g. aurolysin, or indirectly, e.g. staphylokinase. To escape ROS attack, *S. aureus* produces catalases, which inactivate toxic hydrogen peroxides. Moreover, *S. aureus* is able to induce apoptosis of host cells actively, not only to escape the intracellular attack, but also to disseminate thereby throughout the host. Apoptosis of different host cell types can be caused by toxin production and/or by the activation of cellular caspases (38, 62, 65). The majority of *S. aureus* strains produces a polysaccharide capsule that functions as a cloak of opsonins and reduces the opsonophagocytosis by PMN. Moreover the presence of polysaccharide capsules complicates the binding of IgGs that enhance the opsonophagocytic uptake of bacteria. Protein A, another important molecule contributing to immune evasion is blocks IgG-Fc receptor mediated phagocytosis and complement activation by binding to the Fc-part of the immunoglobulin. Direct attack of complement and with that direct lysis and impaired deposition of immune defense molecules on the surface of *S. aureus* cells, is prevented by the thick cell wall, the polysaccharide capsule and biofilm formation. The staphylococcal complement inhibitor (SCIN) is able to block all the complement pathways by stabilizing convertase complexes, to prevent their degradation and thus their activity (49). Another mechanism to evade host attack is to hide intracellular. For a long time, *S. aureus* was considered to be an extracellular pathogen, but meanwhile it could be demonstrated that it is able to hide and grow within endothelial cells, forming small colony variants, resulting in recurrent infections (59).
Fig. 4: Model for innate and adaptive immune response during S. aureus sepsis
In blood S. aureus encounters host phagocytes including monocytes and PMN and is taken up. Binding and phagocytosis induces the release of pro-inflammatory cytokines and chemokines. The blood-flow takes a great proportion of entering bacteria directly to lymphoid organs (e.g. spleen), where they encounter further phagocytes and lymphocytes. To attach to extracellular matrix molecules (ECM) of endothelial cells, bacteria bind host fibronectin by fibronectin binding protein A or B (FnBP-A/FnBP-B), or fibrinogen by clumping factor A (ClfA) and use it as bridging-molecule to the endothelial layer. Upon attachment, they get endocytosed and induce the release of further pro-inflammatory cytokines and a general micro-environmental change including vasodilatation. Hereby and by secretion of proteolytic enzymes, they penetrate through the endothelial layer to sub-endothelial tissue. Taken up by recruited PMNs they may be killed or may induce apoptosis and evade. The adaptive immune response gets activated, when DCs prime naïve Th cells by Ag-presentation; primed Th cells in turn activate primed B cells to secrete antibodies. These pathogen-specific antibodies opsonize bacteria and enhance thereby phagocytosis by macrophages (Mph) and PMN.


Chapter I

IN VIVO SURVIVAL OF TEICOPLAIN-RESISTANT STAPHYLOCOCCUS AUREUS AND FITNESS COST OF TEICOPLANIN RESISTANCE

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ABSTRACT

Glycopeptide resistance, in a set of in vitro step-selected teicoplanin resistant mutants derived from susceptible Staphylococcus aureus SA113, was associated with slower growth, thickening of the bacterial cell wall, increased N-acetylglucosamine incorporation, and decreased hemolysis. Differential transcriptome analysis showed that as resistance increased, some virulence-associated genes became downregulated. In a mouse tissue cage infection model, an inoculum of $10^4$ colony forming units (CFU) of strain SA113 rapidly produced a high bacterial load infection, which triggered MIP-2 release, leukocyte infiltration and reduced leukocyte viability. In contrast, with the same inoculum of the isogenic glycopeptide-resistant derivative NM67, CFUs initially decreased, resulting in the elimination of the mutant in three out of seven cages. In the four cages in which NM67 survived, it partially regained wild-type characteristics, including thinning of the cell wall, reduced N-acetylglucosamine uptake and increased hemolysis, however, the survivors also became teicoplanin hypersusceptible. The elimination of the teicoplanin-resistant mutants and selection of teicoplanin hypersusceptible survivors in the tissue cages indicated that glycopeptide resistance imposes a fitness burden on S. aureus and is selected against in vivo; with restoration of fitness incurring the price of resistance loss.
INTRODUCTION

Over 15 years ago, the first clinical methicillin resistant Staphylococcus aureus (MRSA) isolates with decreased susceptibility to the glycopeptide antibiotic teicoplanin were described (27). In the interceding years, glycopeptide intermediate resistant S. aureus (GISA) isolates have been recovered from most parts of the world (reviewed in 24, 58). GISA is the general term used to describe strains with intermediate glycopeptide MICs ranging from 4-16 µg/ml that are usually isolated from patients after prolonged glycopeptide exposure (54). Resistance arises intrinsically upon glycopeptide exposure, as the result of multiple mutations and/or alterations in gene expression (47, 49, 51). Several of the clinical and laboratory GISA described share phenotypic similarities; most commonly a modification of the cell wall, reducing the amount of glycopeptide able to reach its target at the cell membrane (13, 14, 23, 24, 50). Common GISA features include cell wall thickening, decreased peptidoglycan crosslinking, decreased growth rate and hemolysis, alterations in rates of autolysis and changes in the structure and/or abundance of cell wall teichoic acids (7-9, 13, 41, 42, 49, 50). However, there is still little known about the genetic basis of this phenotype and there is no universal genetic marker typical for all GISA isolates. Certain genetic observations have been frequently documented in both clinical and laboratory derived GISA, such as increased PBP2 and decreased PBP4 expression (10, 23, 38, 43, 48), which are associated with cell wall modifications leading to increased cell wall synthesis and decreased peptidoglycan crosslinking. However, this observation is not true for all GISA strains and these changes in penicillin binding protein abundance are not sufficient in themselves to create a GISA phenotype (23). Several genetic alterations have been shown to only contribute to increased resistance in a single or restricted number of strains, including: overexpression of the global regulator SigB (2, 51) and the two component sensor transducer VraSR (32); defective agr function (36, 46); alterations in the expression of genes encoding autolysins or effecting autolytic function (7, 8, 28, 41, 49) and genes involved in carbohydrate metabolism and cell wall synthesis (29); inactivation of tcaA, a membrane protein of unknown
function (34); and inactivation of \( mprF \) (fmtC), a membrane protein involved in the biosynthesis of the positively charged cell membrane lipid lysylphosphatidylglycerol, was found to increase resistance levels in glycopeptide susceptible strains, but to decrease resistance in GISA (39, 45).

The limited number of global transcriptome analyses performed, have indicated that multiple mutational and regulatory events are causing numerous metabolic changes in GISA (12, 33, 37). The full genetic basis of GISA formation has not been elucidated in any strain, but it appears that there are likely to be several different, as yet undiscovered, loci and pathways involved in resistance formation.

Increased production of peptidoglycan, required to facilitate the construction of a thicker cell wall, is an energetically unfavorable phenotype, as evidenced by the slower growth rate of GISA strains, and is probably the main reason that GISA strains are not isolated more frequently (13, 35, 49). This also contributes to the reported instability of the resistance phenotype in the absence of selection pressure (6, 13). Reversion from a GISA to a GSSA phenotype could be driven by a combination of the reversal of regulatory events and forward mutations, which lead to reclaimed fitness at the cost of decreased resistance. It is speculated that in the absence of drug pressure, mutants with greater fitness emerge spontaneously and dominate the population (13).

The fitness of \( S. \) *aureus* cells inside a host is governed by intrinsic properties such as the rate at which they reproduce and the rate at which they are cleared by host defense mechanisms. Since the fitness of glycopeptide resistant strains in infected hosts is unknown, we chose to investigate the \( in \) \( vivo \) behavior of a teicoplanin resistant strain in a defined murine infection model. The tissue cage model was first described and extensively characterized in the guinea pig (62) and then adapted to the mouse (31). Low inocula, \( 10^3 \) colony forming units (CFU), of \( S. \) *aureus* cause a persistent local infection, which never becomes systemic. The absence of vascularization limits serum factors and the presence of leukocytes, which are attracted by the polymer implant before infection and show weak functional capacity (61), contribute to pathogenesis in this model.
Persistence is facilitated by progressive leukocyte apoptosis and necrosis (30). This model accurately mimics orthopedic implant infections. Because bacteria are inoculated directly into the cage, with no adherence and invasion step through epithelia, the minimal infective dose of staphylococci which is required for a persistent infection reflects virulence. Accordingly, virulence is dependent on the resistance of S. aureus to extracellular phagocyte-dependent killing in the immunocompetent host. Therefore this model differentiates S. aureus strains that have altered susceptibilities to bactericidal mechanisms. The host response is mediated exclusively by phagocytes and comprises defensins, reactive oxygen species, cytokines, chemokines, leukocyte infiltration and apoptosis. S. aureus SA113 was passaged several times on teicoplanin to obtain an isogenic mutant with intrinsically acquired glycopeptide resistance. The stability of the resistance phenotype and accompanying cell wall characteristics were monitored in vitro, prior to and during infection in a mouse tissue-cage infection model, to assess the impact of the GISA phenotype on in vivo survival. A transcriptome comparison of the mutant and wildtype was performed, and the transcription of selected differentially regulated genes was profiled in vitro and from tissue cage isolates.
MATERIALS AND METHODS

Bacterial strains and culture conditions. Strains were routinely cultured at 37°C on sheep blood agar or in brain heart infusion (BHI) broth (BBL™ Becton Dickinson, MD. USA) and stored as frozen stocks in skim milk at -80°C. A series of isogenic strains with increasing glycopeptide resistance levels was generated from susceptible S. aureus strain SA113 (26), by plating dilutions of an overnight culture on BHI agar containing increasing concentrations of teicoplanin. Ten single colonies growing at the highest concentration were selected and subcultured twice on nonselective agar to ensure the stability of the phenotype. Their resistance levels were compared to the susceptible parent on teicoplanin gradient plates. The strain with the highest increase in teicoplanin resistance, 1\(^{st}\) step mutant NM18, was then used to repeat the selection and to obtain the most resistant 2\(^{nd}\) step mutant NM30; which was used to obtain a 3\(^{rd}\) step mutant NM67. To ensure clonality was maintained, all strains were tested by pulsed field gel electrophoresis of Smal-digested chromosomal DNA according to the protocol of Wada (57). Growth rate experiments were performed as previously described (18).

For measurement of N-acetylglucosamine uptake and release, HPLC, autolysis and transmission electron microscopy on cells recovered from tissue cages, 50 µl of tissue cage fluid (TCF) were centrifuged at 4000 rpm for five minutes. The pellet was resuspended in BHI medium and grown overnight at 37°C. The overnight culture was pelleted and transferred into cryovials containing beads in cryopreservative for storage (Pro Lab Microbank Bacterial Preservation System Green, Basel, CH). After inverting vials 4-5 times, cryopreservative was aspirated and the beads were stored at -70°C.

Stability of resistant mutants in vitro. The stability of the teicoplanin resistance phenotype was monitored under in vitro culture conditions. Twenty ml BHI cultures of SA113 and NM67 were either subjected to prolonged stationary culture conditions at 37°C for 8 days, or subcultured daily in 20 ml of fresh broth, using a dilution factor of 10\(^4\), over the 8 day period.
**Resistance tests.** Minimal inhibitory concentrations (MIC) of glycopeptides were determined by E-test (AB-Biodisk, Solna, Sweden) on brain heart infusion (BHI, BBL™) plates with an inoculum of 2 McFarland standard and incubation at 37°C for 48 h. Lysostaphin MICs were determined by broth microdilution in BHI as recommended by the CLSI/NCCLS (25). For population analysis profiles, dilutions of an overnight culture were spread on BHI agar plates containing increasing concentrations of teicoplanin. For population analyses of strains ex vivo, 50 µl of TCF was grown overnight and then adjusted to McFarland 0.5 before plating. The CFU were determined after 48 h incubation at 37°C.

**Hemolysis assays.** Hemolytic activities were compared on sheep blood agar plates. Wells were stamped out of the agar and filled with 100 µl of filtered supernatant, from cultures grown in BHI for 24 h. Plates were incubated overnight at 37°C.

**Tissue cage model.** Male C57BL/6 mice (12–16 weeks old) were anesthetized and sterile cylindrical teflon tissue cages were implanted subcutaneously in their backs, as described previously (31). Two weeks after surgery, sterility of tissue cages was verified, and 200 µl of a suspension from stationary overnight cultures containing 10⁴ CFU, were injected percutaneously. Mice never developed bacteremia and showed no weight change during 8 days of infection.

Mice were anesthetized after days 1, 2, 5 and 8 of infection, and TCF samples of 150 µl were collected by percutaneous aspiration and transferred into tubes containing 15 µl of 1.5% EDTA. The load of planktonic bacteria in TCF was determined by serial dilutions of the samples on BHI agar plates. Leukocytes from TCF were quantified with a Coulter counter. The percentage of viable leukocytes was assessed by trypan blue exclusion. MIP-2 was determined by sandwich ELISA (R&D Systems, Minneapolis). Mice were kept under specific pathogenfree conditions in the Animal House of the Department of Research, University Hospital Basel, and animal experimentation guidelines were followed according to the regulations of Swiss veterinary law.
Statistics. Median and lowest quartiles were calculated from each group of strains on all days of investigation. ANOVA for repeated measures, with Scheffé posthoc tests, was used to compare SA113 and NM67 during infection.

Sampling, RNA isolation, and transcriptional profiling. Differential microarray analysis of the transcriptomes of SA113 and NM67 was carried out on exponentially growing cultures in Luria-Bertani (LB), harvested at an OD$_{600}$ of 2, as previously described (3).

RNA extraction and Northern hybridisation. RNA isolation from bacteria grown in shaking LB cultures and Northern blots were performed as described earlier (34). Ten micrograms of total RNA from each sample were separated through a 1.5% agarose-20 mM guanidine thiocyanate gel in 1 x TBE running buffer (21). ORFs selected for probe amplification and the primers used, are shown in Table 1. All Northerns were performed at least twice on independently isolated RNA samples.

Incorporation and release of N-acetylglucosamine. The incorporation of $^{14}$C-labelled N-acetylglucosamine (Amersham, USA) into cells in resting medium and the release of $^{14}$C-labelled N-acetylglucosamine into the culture medium were measured essentially as described by Hanaki (23), except that the cells were pregrown in BHI.

Cell wall composition. Cell walls were purified from bacteria grown to exponential or stationary phase in BHI broth. Teichoic acids were removed from the cell walls by gently mixing the cell wall suspension in hydrofluoric acid (49%) for 48 h at 4°C. The peptidoglycan was recovered, digested with muramidase (Sigma), reduced, and the muropeptides were separated by HPLC as described by de Jonge et al. (17). Eluted material was detected by its absorbance at 210 nm and principal peaks identified using muropeptide peak libraries from S. aureus COL (5). The degree of muropeptide cross-linking was calculated by
quantification of the relative amounts of monomers, dimers, trimers, and oligomers in the muropeptide digest according to Snowden et al. (52).

**Spontaneous autolysis.** Cells grown to an OD$_{600}$ of 0.7 in BHI were pelleted, washed once with saline and resuspended in 0.01 M sodium phosphate buffer pH 7.0, to an OD$_{600}$ of 0.8. The cell suspension was incubated at 37°C with continuous gentle shaking. Decrease in OD$_{600}$ was monitored every hour for 5 h.

**Zymography.** Exponentially growing cells, cultured in BHI, were harvested at an OD$_{600}$ of 1.0. Autolysins were extracted and analysed according to the method described by Hanaki et al. (23), using cell-wall extracts of *S. aureus* SA113 as the substrate. Band intensities were scanned and quantified. Transmission electron microscopy. Cells grown overnight were processed for thin-sectioning electron microscopy as described by Pante et al. (40). Electron micrographs were recorded with a Zeiss LEO 910 transmission electron microscope.
### Table 1. Primers used in this study

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<th>Gene/orf$^a$</th>
<th>Primer</th>
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<td>spa R</td>
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<td>SA0184 F</td>
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<td></td>
<td>SA0184 R</td>
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<td><em>opp-1A</em> (SA2255)</td>
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$^a$ Indicates the specific gene or open reading frame (orf) for which the primer was designed.
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<th>Primer 2</th>
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RESULTS

Phenotype and in vitro stability of the teicoplanin resistant mutants. A series of three mutants, NM18, NM30 and NM67, with sequentially increasing teicoplanin MICs of 16, 48 and 64 μg/ml, respectively, were generated from the susceptible parent strain SA113 (teicoplanin MIC 3 μg/ml). They showed heterogeneous resistance profiles on teicoplanin, segregating subpopulations resistant to higher concentrations of the drug (Fig. 1). Parallel to the increasing teicoplanin resistance, the vancomycin MIC rose from 4 μg/ml in SA113 to 24 μg/ml in NM67; the minimum doubling time increased from 25.7 ± 1.7 min in SA113 to 37.6 ± 1.4 min in NM67; and hemolysin production, which was clearly visible in SA113, was undetectable in NM67 (refer to Fig. 6B). Initial studies demonstrated that NM67 incorporated, within 120 min, approximately 2.7 times \((n = 3, p < 0.05)\) more \(^{14}\text{C}\)-labelled \(N\)-acetylglucosamine than SA113, a feature of many clinical GISA isolates which is associated with increased cell wall thickness \((20, 23)\). There were no significant differences between SA113 and NM67 in their release rates of \(^{14}\text{C}\)-labelled \(N\)-acetylglucosamine into the culture supernatant (data not shown). The cells of the teicoplanin-resistant strain NM67 were significantly smaller, but their cell wall was significantly thicker (cytoplasm diameter 797.4 ± 21.7 nm; cell wall thickness 54.9 ± 2.3 nm), compared with parent SA113 (961.1 ± 38.3 nm, \(p < 0.001\); 47.3 ± 2.2 nm, \(p < 0.05\)). Muropeptide analysis showed that the degree of peptidoglycan crosslinking in SA113 (71.3 \%) and NM67 (70.9 \%) did not differ significantly.

Glycopeptide resistance phenotypes were stable upon storage of the strains at -80°C and through several subcultures on drug free sheep blood agar plates. However, upon prolonged incubation or repeated daily subculture, over 8 days in liquid culture, decreases in teicoplanin resistance occurred. Loss of resistance was very stochastic with repeated experiments giving widely varying results. After 8 days, resistance had always decreased, with MICs ranging from 6 to 24 mg/ml (data not shown).
Figure 1. Resistance profiles of teicoplanin-selected mutants. Population analysis profile of: filled circle, susceptible strain SA113; filled diamond, 1\textsuperscript{st} step mutant NM18; filled square, 2\textsuperscript{nd} step mutant NM3; and filled triangle, 3\textsuperscript{rd} step mutant NM67.

**Downregulation of virulence-associated genes in NM67.** Transcriptional differences were evaluated between the teicoplanin resistant mutant NM67 and its susceptible parent SA113, in the mid exponential growth phase. In the mutant, there were no genes upregulated by three-fold or more over the parent, despite the relevant increase in cell wall thickness and N-acetylglucosamine uptake. In contrast, 26 ORFs were downregulated more than three-fold in NM67 (Table 2). Downregulated ORFs comprised 12 loci, consisting of seven single gene loci and five multi-gene clusters, most of which are likely to play a role in virulence. These included, lipase (geh), alpha-hemolysin precursor (SA1007), nuclease (nuc), and the secretory antigen (SA2097), which is similar to the secretory antigen precursor ssaA (1); protein A (spa) and IgG-binding surface sprotein (sbi) which
are involved in the evasion of host defenses (60); betA and gbsA playing a role in osmoprotection (4); the icaA operon which is linked to biofilm formation and increased virulence in mouse tissue cage infections (19); and the large operon SA0112-SA120 which is involved in siderophore biosynthesis (15).

Northern blots of RNA harvested in early exponential- and mid-exponential growth phase substantiated the microarray results and in some cases showed at which step during the three step-selection process transcription had decreased. Transcription of spa and opp-1A was already downregulated after the 1st selection step in NM18, the transcript abundance of sbi was slightly lower in the 1st step mutant but decreased more significantly after the 2nd selection step in NM30, while transcription of SA1007 and geh was only downregulated after the 2nd selection step (Fig. 2A). The transcription levels of these five genes correlated with the microarray results, confirming the overall validity of the microarrays. Four of the probes, SA0112, icaA, gbsA and SA0184 produced unreadable Northerns (data not shown), most likely due the large size of polycistronic transcripts, but possibly also due to poor transfer, degradation, or low expression levels. The remaining genes either showed no alteration in transcription, such as SA2097, or gave results that did not agree with the microarray, with the transcript abundance of SA1898 and nuc appearing to have slightly increased in the teicoplanin resistant mutants (data not shown). This discrepancy could indicate periodic fluctuations in gene expression that were not captured in the intervals measured, or could simply reflect subtle differences between the growth conditions or extraction methods used to prepare the RNA for the microarrays and the Northern blots.
Table 2. Genes downregulated in the step selected teicoplanin resistant mutant

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<sup>a</sup> ORF number.

<sup>b</sup> Description/putative function.
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Figure 2. Changes in gene expression upon acquisition and loss of glycopeptide resistance. A) Transcription of differentially expressed genes in SA113 and the three step-selected teicoplanin-resistant mutants NM18, NM30 and NM67, at early- (OD$_{600}$ = 0.5) and mid-exponential phase (OD$_{600}$ = 2). B) Transcription profiles of the same genes from SA113, and NM67 cells recovered from mouse TCF on days 1 and 8 of infection.

Survival in the mouse cage infection model. To assess tissue cage survival, equal numbers of SA113 and NM67 cells were inoculated into mouse tissue cages, and infection was monitored over 8 days. The bacterial load of SA113 steadily increased over the 8 days in the TCF. However, three out of seven mice infected with an identical dose of NM67 completely cleared the bacteria within 5 days. In the remaining four mice, NM67 decreased in numbers after the first day post infection, as in mice clearing the bacteria, (p < 0.01 day 1, p < 0.05 day 2, Fig. 3A) and then resumed growth. The recovering bacteria reached the same final cell density as the wildtype after 8 days (Fig. 3A). This suggested that NM67
was less fit than the wildtype parent, and that it was actively cleared in the tissue cages, giving rise to the outgrowth of a surviving population.

**Host response.** During infection, inflammation in the TCF is marked by granulocyte influx, apoptosis and chemokine release (30, 31). Baseline leukocyte counts of \(1 \times 10^4 \pm 3.1 \times 10^3\) cells/\(\mu\)l, consisting of a larger polymorphonuclear neutrophil (PMN) (91 % ± 3 %) and a smaller monocyte (9 % ± 3 %) fraction were found in uninfected tissue cages, in agreement with previous reports (16, 62). The macrophage inflammatory protein (MIP-2, CXCL2) is a murine neutrophil chemotactic factor with a function similar to IL-8 in humans (55). After infection with SA113, the MIP-2 concentration in TCF steadily increased from day 1 through day 8. NM67 survivors induced two-fold less MIP-2 than SA113 after the eight days. (p < 0.05, Fig. 3B).

MIP-2 is the major leukocyte attractant; accordingly leukocyte infiltration into tissue cages inoculated with SA113 or NM67 showed a time-dependent increase during infection, and correlated with the bacterial load of the TCF (Fig. 3C). Eight days after infection the concentration of leukocytes was two-fold higher in cages containing SA113 than in those with the surviving NM67 (p < 0.05). Mice which were mock-infected, or which were able to clear the infection, showed no increased leukocyte infiltration (Fig. 3C). Since the MIP-2 levels and consequent leukocyte infiltration were proportional to the bacterial load in both strains, it seemed likely that the surviving NM67 cells may have had a similar capacity to induce MIP-2 and a similar chemoattractant potential to SA113.

The leukocyte viability within the TCF during infection was evaluated, because staphylococci are known to kill eukaryotic cells (22). In cages infected with SA113 a significant decline of the proportion of viable leukocytes (from 100% to 36%) was observed on day 8 post infection (p < 0.05). Leukocytes in mice which cleared NM67 did not lose viability. In mice successfully infected with NM67, however, the decrease in leukocyte viability started only two days post infection (Fig. 3D), coinciding in time with the emergence of the surviving NM67 subpopulation.
Figure 3. Bacterial load and host response after infection of tissue cages with strains SA113 and NM67. In vivo bacterial growth and host response over 8 days in tissue cages infected with $10^4$ CFU of S. aureus. SA113 (n = 5, solid circle); NM67 growing in tissue cages (n = 4, solid triangle); NM67 cleared from tissue cages during infection (n = 3, empty triangle); and values in uninfected cages (empty square). Time 0, values before infection. A) bacterial load in TCF, ** p < 0.01, day 1 and * p < 0.05 day 2; B) macrophage inflammatory protein MIP-2 concentrations in TCF, * p < 0.05 day 8; C) leukocyte numbers in TCF, * p < 0.05 day 8; D) viable leukocytes in TCF, * p < 0.05 day 8. Median values are plotted for each group, and the lowest quartiles indicated.
**Host-driven selection of NM67 "survivors".** The clearing or delayed growth of NM67 in the tissue cages, points to a decreased virulence of the teicoplanin resistant mutant. The surviving population that emerged from NM67 may consequently represent the selection and outgrowth of a variant or mutant subpopulation from NM67. Cells isolated on days 1 and 8 post infection were therefore analysed for host-induced changes in their phenotype and gene expression.

**Teicoplanin resistance.** Teicoplanin MICs of NM67 isolates dropped to a median of 48 μg/ml after one day in the tissue cages, and then decreased further to median values of 0.36 μg/ml and 0.5 μg/ml on days 5 and 8, respectively. This was well below the MICs of SA113 isolates, which remained at a constant 2 μg/ml throughout the 8 days. The survivors of NM67 collected from tissue cages after eight days showed a heterogeneous resistance profile, but the majority of the cells had become hypersusceptible compared to SA113 (Fig. 4).
**Figure 4. Teicoplanin resistance profiles after passage in tissue cages.** Median values of population analysis profiles on increasing concentrations of teicoplanin for strain SA113 and NM67 isolated from tissue cages on days 1 and 8. Filled triangles with solid line, strain NM67 on day 1 (n = 5); filled triangles with dotted line, strain NM67 on day 8 (n = 4); filled circles with solid line, strain SA113 on day 1 (n = 4); and filled circles with dotted line, SA113 on day 8 (n = 4).

**Cell size.** On day 1 of infection, cell size and cell wall thicknesses measurements of SA113 and NM67 tissue cage isolates were similar to the original in vitro values; NM67 cells were still significantly smaller with thicker cell walls than SA113 (Fig. 5). After eight days of infection, the cell walls of NM67 survivors became thinner and their cell diameters increased, whereas both overall cell-wall thickness and cytoplasm diameter did not change in wildtype SA113, (Fig. 5A and B). On day 8, the cell wall of NM67 was still slightly thicker (p < 0.05) than that of SA113, however the cytoplasm diameter was now similar in both strains.
Figure 5. Transmission electron microscopy of SA113 and NM67. A) diameter of cytoplasm and cell wall thickness of SA113 and NM67 ex vivo on days 1 and 8 of infection. Bars show the average cytoplasm diameter and cell wall thickness in nm. Mean values from at least 20 electron microscopy pictures, made from bacteria which were harvested from 3 mice infected with either NM67 or SA113 are shown. **, p < 0.01 NM67 vs. SA113 cell wall thickness on day 1; ***, p < 0.001 NM67 vs. SA113 cytoplasm diameter on day 1; *, p < 0.05 NM67 vs. SA113 cell wall thickness on day 8. B) representative TEM pictures of SA113 (A,B) and NM67 (C,D) on days 1 (left) and 8 (right). Scale bars in the bottom right hand corners of the pictures correspond to 50 nm.

N-acetylglucosamine incorporation and turnover. The incorporation of $^{14}$C-N-acetylglucosamine in cells isolated from TCF was significantly (4.5 fold, p < 0.05) higher in NM67 than in SA113 on day 1. Incorporation decreased with time during infection in both strains. However, on day 8 the surviving NM67 cells still showed a higher incorporation rate (2.5 fold, p < 0.05) than SA113 (Fig. 6A). Surprisingly, cell wall turnover remained unchanged, with no significant differences between SA113 and NM67 in the release rates of $^{14}$C-labelled N-acetylglucosamine into the culture supernatant after passage in the tissue cages (data not shown).
Growth rate and hemolysin production. The minimum doubling time of NM67 (37.6 ± 1.4 min) decreased in the tissue cage survivors to mean values of 36.2 ± 1.6 min after day 1 and 32.5 ± 3.6 min after day 8. Meanwhile the minimum doubling time of SA113 (25.7 ± 1.7 min) remained relatively stable, with recovered isolates exhibiting mean minimal doubling times of 26.0 ± 0.6 min and 25.7 ± 1.0 min, on days 1 and 8 of infection, respectively. Hemolysin production, which was undetectable in NM67 in vitro, was partially restored in the NM67 survivors isolated on day 8 of infection. While, hemolytic activities of SA113 supernatants remained relatively stable during the course of infection (Fig. 6B).
Figure 6. Changes in $^{14}$C-N-acetylglucosamine incorporation and hemolysis. $^{14}$C-N-acetylglucosamine incorporation. Filled circle solid line, SA113, and filled triangle solid line, NM67, on day 1; filled circle dotted line, SA113, and filled triangle dotted line NM67, on day 8 after infection. Median values from four cages measured, and lowest quartiles are indicated for each group. ANOVA for repeated measures p < 0.05 (*) NM67 vs. SA113 on days 1 and 8, respectively. B) Hemolysis zones on sheep blood agar. One representative TCF isolate is shown for each strain from days 1 and 8.
**Cell wall properties.** Spontaneous autolysis rates for NM67 and SA113 isolates remained similar over the 8 day infection period (data not shown). Zymography, performed on SA113 and NM67 isolates collected during infection, showed no alterations in abundances of the 62-kDa amidase, the 50-kDa N-acetylglucosaminidase and of an undefined 36-kDa enzyme band in SA113 and NM67 isolates (data not shown). SA113 and NM67 were both similarly lysostaphin susceptible, as were all isolates recovered from the tissue cages (data not shown). Proportions of peptidoglycan monomers and multimers were similar, with peptidoglycan crosslinking degrees of 65.9% on day 1 and 66.1% on day 8 of infection for NM67, and 68.5% and 71.2% on days 1 and 8 of infection, respectively, for SA113. Therefore, physical qualities of the cell wall, such as resistance to osmotic stress, peptidoglycan crosslinking, susceptibility to lysostaphin, and autolysin banding pattern were very similar in wildtype and mutant and did not change remarkably during infection.

**Northern analysis of ex vivo isolates.** In *ex vivo* SA113 isolates, the transcription levels of all ORFs analysed remained the same as the parent. Transcription levels in the *ex vivo* NM67 survivors were more variable. For *spa*, SA1007 and *geh*, transcription levels had increased again after passage *in vivo*, with the signals approaching wildtype levels again by day 1 of infection. However, transcription of *sbi* and *opp-1A* remained low in the NM67 survivors (Fig. 2B).
DISCUSSION
The fitness cost of glycopeptide intermediate resistance in *S. aureus* has been evidenced in several studies by the decreasing growth rates of resistant strains and correlated in several cases with increases in cell wall thickness (42). This fitness burden, imposed by the accommodation of gross alterations in cell-wall morphology, is thought to account for the widely observed instability of the resistance phenotype. However, it has also been previously reported that conditions in a tissue cage infection model promoted the emergence of teicoplanin intermediate resistant mutants in the absence of teicoplanin pressure (56), suggesting that certain *in vivo* conditions favored the development, and thereby potentially the maintenance, of a teicoplanin resistance phenotype.

Here we assed the fitness of the *in vitro* selected teicoplanin-resistant strain NM67 in a murine tissue cage model. Although the MIC for NM67 was above the teicoplanin break point for intermediate level resistance (25), we define NM67 as being glycopeptide intermediate resistant because of the genetic basis of its resistance mechanism, which is distinct from the *van* gene based resistance of clinical VRSA (11). GISA phenotypes and accompanying genetic alterations are known to vary from strain to strain, therefore not all GISA isolates share identical characteristics. NM67 did however show several typical GISA phenotypes such as decreased growth rate, increased cell wall thickness, and enhanced *N*-acetylglucosamine uptake compared to its isogenic, susceptible parent. The tissue cage is a closed *in vivo* system, where neither bacteria nor attracted leukocytes can escape. The balance is in favor of bacterial survival, since phagocytes in tissue cage fluid show a decreased phagocytic and bactericidal activity compared to blood granulocytes (61). This particular situation reflects infection of an orthopedic implant, which explains why such low numbers of wild type *S. aureus* can cause a chronic persistent infection in the mice (31).

In contrast to the parent strain, which replicated over the eight day period, and elicited bacterial load-dependent MIP-2 release, leukocyte infiltration and reduction of leucocyte viability; the teicoplanin resistant mutant was either immediately cleared from the tissue cages or began to lose viability then...
recovered and started to grow after two days. The clearing or delayed growth of NM67 strongly suggested that the original teicoplanin resistant NM67 had lost its ability to cause a persistent infection, and was actively cleared in the tissue cages, while the survivors appeared to have regained wildtype virulence. The survivors had however also become hypersusceptible to teicoplanin, with MICs approximately four-fold less than the susceptible wildtype SA113.

Characteristics of the reemerging NM67 survivors, such as their higher growth rate, the conversion to teicoplanin hypersusceptibility, their partial loss of the thickened cell wall paired with a reduction in the high N-acetylglucosamine incorporation rate, and the restitution of transcription of some of the downregulated virulence determinants, suggested that the survivors were phenotypic revertants of NM67, which arose by forward mutation, since not all wildtype characteristics had been restored. Similar forward mutations producing susceptible variants with slightly altered phenotypes have been observed earlier (13, 43).

Despite maintaining a slightly thicker cell wall and higher N-acetylglucosamine uptake, NM67 survivors isolated on day 8 had lower teicoplanin MICs than SA113 isolates. Interestingly, resistance profiles of the wild-type SA113 remained relatively stable over the 8 day period; spontaneously resistant mutants did not arise as had been described earlier in a similar rat tissue cage infection model (56). Several factors may explain the absence of such spontaneous high resistant mutants in our model. Teicoplanin-resistant mutants were observed in the rat model as late as three weeks after infection; and were stable only when cultured continuously on teicoplanin-containing agar (56), while our study covered just the 8 days needed to reach the maximal numbers of CFU in the murine TC model. Also, we used a susceptible strain (MSSA), while the previous study used a multiresistant, clinical methicillin resistant (MRSA) isolate. The NCTC8325 strain lineage used in this study has no functional IS elements (53), whereas clinical multiresistant MRSA isolates may carry active IS elements, and thus have additional means to form glycopeptide resistant mutants by rearrangements and movement of the IS elements. IS256 transposition was
shown to facilitate mutations contributing to resistance levels in a clinical GISA isolate (34).

Altered autolytic properties are sometimes observed in glycopeptide resistant strains (28, 59). However, neither cell wall crosslinking and turnover, nor the accessibility of the lysostaphin target, were affected by teicoplanin resistance in our in vitro selected strain. Here, glycopeptide resistance seemed to be mainly characterized by the thickened cell wall, although microarray analysis did not detect any significant upregulation of ORFs or any transcriptional changes that might account for these phenotypes, such as transcriptional alterations that have been described for other GISA strains (12, 33, 37). The microarray performed here is unlikely to reflect all transcriptional changes that occurred during the resistance development of NM67 as it was performed on cultures harvested at a single time point during in vitro growth and should therefore be regarded as a snap shot of some of the transcriptional changes that occurred during resistance formation. More detailed transcriptional analyses would be required to obtain a global picture of the differences between these strains.

Altered virulence factor expression has been previously described in other GISA strains; spa transcription was decreased in Mu50 (33) and fibronectin binding protein production decreased in strain 14-4 (44). The decreased in vivo survival of NM67 may have been predicted by the gradual turning down of gene expression of virulence-associated genes as increased resistance was selected. It is likely that the downregulation of genes involved in biofilm formation, lipase, haemolysin and secretory antigen production and evasion of host immune responses, contributed to the poor survival of NM67 in vivo. The downregulation of virulence genes was probably a consequence of, but unlikely to be the cause of increased teicoplanin resistance.

Transcription of some of the virulence associated genes was partially restored upon loss of the resistance phenotype in the in vivo selected survivors. This possible link between resistance profile and the transcription of virulence genes may be one explanation for why the resistance phenotype was lost so rapidly in the tissue cages that did not clear the teicoplanin-resistant bacteria. In these
cases the mutation(s) causing reversion of resistance and restoration of virulence gene expression must have occurred before the bacteria were cleared.

In conclusion, intrinsic glycopeptide resistance acquisition can impose a fitness burden on S. aureus, creating selection pressure for the loss of the resistance phenotype in the absence of glycopeptides. In this study, the glycopeptide resistance level of NM67 gradually decreased upon in vitro culture in glycopeptide-free media; however, in vivo, in the presence of the host defense system, resistance was lost extremely rapidly due to the elimination of the resistant strain and subsequent selection for fitter, susceptible variants. The link between resistance acquisition and the decreased expression of virulence associated genes is likely to contribute to the strong selection for clearance of the resistant strain in vivo. It is also possible that the decreased growth rate, which accompanied the high level resistance, had also contributed to the poor in vivo survival of NM67. Compensatory mutations leading to decreased cell wall thickness indicate that metabolism reverts to normal, with the concomitant increase in virulence factor production indicating that the easiest way to restore fitness and virulence is at the expense of resistance.
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Chapter II

VIRULENCE OF THE TWO PREDOMINANT HOSPITAL-AQUIRED MRSA CLONES IN FRANCE COMPARED WITH THAT OF MSSA ISOLATES IN A MURINE SEPSIS MODEL

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# H. Karauzum and T. Ferry contributed equally to this study

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ABSTRACT

Clinical studies comparing the virulence of MRSA and MSSA strains, which cause sepsis, are hampered by confounding factors in patients. We used a mouse sepsis model a) to compare mortality of the two prevalent pandemic SCCmec IV MRSA clones in France (Lyon clone MRSA ST8 and a ST5 clone) and of MSSA with variable genetic background, which are prevalent in France in causing blood stream infections, b) to understand the contribution of SCCmec and of the two different sequence types ST5 and ST8 to outcome. All 44 isolates (10 MRSA ST8, 8 MSSA ST8, 8 MRSA ST5, 8 MSSA ST5 and 10 MSSA var. ST) were characterized by MLST, agr, spa and capsule typing, by measuring in vitro doubling time and resistance, toxin genes and proteins, and adhesin gene content. Infection with MRSA ST8 isolates caused a slightly, infection with ST5 isolates a significantly higher mortality than infection with MSSA expressing variable ST (48 vs. 29%, ns and 70 vs. 29%, p<0.001). However, the same 10 MRSA ST8 isolates caused a lower mortality than clonal MSSA ST8, (48 vs. 73%, P <0.001). This was not related to the presence of SCCmec, since for MRSA of ST5, mortality was similar as that induced by MSSA ST5 (79 vs. 59%, ns). Despite the same genetic background individual clonal MRSA isolates had heterogeneous virulence properties. No specific virulence factor determined in vitro, was found related to mortality in mice. In conclusion, in a bacteraemic model, lethality varied with the ST in the presence or absence of SCCmec. Within a ST, clonality was not associated with a homogenous outcome in the mouse model.
INTRODUCTION

*S. aureus* is a pathogen expressing multiple virulence factors such as adhesins, toxins and immune evasion molecules that enable the bacteria to induce a wide variety of infections. Hospital-acquired methicillin-resistant *S. aureus* (MRSA) arose in 1961 through the acquisition of the SCCmec element and became one of the most frequent pathogens responsible for hospital-acquired infections worldwide, particularly in intensive care units (ICU). Molecular characterization by multilocus sequence typing (MLST) of clinical MRSA isolates in a given area revealed that most of them are genetically related, i.e. they share the same sequence type (ST) which belongs to one of the five major clonal complexes (CC). In France, we recently described and genetically characterized the healthcare-associated MRSA isolates. The major clone, named Lyon clone, shares ST8 that belongs to CC8, and a minor one, which is related to the New-York-Japan clone and is spreading since a few years, shares ST5 that belongs to CC5 (6).

Differences in pathogenicity and virulence among MRSA and MSSA isolates may exist but remained an unresolved question. Studies of MRSA virulence in human suggest a greater burden for MRSA infections, in term of length of hospitalization and mortality rate, but these studies are impaired by multiple confounding factors. First, differences in patient populations, that have different co-morbid conditions, and differences in therapeutic options that are more or less bactericidal, limit conclusions about a putative increased virulence associated with methicillin resistance. Second, each pandemic MRSA clone may have specific virulence properties, as has been demonstrated for the pandemic MRSA clone in Brazil (CC8, ST239), which has an enhanced ability to adhere and invade epithelial cells *in vitro* in comparison with sporadic MRSA isolates (1).

The aims of this study were to: (i) compare the mortality rate induced by isolates belonging to pandemic MRSA clones in France with mortality caused by MSSA isolates in an inbred mouse model of sepsis, thus eliminating confounding factors associated with host variation and antimicrobial therapy; (ii) to investigate the
relationship between different virulence factors expressed by each *S. aureus* lineage and mouse mortality.
MATERIALS AND METHODS

S. aureus isolates.

We used MRSA isolates that allowed us to describe the major MRSA clone in our hospital. During a survey in ICUs of Edouard Herriot Hospital (a 1100-bed university hospital located in Lyon, France) we collected 17 MRSA isolates responsible for bloodstream infections and characterized two different MRSA clones (8). A major one, called Lyon clone (13 of the 17 isolates shared agr type 1 allele, ST8, CC8, spa type t008 or relatives and were positive for the sea gene), and a minor one related to the New-York-Japan clone (3 of the 17 isolates shared agr type 2 allele, ST5, CC5, spa type t002 or relatives, SCCmec type IV and were positive for the tst gene and the egc locus). Epidemiological studies (unpublished) revealed that the Lyon clone was the predominant MRSA clone in France whereas the ST5 MRSA clone was emergent and spreading throughout France since few years. We randomly selected for the analysis 10 of the 13 MRSA ST8 Lyon clone isolates, the three MRSA ST5 positive for the tst gene and the egc locus, and 10 of the 19 MSSA isolates with various STs (one ST8, four ST15, one ST45, one ST97, one ST217, one ST463 and one ST465) also responsible for bloodstream infection in the same area in the same period of time. To analyze the effect of the SCCmec cassette and the effect of the genetic background on the virulence, we completed the first strain selection by adding eight MSSA ST8 and eight ST5 isolates. These isolates were also from bloodstream infections and were collected by the National Center for Staphylococci located in Lyon, France, either during a national survey (in 2006-2007) or spontaneously referred to the Center (in the 2003-2007 period). Five MRSA ST5 isolates were also taken from the national survey to complete the first selection of three MRSA ST5. Finally, a total of 44 S. aureus isolates were included in the study and grouped as follows: 10 MRSA ST8, 10 MSSA with variable ST (MSSA var. ST), 8 MSSA ST8, 8 MRSA ST5 and MSSA ST5. MRSA ST8 and MSSA ST8 were positive for the sea gene; MRSA ST5 and MSSA ST5 isolates were positive for the tst gene and the egc locus.
Characterization of the genetic background and virulence factors.
All strains were characterized by MLST, spa typing and accessory gene regulator (agr) allele typing (1-4) as previously described. Capsular typing was based on serotyping and PCR as previously described (21). Each isolate was genetically characterized by adhesin gene profiling (bbp, cna, eno, ebpS, clfA, clfB, fnbA and fnbB), toxin gene content (tst; staphylococcal enterotoxins and enterotoxins-like sea, seb, sec, sed, see, seh, selk, sell, selm, selo, selp, selq, selr). Positive isolates for selm and selo were considered to be also positive for the egc locus, as these genes belong to the egc operon. Delta-toxin production was determined in an agar plate assay testing synergy with a beta-toxin reference strain as previously described; isolates without delta-toxin production were considered to have dysfunction of the locus agr (18). Production of alpha-toxin was determined by incubating the strains overnight on sheep blood agar plates at 37°C. Superantigen expression was measured by using the RIDASCREEN SET A, B, C, D and E (R-Biopharm AG, Darmstadt, Germany). Finally, bacterial doubling time was determined as previously described with minor modifications (7). Overnight cultures were diluted 1:100 in 5 ml of BHI medium and grown for 3 h at 37°C with shaking (180 rpm). These cultures were seeded at 1:100 dilution and a 1:4 culture-to-flask ratio, then incubated at 37°C and 180 rpm for 4 h. Optical density (OD) was measured every 30 min for 4 h. Values were converted into log₂ values and the doubling time was calculated as the reciprocal of the slope.

Determination of the virulence in mice sepsis model.
C57BL/6 mice were kept in specific-pathogen-free conditions in the Animal House of the Department of Research, University Hospital Basel, Switzerland and all experiments were performed according to Swiss veterinary law. Before intravenous challenge, S. aureus isolates were grown freshly from frozen stock cultures (-70°C) prepared using the Microbank system (Pro Lab Microbank Bacterial Preservation System Green, Basel, CH). One cryoculture bead per strain was incubated in 1 ml of brain-heart infusion (BHI) broth (BBL Becton Dickinson, Maryland) for 7 h at 37°C. Cultures were then diluted 1:100 in 5 ml of
BHI and incubated overnight at 37°C. The bacteria were then centrifuged at 4000 g for 10 min, and the pellet was washed twice and resuspended in pyrogen-free 0.9% NaCl before use. Female 6-8 weeks old C57BL/6 mice were injected with MRSA and MSSA inocula ranging from $5.1 \times 10^7$ to $1.4 \times 10^8$ CFU (median) in a volume of 200 µl via the caudal vein. Control mice were injected with 200 µl 0.9% NaCl. Inocula for the different groups of strains were not significantly different: For MRSA ST8 the median inoculum was $1.4 \times 10^8$ CFU; for MSSA var. ST it was $6.5 \times 10^7$ CFU; for MSSA ST8 it was $6.8 \times 10^7$ CFU; for MRSA ST5 it was $6.8 \times 10^7$ CFU and for MSSA ST5 it was $5.1 \times 10^7$ CFU; control mice were injected with 200 µl of 0.9% saline. Weight change and disease severity were determined on day 1 to 9 after infection. Disease severity was assessed by a scoring system (0, normal activity; 1, trembling, weakness; 2, piloerection, decreased activity; 3, strongly decreased activity; 4, no movement). The mice were killed by intraperitoneal injection of 100 mg/kg pentobarbital (Abbott Laboratories) when they reached a score of 4 and surviving mice were sacrificed on day 9 after infection. At least 4 mice were used per group.

**Data analysis.** Data were analyzed with Graph Pad Prism 4 software. $P$ values < 0.05 were considered to denote statistical significance. Survival differences between mice challenged with the different groups of strains were analyzed during the infection process with the log rank test and a Kaplan-Meier survival curve. Overall mortality between groups was compared by Chi-square contingency test.
RESULTS

Virulence properties in the murine sepsis model, and comparison between groups of strains

Comparison of MRSA ST8 and MRSA ST5 groups with MSSA var. ST
The collection of MRSA ST8 and ST5 as well as that of MSSA var. ST isolates simulate the distribution of *S. aureus* in clinical settings in French ICUs. The infection of mice with these strains revealed a trend to a higher mortality for the MRSA ST8 group in comparison with the MSSA var. ST group, both by comparing survival curves and by comparing the overall mortality occurred during infection (Fig. 1A). These differences were significant after including further 48 and 41 mice that were infected with a wider range of inocula of MRSA ST8 and MSSA var. ST respectively (*P* < 0.011). The overall mortality induced by the MRSA ST5 group was extremely high (79%), it was significantly higher than that induced by the MSSA var. ST group (29%) and occurred early between day 1 and 4 of sepsis (Fig. 1B).
Fig 1. Percentage survival of mice infected with MRSA and MSSA isolates
A) Survival of 44 and 55 mice infected with 10 MRSA ST8 isolates (closed circles; $1.4 \times 10^5$ CFU/mouse) and 10 MSSA isolates of var. ST (open triangles; $6.5 \times 10^7$ CFU/mouse) respectively (log rank test, $P = 0.072$). B) Survival of 42 and 55 mice infected with 10 MRSA ST5 (closed squares; $6.8 \times 10^7$ CFU) and 10 MSSA isolates of var. ST (open triangles; $6.5 \times 10^7$ CFU) respectively (log rank test, $P < 0.0001$). ≥ 4 mice were infected with each strain. Statistics of overall mortality was determined by Chi-square test.

Effect of the SCCmec cassette on virulence in ST5 and ST8 lineages
The comparison of clonal MRSA with variable MSSA did not allow any conclusion on the role of SCCmec for virulence. Therefore outcome of sepsis was compared in C57BL/6 mice infected with MRSA or MSSA from the same ST. In this comparison the MRSA ST8 group induced a lower mortality than the MSSA ST8 group (Fig. 2A). Conversely, mortality with MRSA ST5 was higher than with the MSSA ST5 group, although the overall value in groups with ST5 observed at day nine was not significantly different (Fig. 2B).

Thus SCCmec had a variable influence upon outcome depending on the ST lineage. This was also confirmed by a comparative analysis of survival curves after infection with MRSA and MSSA of the two lineages. In MRSA, the ST5 lineage, compared to the ST8 lineage, conferred a significantly higher mortality (Fig. 2C). In MSSA, the reverse was observed. ST8 conferred a higher mortality
compared with that of the ST5, although overall mortality was not significantly different (Fig. 2D).

Fig. 2 Percentage survival of mice infected with MRSA and MSSA isolates
A) Survival of 44 and 41 mice infected with 10 MRSA ST8 (closed circles; $1.4 \times 10^8$ CFU) and 8 MSSA ST8 isolates (open circles; $6.8 \times 10^7$ CFU) respectively ($P = 0.0012$). B) Survival of 42 and 32 mice infected with 8 MRSA ST5 (closed squares; $6.8 \times 10^7$ CFU) and 8 MSSA ST5 (open squares; $5.1 \times 10^7$ CFU) respectively ($P < 0.001$). C) Survival of 44 and 42 mice infected with 10 MRSA ST8 (closed circles; $1.4 \times 10^8$ CFU) and 8 MRSA ST5 isolates (closed squares; $6.8 \times 10^7$ CFU) respectively ($P < 0.0001$). D) Survival of 41 and 32 mice infected with 8 MSSA ST8 (open circles; $6.8 \times 10^7$ CFU) and 8 MSSA ST5 (open squares; $5.1 \times 10^7$ CFU), respectively ($P = 0.0059$). 4 mice were infected with each strain. Chi-square test was used to determine overall mortality.
Association between virulence factors, MRSA and MSSA, sequence type and mouse mortality

**Virulence factors of MRSA ST8 and MRSA ST5 and MSSA var. ST**
Isolates belonging to the MRSA ST8 and ST5 groups’ harbored capsular type 5, whereas four of the MSSA var. ST group expressed capsular type 5, the six remaining isolates had capsular type 8. Isolates belonging to the MRSA ST8 and ST5 groups harbored genes encoding SEA and TSST-1, respectively, whereas none of the MSSA var. ST isolates harbored any of these genes. Finally, the group of MRSA ST8 showed a slightly slower *in vitro* growth than the group of MSSA var. ST with a mean doubling time of 34.60 ± 2.70 min compared to 29.20 ± 1.15 min (*P* = 0.08). Doubling times of isolates belonging to the MRSA ST5 group were similar with 30.00 ± 3.88 min (*P* = 0.83).

**Mouse mortality induced by individual isolates of ST5 and ST8 lineages**
Three of the 10 isolates belonging to the MRSA ST8 group were never fatal whereas five were fatal for 80 to 100% of the mice. As shown in Fig. 3, no specific virulence factor was able to discriminate between lethal and non-lethal isolates. Similarly, two isolates belonging to the MSSA ST5 group were never fatal and did not lack any particular adhesin or toxin, which could explain the reduced virulence (Fig. 4).
Fig3
Fig. 4
DISCUSSION

In this study we found that, MRSA isolates belonging to the Lyon clone, expressing ST8, which is the main clonal population of MRSA prevailing in a large French hospital, collectively exhibited a lower virulence in a murine sepsis model than MSSA isolates of the same ST8. This observation was mitigated in the comparison of MRSA ST8 and MSSA with variable ST. We further found that lower virulence of MRSA was not due to the acquisition of SCCmec per se, since MRSA ST5 showed virulence as strong as MSSA ST8. Finally, individual clones of MRSA or MSSA either ST5 or ST8 showed a variable virulence, and no relationship between known virulence factors and lethality in the sepsis model was found.

The Lyon clone MRSA isolates showed a heterogeneous low level of resistance to oxacillin. Acquisition of the SCCmec type I element has previously been shown to slow the S. aureus doubling time in vitro (7). However, in the present study, we found that clonal MRSA and MSSA isolates of either ST 8 or ST 5 had similar doubling times, possibly indicating that a potential initial fitness burden (12) due to SCCmec acquisition was overcome by compensatory mutations, or that only clones retaining rapid growth survived (2, 13).

In the sepsis model the MRSA ST8 clone isolates killed less mice than the MSSA ST8 isolates. This observation supports the previous results of Mizobuchi et al., who compared the virulence of 13 highly resistant MRSA and 7 MSSA isolated from patients with various clinical syndromes (14) and found that MRSA were less virulent than MSSA; however, it is noteworthy that the strains were not genetically characterized. Peacock et al. found that a strain belonging to an epidemic MRSA clone showed similar lethality to three clinically significant MSSA strains after intraperitoneal or intravenous inoculation to mice (17). Our comparative study of MRSA and MSSA infection in mice is strengthened by the genetic homogeneity of the MSSA isolates. Our findings highlight that clonal isolates have to be compared when the effect of SCCmec upon virulence is to be identified. Indeed, in our initial series in which lethality induced by MRSA ST8 was compared to that induced by the genetically variable MSSA isolates an
apparently higher virulence of MRSA ST8 was observed. Nevertheless the acquisition of SCCmec type IV per se is not associated with a loss of virulence, since our MRSA isolates ST5 were as virulent as MSSA ST8.

When investigating virulence factors, which could contribute to death, surprisingly, MRSA and MSSA isolates with a homogenous genetic background to the level of our present knowledge had a heterogeneous outcome. Capsular polysaccharides contribute to staphylococcal virulence. Most clinical S. aureus isolates have serotype 5 (CP5) or serotype 8 (CP8) capsules. By comparing the biological activities of isogenic mutants with CP5, CP8 or no capsule in a mouse model, Watts et al. showed that CP5-positive strains were more virulent than both CP8-positive strains and CP-negative strains (22). We found no correlation between CP5 and mouse lethality. Among the MRSA tested here, all of which were CP5-positive, three strains were non-lethal for mice.

When interpreting our mouse lethality data it is important to remember that mice are susceptible to certain virulence determinants (hemolysin, leukocidin, etc.), but are less susceptible than men to superantigenic toxins. Mouse susceptibility to these toxins can be enhanced by D-galactosamine administration (4, 19). We had previously assumed that SEA was involved in the pathogenesis of S. aureus septic shock, as we had found that sea was more frequent in blood isolates from patients with septic shock than from patients without septic shock (9). However, all 10 Lyon clone isolates tested here harbored the sea gene and produced SEA in vitro, but three isolates were non lethal for mice, suggesting that SEA is not a major MRSA virulence factor. In contrast, Nilsson et al. demonstrated that vaccination with genetically modified SEA lacking superantigenic properties conferred protection in a non-sensitized murine sepsis model induced by an S. aureus strain expressing sea in vitro (16). The sea gene has been detected in several pandemic MRSA clones (CC8 and CC30) (8). Recently, Diep et al. demonstrated that horizontal transfer of virulence genes such as sea was epidemiologically associated with the emergence of new virulent strains of both hospital- and community-acquired MRSA (5). It cannot be excluded that SEA was differentially expressed in vivo in lethal and non-lethal isolates.
Five of the 10 MRSA ST8 isolates tested here produced alpha-toxin and harbored genes encoding gamma-toxin. Alpha-toxin has been shown to cause epithelial damage in a model of corneal and tracheal staphylococcal infection (3, 10). In addition, in a rat model of heart failure, alpha-toxin provoked coronary vasoconstriction and cardiodepression, both of which can occur in the course of sepsis (11). Gamma-toxin had a pro-inflammatory effect in a rabbit endophthalmitis model (20). However, only alpha- and gamma-toxin jointly promoted *S. aureus* virulence in a septic arthritis model (15). Thus, toxins may play an important role in local infection, whereas there is no firm evidence that alpha toxin or gamma-toxin are directly involved in septic shock.

In conclusion, MRSA isolates of the Lyon clone expressed heterogeneous methicillin resistance and harbored heterogeneous intrinsic bacterial characteristics and virulence properties. This heterogeneity may be linked not only to one specific virulence factor, but to differences in global regulation or to single nucleotide polymorphisms not detected in our study. Lastly, whole genome sequencing of the clonal isolates with differing outcome will allow identifying the hitherto unknown genes responsible for high virulence.
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Chapter III

HOST-PATHOGEN INTERACTIONS
USING CLONALLY RELATED METHICILLIN-RESISTANT
STAPHYLOCOCCUS AUREUS ISOLATES
INTRODUCTION

In the third part of my thesis, I investigated the phenotypic properties of, and immune response to clonal MRSA isolates, which elicited a variable outcome in the murine sepsis model. Since we could not attribute their variable lethality to toxins or adhesin genes, we searched for a confirmatory typing method and applied MLVA. Further we investigated other pathogen properties, which would help explain their variable in vivo behavior, such as clumping. We also investigated chemotaxis, susceptibility to PMN killing, apoptosis of PMN and T, B and dendritic cell response in liver and spleen hoping that host reaction to lethal and non-lethal isolates would be different and that through the different host reactions we would be able to reveal virulence factors relevant for the lethal effects observed in the mouse model.

The inter-human dissemination and invasion in human infection of S. aureus is relying on the expression of microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) in the host, such as ClfA/B, FnBP and Cna.

Role of ClfA was studied, since it is known to mediate clumping of bacteria in the presence of fibrinogen in vitro (8), due to the dimeric structure of fibrinogen enabling two ClfA molecules to bind simultaneously. The interactions occur between the N-terminal A domain of ClfA and the gamma chains of fibrinogen (15). Furthermore fibrinogen can serve as a bridging molecule to attach to host cells (14). Bacterial clumping, often found in abscesses, is probably a mechanism to make bacteria more resistant to host defense, such as opsonization and phagocytosis (10, 11). Moreillon et al. showed a S. aureus clfA-defective mutant had decreased adherence in vitro and also a decreased virulence in a rat endocarditis model (17).

Next, chemotaxis of PMN was investigated since S. aureus is known to secrete proteins that thwart PMN recruitment. The chemotaxis inhibitory protein (CHIPS), a 14.1 kDa protein, is known to inhibit chemotaxis by binding to formyl peptide (FPR) and C5a receptor and thus competing out their physiological ligands (3). The extracellular adherence protein (Eap), a 60 kDa protein inhibits leukocyte chemotaxis by binding directly to host adhesive proteins, like ICAM-1, expressed
on endothelial and epithelial cells, and thus hinders the interaction with leukocytes necessary for diapadesis and extravasation (2).
Since several groups demonstrated, that upon internalization S. aureus secretes certain pore-forming toxins that lead to the activation of caspases of the host cells and to apoptosis, we measured apoptosis in vitro (6, 16, 24). PMN undergo constantly spontaneous apoptosis and signal macrophages to recognize and remove them, an important process to prevent host tissue damage and inflammation (20). This spontaneous apoptosis, was shown to be accelerated upon uptake of S. aureus (12, 13).
MATERIALS AND METHODS

Preparation of bacteria and supernatants

Bacteria were grown freshly from frozen stock cultures (-70°C) prepared using the Microbank system (Pro Lab Microbank Bacterial Preservation System Green, Basel, CH). One cryoculture bead per strain was incubated in 1 ml of brain-heart infusion (BHI) broth (BBL Becton Dickinson, Maryland) for 7 h at 37°C. Cultures were then diluted 1:100 in 5 ml of BHI and incubated overnight at 37°C. The bacteria were then centrifuged at 4000 g for 10 min, and depending on further usage, the pellets were taken up immediately or after washing twice in 0.9% NaCl.

For sepsis, killing, and apoptosis experiments, the pellet was washed twice and re-suspended in pyrogen-free 0.9% NaCl before use.

For chemotaxis, bacteria were grown from cryoculture beads as described above, but instead of BHI, synthetic medium was used. The overnight cultures were centrifuged at 4000 g for 10 min, the supernatants (SUP) were filtered through 0.22 μm filters (Millex- MP, Millipore) and dialyzed against PBS (Phosphate Buffered Saline, Laboratorium Dr. Bichsel AG, Interlaken) using 3.5 kDa membrane tubings (Spectra/Por, Spectrum Laboratories, Inc., Canada). Aliquots of dialyzed and non-dialyzed supernatants were frozen at –70°C.

For adhesion and clumping assays, bacteria were grown from cryoculture beads as described above and were washed twice in PBS. To yield a concentration of about 5 x 10^8 CFU, the pellet was taken up in 2 ml of PBS.

For the peritonitis model, bacteria were grown from cryoculture beads as described above, but instead of BHI, Iscove’s modified Dulbecco’s medium (IMDM) without phenol red was used (GIBCO, Invitrogen).
Multi-locus variable–number tandem repeat analysis (MLVA)
MLVA was performed as described previously (19). MW2 was used as a reference strain. The primers used for the assay are described below.

- **mecA** (F-CATTGATCGCAACGTTCAATTT) (R-TGGTCTTCTGATTCTCGGA);
- **sspA** (F-ATCMATTTCGMAAYGATGACCA) (R-TTGTCCTGAATTTGTTATCGCC);
- **spa** (F-AGCACCATAAGAAGAAAGACAA) (R-GTATAACGACTGTACTCCGT);
- **sdrCDE** (F-TTACCGATCATGATGATTCA) (R-CAYTACTCTGTCTGTAATGCTT);
- **clfB** (F-ATGGTGATTCAGCAGAAATCC) (R-CATTATTTGGTGTGTAACCTT);
- **clfA** (F-GATTCTGACCGAGTTCA) (R-CTGTATCTGGTAATGTTCTTT);
- **fnBP** (F-GGTCAAGCRCAGAGGCACART) (R-AATAATCCCGCGAACAACAT);
- **sasA** (F-TTGAACATTTCGAATATACAGT) (R-TCGATGACTGTCACTTAATGATG);
- **plsR2** (F-AATTACAACGCCTCAAGCTG) (RGCACCATGGATGATTACTTC);
- **cna** (F-AAATGACAAAAATGGCAAG) (R-CAGGTTCAGTTGTTGCTT)

Bacterial adhesion to solid-phase fibrinogen
Flat-bottom 96-well microtiter plates were coated with 10 μg/well human fibrinogen for 2 h at 37°C. To block unspecific binding sites, 100 μl of a 2% solution of bovine serum albumin (BSA) in PBS was added per well and incubated at 4°C overnight. After four washing steps with PBS-Tween (0.05%), a
suspension of 50 µl of bacteria, prepared as described above, corresponding to 5 x 10^8 CFU was applied to each well. Control wells were supplied with sterile PBS. The plates were incubated for 2 h at 37°C with gentle shaking. After four washing steps, bound bacteria were stained with 100 µl of 0.5% crystal violet in 70% methanol per well. After 15 min of incubation, the plates were rinsed with water until all excess stain was removed. Bound bacteria were detached with 100 µl of 33% acetic acid per well and the absorbance was determined at 590 nm in an ELISA reader. Control wells were considered as background levels.

**Clumping assay with soluble fibrinogen**

Strains were grown as described above. 50 µl aliquots corresponding to 5 x 10^8 CFU were transferred to a 96-well microtiter plate (Beckton Dickinson) containing serial twofold dilutions of human fibrinogen (Endotell, Switzerland) in PBS starting with 10 µg/50µl. After shaking 2 h at room temperature, each well was controlled for visible clumps or clots by microscopy. The clumping titer of each strain was determined as the highest dilution factor of fibrinogen that still resulted in visible clumping of the bacteria.

**Isolation and preparation of human PMN**

Human blood was taken from voluntary healthy donors and collected in EDTA. PMN were isolated by dextran sedimentation and density gradient centrifugation on a discontinuous Percoll gradient with 53% and 67% Percoll in PBS as described previously (9). The interface between the two Percoll layers containing the PMN was collected and contaminating erythrocytes were removed by hypotonic lysis. Cell numbers were determined by Türk staining.

**Chemotaxis of PMN**

Human PMN and bacterial supernatants were prepared as described. Chemotaxis of human PMN was determined assessing their migration towards bacterial supernatants using a 48-well microchemotaxis chamber as previously described (21). The wells of the lower chamber were filled with 28 µl of bacterial
supernatants, N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP), synthetic medium, or HHG⁺Ca²⁺ in quadruplicates. A 3 μm pore size polyvinylpyrrolidone-free polycarbonate filter membrane (Nucleopore, Sterico AG, Switzerland) was placed between lower and upper wells, which were filled with 45μl/well (2.5 x 10⁶ PMN/ml in Hanks Hepes with Ca²⁺ with Glucose). The mounted chambers were incubated 30 min at 37°C in 5% CO₂ humidified air. After incubation, chambers were disassembled, the membrane fixed and stained with Wright-Giemsa (Diff Quick, Medion Diagnostics, Switzerland). The number of cells, which migrated through the filter-pores, was determined by microscope. Migration of PMN was expressed as the mean percentage of cells migrated per well. Six replicates per sample were counted.

**Killing of bacteria by human PMN**
Bacteria and human PMN were prepared as described above. Bacteria were co-cultured with human PMN in a 1:1 ratio of 5 x 10⁶ CFU/ml: 5 x 10⁶ PMN/ml in D-PBS CaCl₂⁺, MgCl₂⁺ (GIBCO) enriched with 10% complete normal human serum. The samples were incubated at 37°C for 3 h under continuous rotation at 200 rpm. At time points 0’, 30’, 60’ and 180’ aliquots were sampled and diluted in dH₂O (pH11) to lyse PMN; further dilutions were plated onto BHI agar plates to quantify surviving bacteria. Bacterial growth in the absence of PMN was determined as positive control.

**Apoptosis of human PMN by bacteria**
Human PMN were prepared as described above. Bacteria were co-cultured with human PMN in a 1:1 ratio of 5 x 10⁶ CFU/ml: 5 x 10⁶ PMN/ml in D-PBS CaCl₂, MgCl₂ (GIBCO) enriched with 10% complete normal human serum. The samples were incubated at 37°C for 6 h under continuous rotation at 200 rpm. Cells were sampled at time points 0’ and 360’, centrifuged and the pellet was resuspended in Hepes buffer containing the dyes Annexin V and 7AAD. After 15 min of incubation at room temperature, samples were acquired with the flow cytometer. For analysis, gates were set on live (double negative staining), apoptotic
(Annexin V+/7AAD-), late apoptotic/early necrotic (double positive staining) and necrotic (Annexin V-/7AAD+) cells.

**Animal models**

Mice were kept in specific-pathogen-free conditions in the Animal House of the Department of Research, University Hospital Basel. All experiments were performed according to Swiss veterinary law.

**Intravenous challenge**

Female 6-8 week old Balb/c mice were inoculated with 200 µl of a suspension containing 2.9 x 10^6 CFU of bacteria intravenously via the caudal vein. Control mice were injected with 200 µl of 0.9% NaCl. The mice were monitored for nine days. On day 1, 4 and 7 mice were euthanized by CO₂ inhalation. Organs were taken out and used for further cell preparations.

**Intraperitoneal challenge**

Female 14-16 week old C57Bl/6 mice were intraperitoneally inoculated with 2 ml of filtered culture supernatants using 25-G needles. Control mice were injected with 2 ml culture medium. After 6 h, mice were euthanized by CO₂ inhalation and 6 ml of RPMI containing 10% FCS were injected into the peritoneal cavity. Exudates were collected with 23-G needles from the peritoneum and leukocyte numbers were quantified using a Coulter Counter (Coulter Electronics).
RESULTS

To certify the clonal nature of the 10 MRSA isolates used in chapter I, MLST was complemented by multi-locus variable-number tandem repeat (VNTR) analysis (MLVA). In our study, a total of 9 primer pairs were selected to map repeat-containing genes that are encoding potentially important pathogenicity and virulence factors of *S. aureus* (4, 5). Since strains used in this study were MRSA isolates, a primer pair was selected to confirm the presence of *mecA*. The multiplex PCR profiles of 10 MRSA isolates that were formerly characterized by MLST and *spa* typing (described in chapter II) were analyzed by using a micro-capillary electrophoresis device (BioAnalyzer). Numerical files were then imported in specifically developed software performing UPGMA clustering that revealed genetic distance far below a cut-off value of 0.2 confirming high clonal relatedness among the isolates (Fig.1).
Fig. 1: MLVA dendrogram of MRSA ST8 isolates
Dendrogram based on the allelic profiles of 10 MRSA isolates compared to MW2 strain as reference. Red lines show the position of PCR products after normalization of PCR profiles.
Adhesion of MRSA ST8 to human solid-phase fibrinogen and clumping in the presence of soluble fibrinogen

We assessed the binding capacities of the 10 MRSA ST8 isolates to human solid-phase fibrinogen and the degree of clump formation in the presence of soluble fibrinogen and tested whether the variable fibrinogen binding or clumping capacity was related to virulence of these isolates in vivo (Fig. 2). The adhesion to fibrinogen among isolates was heterogeneous. A clfA/B-deficient mutant, used as negative control, showed nearly no adhesion (striped white bars). Newman strain was used as a positive control and elicited adhesion (striped grey bars). A specific adhesion pattern, discriminating lethal (closed bars) or non-lethal (open bars) isolates was not detected (Fig. 2A).

Clumping of the isolates in the presence of human soluble fibrinogen was very heterogeneous among the isolates. The clfA/B-deficient mutant showed no clumping at all, while the Newman strain had a high clumping titer (striped grey bars) (Fig. 2B). Strain 0291, which yielded a very low adhesion, was also impaired in its clumping ability. This relationship between adhesion and clumping ability was not observed for the other strains.
Fig. 2: Bacterial adhesion to human solid-phase fibrinogen and clumping in presence of soluble fibrinogen by 10 clonal MRSA isolates

A) Bacterial adhesion to human solid-phase fibrinogen was assessed by OD at 590nm. Median values of three independent experiments with quadruplicates per isolate are shown. B) Clumping of bacteria in the presence of soluble fibrinogen was determined by the clumping titer. Median values of five independent experiments are shown. Non-lethal (open bars) and lethal (closed bars) isolates. A clfA/B-deletion mutant (striped white bars) and Newman strain, strongly expressing ClfA/B (striped grey bars) were used as negative and positive controls respectively.
Chemotaxis of human PMN towards supernatants of MRSA ST8 isolates

We studied the chemotactic capacity of bacterial supernatants of single lethal and non-lethal MRSA ST8 isolates. These supernatants were used non-dialyzed or dialyzed to eliminate small chemotactic molecules such as fMLP. Medium alone was used as negative control and induced 21% of PMN migration. PMN migration towards fMLP in medium was used as a positive control and induced 73% of PMN migration (Fig. 3A). PMN migration towards non-dialyzed supernatants of bacterial overnight cultures was ranging from 12 to 36% (Fig. 3B, closed bars) and was less than PMN migration towards dialyzed supernatants of bacterial overnight cultures, which was ranging from 56 to 76% (Fig. 3B, open bars). Inhibition of PMN migration was observed in some strains when non-dialyzed supernatants were compared to sole medium. However, the extent of migration among dialyzed and non-dialyzed supernatants could not be related to virulence of the isolates in the sepsis model.
Fig. 3: Chemotaxis of human PMN towards bacterial supernatants of O.N cultures from 10 clonal MRSA isolates
A) Percent PMN migration towards medium (grey bar) and fMLP (striped bar)
B) Percent PMN migration towards non-dialyzed (closed bars) and dialyzed (open bars) supernatants of 10 MRSA ST8 O.N. cultures. Strain numbers: 0012, 0141, and 0143 were non-lethal in the sepsis model. Median values of 5 replicates from two independent experiments are shown.
Chemotaxis of leukocytes towards MRSA ST8 supernatants in a murine peritonitis model

We were interested if the 10 MRSA ST8 isolates had a variable ability to attract leukocytes *in vivo*. Thus, we determined the number of leukocytes entering the peritoneum 6 h after injecting bacterial supernatants i.p.. We observed variable migration of leukocytes ranging from baseline levels of about 500 leukocytes/μl to maximum levels of about 2000 leukocytes/μl. Supernatants of lethal (closed bars) and non-lethal (open bars) strains behaved similarly in their capacity to induce leukocyte attraction (Fig. 4).

![Bar chart](image)

**Fig. 3: Number of leukocytes/μl after intraperitoneal injection of bacterial supernatants from 10 clonal MRSA isolates**

Leukocyte numbers in peritoneal fluid were determined 6 hours after i.p. injection of 2 ml bacterial supernatants of lethal (closed bars) and non-lethal (open bars) strains. IMDM was used as negative control (grey bars). Median values of three mice per isolate of one experiment are shown.
Killing of MRSA ST8 isolates by human PMN

We were interested if certain isolates of MRSA ST8 were more resistant to killing than others and if there was a possible relation between susceptibility to killing and virulence in the former described murine sepsis model. The multiplicity of infection (MOI) was ranging from 0.03-2 (bacteria): 1 (PMN). Susceptibility to killing by human PMN was very heterogeneous among the 10 MRSA ST8 isolates, especially within the first 30 minutes (closed bars), where survival of bacteria ranged from 30 to 90%. After 60 minutes (striped bars), surviving bacteria ranged from 5 to 40%. After 180 minutes (open bars), killing was most efficient and not more than 3% of bacteria were detectable. Kinetics of killing was not related to virulence in the sepsis model (Fig. 5).

![Graph showing survival of bacteria over time for different MRSA isolates](image)

**Fig. 5: Killing of 10 MRSA ST8 isolates by human PMN**

Percentage of surviving bacteria, which were lethal (0019, 0020, 0064, 0065, 0234, 0291) and non-lethal (0012, 0141, 0143) in the murine sepsis model, was determined after 30 min (closed bars), 60 min (striped bars), and 180 min (open bars) of co-culture of bacteria with human PMN. Results are from one experiment and therefore have to be considered as preliminary data.
**Apoptosis of human PMN induced by 10 MRSA ST8 isolates**

To elucidate, if the variable virulence of the MRSA ST8 isolates was related to either enhanced or diminished capacity to induce apoptosis, we measured the percentage of apoptotic human PMN induced by these bacteria (Fig. 6). Under our experimental conditions, about 50% of the untreated PMN were undergoing spontaneous apoptosis, while cyclohexamide treatment, used as a positive control, yielded 60% of apoptotic cells after 6 h. The fraction of apoptotic PMN in the presence of bacteria ranged from 18 to 27% after 6 h. These results demonstrate clearly, that MRSA ST8 isolates decelerate apoptosis at least 2 fold, when compared to spontaneous apoptosis of untreated PMN. The percentage of apoptotic cells was similar for lethal and non-lethal isolates.

**Fig. 6: Apoptosis of human PMN induced by MRSA ST8 isolates**

Percent apoptotis of PMN was induced similarly by both, lethal (0019, 0020, 0064, 0065, 0342, 0234, 0291) and non-lethal (0012, 0141, 0143) isolates after 6 hours of co-culture (open bars). Closed bars: 0 min: PMN in PBS served as negative, cyclohexamide-treated PMN as positive controls.
Cellular host response against lethal and non-lethal isolates during sepsis in liver and spleen of Balb/c mice

As we were interested in the innate and adaptive immune response to lethal and non-lethal MRSA ST8 isolates, Balb/c mice were infected with a lethal and a non-lethal isolate or injected with NaCl; 1, 4, and 7 days after infection the cellular response in liver and spleen was determined. CD4\(^+\) and CD8\(^+\) T cells, among them activated cells, thus CD4\(^+\)CD69\(^+\) and CD8\(^+\)/CD69\(^+\) T cells, as well as B cells, myeloid cells, and PMN were detected. In addition, TCR gamma-delta- (\(\gamma\delta\)) T cells, among them activated cells, thus TCR \(\gamma\delta\)^+CD69^+T cells were determined in the spleen. Mean fluorescent intensity (MFI) was measured for activated T cells. Among myeloid cells, highly Mac1-expressing cells were regarded as DCs. The response on day 4 after infection was most striking for all cells in both organs (Fig. 7 + 8).

In the liver, infection of mice with the lethal or non-lethal isolate did not change the percentage of CD4\(^+\)-T cells compared to mock-treated mice. Nevertheless, the lethal isolate induced activation of 80% of CD4\(^+\)-T cells already on day 4, as indicated by the up-regulation of CD69 (Fig. 7A). Mice infected with a lethal isolate, showed a strong decrease in the percentage of CD8\(^+\)-T cells own to 5% on day 4. More than 50% of this 5% - population was activated, as indicated by the up-regulation of CD69 and the MFI of CD8\(^+\)/CD69\(^+\)-T cells was enhanced (Fig. 7B). The B cell fraction decreased more strongly, after infection with the lethal than with the non-lethal isolate. The infection with the lethal isolate induced a stronger increase of DCs, than infection with the non-lethal isolate when compared with cells of mock-treated mice. On day 4, both isolates induced a PMN infiltration into the liver, but the percentage of PMN among liver cells was higher for the lethal than for the non-lethal isolate (Fig. 7C).
Liver

A)

B)

C)

Fig. 7 Cellular response towards infection detected in liver
Response of T cells (A+B), B cells, myeloid cells, and PMN (C) NaCl injection (grey bars) and infection with a lethal (black bars) and a non-lethal (white bars) isolate in the liver of Balb/c mice on days 1, 4, and 7. A) CD4⁺-T cells, CD4⁺/CD69⁺-T cells and MFI of CD4⁺/CD69⁺-T cells. B) CD8⁺-T cells, CD8⁺/CD69⁺-T cells and MFI of CD8⁺/CD69⁺-T cells. C) B cells, myeloid cells, and PMN
In the spleen, similar results were obtained. Infection of mice with lethal or non-lethal isolates did not change the total percentage of CD4⁺-T cells compared to mock-treated mice. Nevertheless, both isolates induced activation of 20% of CD4⁺-T cells on day 4 as indicated by the up-regulation of CD69 (Fig. 8A). The MFI for CD4⁺/CD69⁺- T cells of mice infected with the lethal isolate was 1.6 fold higher than of those infected with the non-lethal isolate (Fig. 8A). Both, lethal and non-lethal isolates induced a modest activation of CD8⁺-T cells on day 4 without a change in the proportion of CD8 T cells (Fig. 8B). The lethal isolate induced a strong decrease in γδ⁺-T cell fraction on day 4. About 40% of the remaining γδ⁺-T cell cells were activated as indicated by the up-regulation of CD69 (Fig. 8C). B cells of mice infected with bacteria showed a decrease on day 1 of infection compared to mock-treated mice. Both isolates induced an increase in the percentage of DCs on all days of infection, which was stronger for the lethal than non-lethal isolate on the days 4 and 7. Also on day 4 and 7, both isolates induced a PMN infiltration into the spleen, but the percentage PMN among spleen cells was higher for the lethal than for the non-lethal isolate (Fig. 8D).
**Fig. 8 Cellular response towards infection detected in spleen**

Response of T cells (A+B+C), B cells, myeloid cells, and PMN (D) NaCl injection (grey bars) and infection with a lethal (black bars) and a non-lethal (white bars) isolate in the spleen of Balb/c mice on days 1, 4, and 7. A) CD4⁺-T cells, CD4⁺/CD69⁺-T cells and MFI of CD4⁺/CD69⁺-T cells. B) CD8⁺-T cells, CD8⁺/CD69⁺-T cells, and MFI of CD8⁺/CD69⁺-T cells. C) γδ(GD)⁺-T cells, γδ⁺/CD69⁺-T cells and MFI of γδ⁺/CD69⁺-T cells. D) B cells, myeloid cells, and PMN.
Sepsis induced by MRSA ST8 isolates in Balb/c mice

Due to their genetic background, certain mouse strains are more susceptible to infections than others (18). This is also the case for Balb/c mice, which are known to be more susceptible to bacterial infections than C57BL/6 mice. To assure that the observed pathogenicity of the different isolates was pathogen-dependent in C57BL/6 mice (see chapter II) and not due to host related factors, we used 8 of 10 MRSA ST8 isolates (5 lethal and 3 non-lethal strains, as determined in C57BL/6 mice) to induce sepsis in Balb/c mice. Isolates, which were lethal in C57BL/6 mice showed a significantly higher mortality than non-lethal isolates ($P = 0.045$), indicating the virulence pattern was similar in C57BL/6 and Balb/c mice. As expected, Balb/c mice were more susceptible and even isolates, which were non-lethal in C57BL/6 mice, were lethal in Balb/c mice. However, the strains, which were lethal in C57BL/6 mice caused also significantly higher mortality in Balb/c mice (Fig. 9).

![Figure 9. Percentage survival of Balb/c mice infected with MRSA ST8 isolates](image)

Percentage survival of Balb/c mice infected with 5 isolates, which were lethal (closed circles) and 3 isolates, which were non-lethal (open circles) in C57BL/6 mice. Median inoculum of $2.9 \times 10^5$ CFU/mouse. (Log-rank test in Kaplan-Meier analysis, $P = 0.045$). 3 mice were infected per isolate.
DISCUSSION

The clonality of 10 MRSA isolates, which were characterized earlier by different molecular typing methods, was confirmed by MLVA. Despite their clonality, the 10 MRSA isolates induced infection with a variable outcome in the murine sepsis model. To investigate the variability in outcome, we studied further pathogen phenotypes and evaluated differences in the host response towards these isolates.

We studied first the adhesion and clumping capacity of our isolates, and did not find any relation between adhesion, clumping, and virulence in the murine sepsis model. Wann et al. demonstrated, that fibronectin-binding protein A (FnBPA) is able to bind fibrinogen via its N-terminal A domain and to compensate binding to soluble fibrinogen in the absence of ClfA/B (23). However, such an effect was not detectable in the clfA/B-deletion mutant, which showed very low adhesion, but no clumping. Since all isolates were harboring the fnbp and clf genes, the heterogeneous adhesion and clumping profile possibly depended on a heterogeneous adhesin protein expression.

The ability to attract PMN was similar for all 10 MRSA supernatants. Dialyzed supernatants of overnight cultures enhanced chemotaxis of PMN up to 5 fold, suggesting that inhibitory molecules were eliminated by dialysis. Since a membrane pore size of 3.5 kDa was used for dialysis, the 14.1 kDa inhibitory protein CHIPS was still present in the dialyzed supernatants, and was therefore unlikely to play an important role; it is proposed that other secreted unknown molecules might inhibit PMN migration. In contrast to our results, Veldkamp et al. demonstrated that chemotaxis towards chemotactants like fMLP or C5a was strongly reduced after pre-incubation of PMN with dialyzed supernatants. But since this group used a 10 kDa pore membrane, it is possible that those inhibitory molecules were in the range from 3.5 to 10 kDa and therefore no more present in our supernatants (22). Due to its size of 60 kDa, also Eap was still present in our dialyzed supernatants. But since Eap is mainly inhibiting extravasation by interfering with receptors on endothelial and epithelial cells its
inhibitory function is negligible in our in vitro model, which was only containing PMN (7). Killing of bacteria by PMN was very heterogeneous within the first 30 min, but efficient for all isolates after 3 h. However, since the MOI was variable among the isolates, a clear interpretation of the results is difficult. Several groups showed that apoptosis was faster upon phagocytosis of S. aureus than spontaneous apoptosis and that it occurred already one hour after phagocytosis (12, 13). In contrast to these findings, we demonstrated, that apoptosis of PMN, when co-cultured with S. aureus, did not occur earlier than 6 hours of incubation. Interestingly, bacteria did not accelerate but were decelerating the percentage of spontaneous PMN apoptosis.

Compared to the well-studied innate immune response, little is known about adaptive immunity against S. aureus infections. Though, in our study, we detected differences in T and B cell responses to a lethal versus a non-lethal isolate.

Since antigen-specific antibodies are not available in the early phase of infection, so called ‘natural antibodies’, which are secreted by B-1-B cells, might provide support to the defense against invaders (1). The decrease of B cell numbers observed in the spleen on day 1, might originate from B1-B cells migrating into the periphery and providing unspecific antibodies. Though, to confirm their presence a detection of their surface marker CD5 would be adequate.

Our data of liver and spleen suggest that the infection with the lethal isolate induced a higher level of activation in CD4⁺-T cells than the infection with the non-lethal isolate. Considering that at day 4 adaptive immunity is not yet available it is possible that the activated CD4⁺-T cells are a particular subset of T cells such as NKT cells that do not require priming.

Since variations in the response to the lethal and non-lethal isolates seem to contribute to the different outcome of infection, the role of different cell subsets will be investigated in more detail in a further study.

In summary PMN chemotaxis, susceptibility to PMN killing, and apoptosis did not differ between lethal and non-lethal isolates, thus the innate host response did
not allow any conclusion on pathogen properties. Pilot measurements of the specific immune response point to a possible relation between the extent of T cell activation, the decrease in B cell number and lethal outcome. Further investigations are needed to relate and identify the bacterial antigens in the lethal strain with the strong T cell activation.


pulmonary infection with Listeria monocytogenes: differential susceptibility of BALB/c, C57BL/6 and DBA/2 mice. Microbes Infect 7:600-11.


CONCLUDING REMARKS

The main focus of this thesis is the problematic issue of antibiotic resistance in *Staphylococcus aureus* and its still unknown impact on virulence in localized and systemic infections. Since *S. aureus* per se is a pathogen that inherits a multitude of virulence factors enabling resistance to the host in multitudinous ways, the ability to evolve and acquire resistance mechanisms to different classes of antibiotics enhances its pathogenicity to a greater extent (6). The development of antibiotic resistance types in *S. aureus* that were addressed in this study, are illustrated in the following scheme:

MRSA evolve from successful MSSA clones by the acquisition of SCCmec and bear the risk to become GISA upon exposure to glycopeptides or to become GRSA upon acquisition of vanA resistance from *Enterococci* (2, 4, 7).

Due to the instability of *Tn1547* in *S. aureus*, GRSA are a rare alternative to GISA. Though, this will change and will become a further serious issue once this mobile genetic element, carrying the *van* genes of *Enterococci*, will stably insert into the *S. aureus* genome. In contrast to GRSA, GISA is already frequently isolated from patients. In our first study, we could show that a development of the GISA phenotype is associated with a fitness cost of the pathogen. Thus, with our *in vitro* generated GISA strain, we confirmed several phenotypic alterations that were also observed in clinical isolates. In addition, we demonstrated that the acquisition of resistance was associated with a decreased expression of virulence genes. Interestingly, the advantage of this phenotype in the presence of glycopeptides turned into a disadvantage in the absence of glycopeptides, especially in an infected host. The absence of selective drug pressure and the presence of host factors resulted in a restoration of fitness and virulence at the cost of resistance. Compensatory mutations that were underlying the restored
fitness in our study mainly affected the resistance phenotype, indicating the next upcoming problem besides the emergence of GRSA: the emergence of fitter GISA variants that are able to compensate their fitness burden while keeping both their resistance and their virulence.

In our second study, we were confronted with two pandemic MRSA clones that had already proven their success by their worldwide dissemination. Isolates of both clones were carrying SCCmec IV, which was shown not to impair the fitness of the bacterium as it was observed for its SCCmec I counterparts in vitro (3, 5). Moreover, the characteristic to accumulate further resistance and virulence determinants typical for strains carrying SCCmec II and III, forming them to highly virulent and multi-resistant pathogens, is absent in SCCmec IV strains. Thus, the high virulence of certain MRSA clones in our study, reflected in mouse mortality, and their success, reflected in their dissemination, is suggesting a crucial promoting role of their toxins and adhesins. Avid adhesive properties were already shown to promote epidemicity as shown for the epidemic Brazilian clone (1). However, the variable virulence among isolates of the same clone inheriting the same toxin and adhesin gene profile questions the role of toxins and adhesins as key players for pathogenicity. Despite the high discriminatory potential of the molecular typing methods that are used, the influence of host factors and drug treatment on the pathogen during an infection is unknown. Therefore, a characterization of these isolates by whole genome sequencing would be more adequate to detect differences, which are decisive for the different outcome in mortality and the different specific host response to lethal and non-lethal isolates, as it was described in the last chapter of this thesis. Identification of the presence or the absence of certain factors leading to the different stimulation of host cells, might also help to understand, why the host is on the one hand able, but on the other hand unable to cope with the pathogen.


