Insights into selected genomic adaptation strategies of *Staphylococcus aureus*

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Declaration of Independence

Herewith I certify that I have prepared and written my thesis entitled “Insights into selected genomic adaptation strategies of *Staphylococcus aureus*” independently and that I have not used any sources and aids other than those indicated by me. I also declare that I have not submitted the dissertation in this or any other form to any other institution as a dissertation.

Contents

Summary

Staphylococcus (*S.*) *aureus* is a ubiquitous Gram-positive, spherical bacterium commonly isolated from humans, domestic-, livestock-, wildlife-, and even aquatic animals. The bacteria also survive and continue their growth on abiotic material in natural and artificial environments. Transmission of the immobile bacterium often occurs through direct contact with infected living beings or contaminated objects, whereby *S. aureus* is continuously forced to adapt to altered environmental conditions, including various host species. To overcome these challenges, members of the Genus *Staphylococcus* possess numerous strategies that include genomic alterations as well as the uptake or loss of mobile genetic elements (MGEs).

The enormous adaptive capabilities and tendencies to accumulate genes encoding resistance traits define *S. aureus* as a pathogen of great clinical importance in human and veterinary medicine. Further insights into the adaptive strategies of this notorious pathogen are crucial, especially regarding the development of novel treatment options in case of infection. Against this background, changes in the essential regulatory quorum sensing (QS) system that promote bacterial niche adaptation were identified and characterised in this cumulative work. In addition, the genetic structure and putative role of the MGE mediating broad-spectrum β-lactam resistance in methicillin resistant *S. aureus* (MRSA) was investigated in detail using livestock-associated (LA)-MRSA as a prime example for adaptation to challenging environments.

The data presented in Publication I revealed core genome alterations within the accessory gene regulator (*agr*) system, especially in the *agr*A and *agr*C genes, that led to variation or even silencing of the bacterial QS capabilities. Moreover, these changes occurred independently in strains with completely different genomic backgrounds of various animal origins across Europe as well as in closely related human isolates, clearly indicating the importance of QS regarding adaptive changes and not at least bacterial evolution. These adaptive modulatory changes enable the bacteria, beyond others, to survive among competing staphylococci belonging to the resident microbiota, simultaneously reducing their perceptibility by the host's immune system while enhancing their biofilm formation capabilities. Consequently, *S. aureus*, lacking a functional *agr* system, saves the energy otherwise spend on QS and can conquer challenging environments since this deficit obviously promotes inter-species spread between different animal species, and, not at least, humans.

The second part of this cumulative thesis focuses on a specific kind of MGEs, the Staphylococcal Cassette Chromosome (SCC) *mec* elements. A mechanism leading to the loss of a complete SCC*mec* element from its genomic integration site was reconstructed in

detail for an MRSA sequence type (ST) 398 strain and described in Publication II. The investigated excision of the complete SCC*mec*Vc element is suggested to be the result of homologous recombination within a repetitive sequence between the end of intergenic spacer region 1 and the first 48 bp of the large serine recombinases (*ccr*). Furthermore, the presence of an operon that encodes the machinery for autonomous replication of the deleted SCC*mec*Vc element was revealed.

Livestock-associated environmental conditions that are in particular associated with industrial pig farming were previously discussed as a potential trigger for the loss of SCC*mec* elements. To mimic conditions commonly associated with pig farming, LA-MRSA were exposed to porcine serum, an increased ammonia concentration and a combination of both for 10 min or 60 min. Since *ccr* genes are known for their capability to induce the elements' uptake and loss, the growth conditions evaluated here did not significantly induce *ccr* transcription compared to the unchallenged controls.

A detailed study of the deletion leading to the loss of a complete and functional SCC*mec* at defined genomic sites, on the other hand, strongly suggests a complex biological role of this particular event. While the induction of the *ccr* genes seems to depend on a massive cell- or DNA damage that leads to a strong activation of the SOS response, a far more efficient pathway for the horizontal spread of SCC*mec* is the occasional loss of complete elements at certain redundant "breaking points", as described in Publication II. Since SCC*mec* elements possess the machinery for autonomous replication, this pathway might be of utmost epidemiological importance for the emergence, maintenance and spread of methicillin resistance.

Both adaptation strategies investigated in this cumulative work highlight that the evolution of *S. aureus* will continue unabated. However, the unwearyingly evolution of these bacteria will lead to further problems in combating infections caused by *S. aureus*. The development of completely new treatment options, which consider the current knowledge on adaptation strategies of *S. aureus,* is therefore of clinical importance. Additionally, a holistic one-health approach that engages scientists from human and veterinary medicine as well as agricultural and environmental sciences is necessary to prevent further trans-sectoral transmission and spread by reducing the anthropogenic selective pressure that leads to resistance accumulation in bacteria.

Zusammenfassung

Staphylococcus (*S.*) *aureus* ist ein ubiquitär vorkommendes Gram-positives, kugelförmiges Bakterium, welches vielfach aus Proben von Menschen, Haus-, Nutz- und Wildtieren sowie von Meeressäugern isoliert wird. Das Bakterium kann auch auf abiotischem Material in natürlicher und künstlicher Umgebung überleben und dort sein Wachstum fortsetzen. Die Übertragung des unbeweglichen Bakteriums erfolgt häufig durch den direkten Kontakt mit infizierten Lebewesen oder kontaminierten Gegenständen, wodurch *S. aureus* ständig gezwungen ist, sich an veränderte Umweltbedingungen, einschließlich verschiedener Wirtsarten, anzupassen. Um diese Herausforderungen zu bewältigen, verfügen die Mitglieder des Genus *Staphylococcus* über zahlreiche Strategien, zu denen genomische Veränderungen sowie die Aufnahme oder der Verlust von mobilen genetischen Elementen (MGE) gehören.

Die enormen Anpassungsfähigkeiten und die Tendenz zur Akkumulation von Genen, die für Resistenzeigenschaften kodieren, machen *S. aureus* zu einem Erreger von großer klinischer Bedeutung in der Human- und Veterinärmedizin. Weitere Erkenntnisse über die Anpassungsstrategien dieser Bakterien sind im Hinblick auf die Entwicklung neuer Behandlungsmöglichkeiten im Falle einer Infektion von entscheidender Bedeutung. Vor diesem Hintergrund wurden in dieser kumulativen Arbeit Veränderungen im regulatorisch wichtigen „Quorum-Sensing“ (QS)-System identifiziert und charakterisiert, welche die bakterielle Nischenanpassung unterstützen. Ergänzend dazu wurde die genetische Struktur und die mutmaßliche Rolle des MGE, welches die Breitspektrum-β-Lactam-Resistenz bei Methicillin-resistentem *S. aureus* (MRSA) vermittelt, eingehend untersucht, wobei hier der mit Nutztieren assoziierte (engl. livestock-associated (LA)) MRSA als Paradebeispiel für die Anpassung an schwierige Umgebungen dient.

Die in der Publikation I vorgestellten Daten zeigen genomische Varianten innerhalb des akzessorischen Genregulatorsystems (*agr*), insbesondere in den *agr*A- und *agr*C-Genen, die zu einer Veränderung oder sogar Unterdrückung der bakteriellen QS-Fähigkeiten führten. Darüber hinaus traten diese Veränderungen unabhängig voneinander in Stämmen mit völlig unterschiedlichen phylogenetischem und geografischem Hintergrund, bei unterschiedlichen Tierarten sowie in eng verwandten menschlichen Isolaten auf, was eindeutig auf die Bedeutung von QS für adaptive Veränderungen und nicht zuletzt für die bakterielle Evolution hinweist. Diese adaptiven modulatorischen Veränderungen ermöglichen es den Bakterien, unter anderem unter konkurrierenden Staphylokokken der residenten Mikrobiota des Wirtes zu überleben und gleichzeitig ihre Perzeption durch das Immunsystem zu verringern, während die Fähigkeiten zur Biofilmbildung verstärkt werden. Infolgedessen verfügen

S. aureus, denen ein funktionsfähiges *agr*-System fehlt, über einen energetischen Vorteil und können schwierige Umgebungen erobern, da dieses Defizit offensichtlich die Ausbreitung zwischen verschiedenen Tierarten und nicht zuletzt dem Menschen fördert.

Der zweite Teil dieser kumulativen Arbeit konzentriert sich auf eine spezielle Art von MGEs, die „Staphylococcal Cassette Chromosome“ (SCC) *mec*-Elemente. Ein Mechanismus, der zum Verlust eines kompletten SCC*mec*-Elements von seiner genomischen Integrationsstelle führt, wurde für einen MRSA Sequenztyp (ST) 398-Stamm im Detail rekonstruiert und in Publikation II beschrieben. Die untersuchte Exzision des vollständigen SCC*mec*Vc-Elements ist wahrscheinlich das Ergebnis homologer Rekombination innerhalb einer repetitiven Sequenz zwischen dem Ende der „intergenen Spacer“-Region 1 und den ersten 48 bp der großen Serin-Rekombinasen (*ccr*). Außerdem wurde das Vorhandensein eines Operons nachgewiesen, welches die Maschinerie für eine autonome Replikation des deletierten SCC*mec*Vc-Elements kodiert.

Mit der Tierhaltung einhergehende Umweltbedingungen, die insbesondere mit der industriellen Schweinehaltung assoziiert sind, wurden zuvor als möglicher Auslöser für den Verlust von SCC*mec*-Elementen diskutiert. Um Bedingungen nachzuahmen, die üblicherweise mit der Schweinehaltung in Verbindung gebracht werden, wurden LA-MRSA jeweils 10 Minuten oder 60 Minuten mit Schweineserum und einer erhöhten Ammoniakkonzentration sowie einer Kombination aus beidem behandelt. Die *ccr*-Gene sind dafür bekannt, dass sie die Aufnahme und den Verlust von Elementen induzieren können, jedoch führten die hier untersuchten Wachstumsbedingungen im Vergleich zu den unbelasteten Kontrollen zu keiner signifikanten Induktion der *ccr*-Transkription.

Eine detaillierte Untersuchung des Vorgangs, welcher zum Verlust eines vollständigen und funktionsfähigen SCC*mec* an bestimmten Stellen im Genom führen kann, deutet dagegen auf eine komplexe biologische Rolle dieses Ereignisses hin. Während die Induktion der *ccr*-Gene von einer starken Aktivierung der SOS-Antwort in Folge einer massiven Zell- oder DNA-Schädigung abzuhängen scheint, ist ein weitaus effizienterer Weg für die horizontale Verbreitung von SCC*mec* der gelegentliche Verlust kompletter Elemente an bestimmten redundanten „Sollbruchstellen“, wie in Publikation II beschrieben. Da SCC*mec*-Elemente die Maschinerie zur autonomen Replikation besitzen, könnte dieser Weg von größter epidemiologischer Bedeutung für die Entstehung, Aufrechterhaltung und Verbreitung der Methicillin-Resistenz sein.

Beide Anpassungsstrategien, die in dieser kumulativen Arbeit untersucht wurden, machen deutlich, dass die Evolution von *S. aureus* ungebremst weitergehen wird. Die unermüdliche Evolution dieser Bakterien wird jedoch zu weiteren Problemen bei der Bekämpfung von

S. aureus verursachten Infektionen führen. Die Entwicklung völlig neuer Behandlungsmöglichkeiten, die das aktuelle Wissen über die Anpassungsstrategien von *S. aureus* berücksichtigen, ist daher von klinischer Bedeutung. Darüber hinaus sollte für die Zukunft ein ganzheitlicher „One-Health“-Ansatz in Betracht gezogen werden, der Wissenschaftlerinnen und Wissenschaftler aus der Human- und Veterinärmedizin sowie den Agrar- und Umweltwissenschaften einbezieht, um eine weitere sektorübergreifende Übertragung und Ausbreitung resistenter Bakterien zu vermindern, indem der anthropogene Selektionsdruck, welcher zu einer Resistenzakkumulation führt, verringert wird.

List of Figures and Tables

Figures

Tables

Abbreviations

aa	amino acid
agr	accessory gene regulator
AgrA	response regulator
AgrB	transmembrane endopeptidase
AgrC	sensor histidine kinase
AgrD	peptide precursor of AIP
AIP	autoinducing peptide
*arc*C	gene for carbamate kinase
*aro*E	gene for shikimate dehydrogenase
att	attachment site
*bla*I	gene for β-lactamase repressor
*bla*R1	gene for β-lactamase inducer
*bla*Z	gene for β-lactamase
bp	base pair
CA	community-associated
CC	clonal complex
Cch	helicase
CCPol	putative A-family DNA polymerase
ccr	cassette chromosome recombinase
chip	gene for chemotaxis inhibitory protein
DNA	deoxyribonucleic acid
DR	direct repeat
*drp*35	gene for lactonase
DUF	domain of unknown function
dus	gene for tRNA-dihydrouridine synthase
*glp*F	gene for glycerol kinase
gmk	gene for guanylate kinase
*gra*SR	gene for a two-component regulatory system
HA	healthcare-associated
HGT	horizontal gene transfer
HIV	human immunodeficiency virus
hla	gene for α-hemolysin
hlb	gene for β-hemolysin
HMG-CoA	Hydroxy-3-methylglutaryl-Coenzyme A
IEC	immune evasion cluster

IS	insertion sequence
ISS	integration site sequence
IWG-SCC	International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements
J-region	joining / “junkyard”-region
kb	kilobase (pair)
LA	livestock-associated
LexA	repressor of SOS genes involved in DNA reparation
log2FC	log2(fold-change)
LP1413	short ssDNA-binding protein
M.	*Macrococcus*
Mbp	megabase pair
*mec*A	gene for methicillin resistance
*mec*C	gene for methicillin resistance
*mec*I	gene for methicillin resistance repressor
*mec*R1	gene for methicillin resistance sensor inducer
MGE	mobile genetic element
MIC	minimum inhibitory concentration
MLST	multilocus sequence typing
MP	“middle” protein
MRSA	methicillin resistant *Staphylococcus aureus*
MSSA	methicillin susceptible *Staphylococcus aureus*
MVA	mevalonate
*mva*S	gene for HMG-CoA synthase
nt	nucleotide
ORF	origin of replication
*orf*X	former designation of *rlm*H
*orf*Y	former designation of *dus*
PBP	penicillin-binding protein
PCR	polymerase chain reaction
PSM	phenol soluble modulin
pta	gene for phosphate acetyltransferase
PVL	Panton-Valentine Leucocidin
QS	quorum sensing
RecA	bacterial recombinase A
*rlm*H	gene for ribosomal RNA large subunit methyltransferase H
RNA	ribonucleic acid

Rot	repressor of toxins
*rpo*B	gene for a subunit of the RNA polymerase
S.	*Staphylococcus*
sak	gene for staphylokinase
SaUGI	staphylococcal uracil-DNA glycosylase inhibitor
SCC	Staphylococcal Cassette Chromosome
SCC*mec*	Staphylococcal Cassette Chromosome *mec*
scn	gene for staphylococcal complement inhibitor
SigB	alternative sigma factor
SNP	single nucleotide polymorphism
spa	gene for staphylococcal protein A
Spx	global transcriptional regulator
ssDNA	single-stranded DNA
SspB	type I extracellular signal peptidase
ST	sequence type
tpi	gene for triosephosphate isomerase
TSS	transcription start site
U.S.	United States
UV	ultraviolet
*van*A	gene for vancomycin resistance
VISA	vancomycin intermediate *S. aureus*
*vra*SR	gene for a two-component regulatory system
VRSA	vancomycin resistant *S. aureus*
*wal*KR	gene for a two-component regulatory system
WHO	World Health Organization
*yqi*L	gene for acetyl coenzyme A acetyltransferase

1 Literature review

1.1 A brief historical overview of the discovery of staphylococci

In the 19th century, the particular nature of infectious diseases caused by bacteria was poorly understood, and subsequent deaths were insufficiently investigated [1]. Although the Dutch scientist Antoni van Leeuwenhoek saw and described microorganisms using a light microscope's magnifying skills 200 years earlier [2], scientists were still divided into two fractions [1]. One group favoured the idea that noxious emanations ("miasmas"), originating from mouldering, putrefying, and decomposing sources, were responsible for the onset of illnesses [3]. Other scientists followed the idea that small germs, invisible to the eye, were the actual cause of infectious diseases. The Hungarian surgeon Ignaz Semmelweis and the London physician John Snow pioneered the germ theory [1, 3]. However, it was not until 1857, when the work of Louis Pasteur became public, that the latter approach gained acceptance [1]. Pasteur's work demonstrated that microorganisms are responsible for the fermentation, spoilage, or contamination of organic substances, thereby proving that bacteria play an essential role in the development of diseases (reviewed in [1]).

In subsequent years, a large number of microorganisms were discovered and described, and the importance of certain bacterial species as causative agents of distinct infectious diseases became increasingly apparent. For instance, the German surgeon Theodor Billroth discovered spheres arranged in chains while investigating wound smears, which he denominated as streptococci [4]. A few years later, the German physician Robert Koch characterised the causative agent for the until then deadly infectious disease called phthisis (today referred to as tuberculosis [5]), the bacterium *Mycobacterium tuberculosis*. He further recognised an association between bacteria and infectious diseases. After developing solid nutrient media for cultivating bacteria on a plate, introducing new staining methods, and animal experiments, Koch finally demonstrated a causal relationship between a pure bacterial culture and a specific infectious disease [6]. These prerequisites became known as Koch's postulates and, briefly summarised, included the following four criteria [6]:

1. The bacteria must be detectable at the infected site of either humans or animals.
2. The organisms need to grow on solid nutrient media as a pure culture.
3. A pure culture of the organism must be capable of causing the respective disease in animals.
4. Re-growth of bacteria that caused the disease is possible from samples taken from infected sites of the animals.

Despite the continuously increasing knowledge about the importance of bacteria as causative agents of infections at that time, cleanliness and hygiene left much to be desired. The operating rooms, surgical instruments, surgical gowns, and even the surgeons' hands were hardly cleaned between patients subjected to medical care. The risk of individual patients dying during or after surgery was very high, not at least because suppuration of infected incisional sites was considered an important stage of wound healing [7].

Inspired by the work of Louis Pasteur, the British surgeon Joseph Lister pioneered the principles of antisepsis. The first antiseptic agent he applied to patients' skin before surgery was carbolic acid. As a result, he significantly improved wound healing while pus formation was largely avoided [1, 8]. Around 1880, Alexander Ogston, a British surgeon, was intrigued by Lister's observations on wound healing. He intended to reveal the reason for the effective prevention of wound infections by using Lister's antiseptics before surgery [7]. For this purpose, Alexander Ogston took a swab from an abscess of one of his patients. Examining the smear with a light microscope, he saw "micrococci" arranged in groups or chains [4, 7]. Through further research, including trials with guinea pigs, mice, and eggs, Alexander Ogston proved that these "micrococci" were responsible for pus formation. In addition, he recognised that different forms and shapes of microorganisms were present in the investigated samples [4, 7]. Some coccoidal bacteria were arranged in chains, which Theodor Billroth had already described as streptococci several years earlier. However, Alexander Ogston additionally identified spherical bacteria that appeared in groups, which he termed staphylococci, derived from the Greek word "Staphylé" for grape and "Kókkos" for seed or berry [7, 9, 10].

Pigmentation differences among distinct staphylococcal colonies were recognised a few years later, in 1884, by the German physician Friedrich Julius Rosenbach. He designated the pigmented colonies after the Latin word "*aureus*" for gold as *Staphylococcus* (*S.*) *aureus* and the non-pigmented bacteria as *S. albus* (today referred to as *S. epidermidis*) after the Latin word "*albus*" for white [10, 11].

1.2 Taxonomy and characteristics of staphylococci

Staphylococci are immobile, spherical bacteria that belong to the family of *Staphylococcaceae* [12]. The bacteria are 0.5 to 1.5 µm in size, resilient to dehydration, have a growth optimum at 37°C, and exhibit a high pH tolerance ranging from pH 4.0 to pH 10.0 [6, 13]. In bacterial smears, staphylococci are typically arranged in irregular grape-like clusters. However, the cocci also occur in short chains, in pairs, or even as single cells (Figure 1) [14].

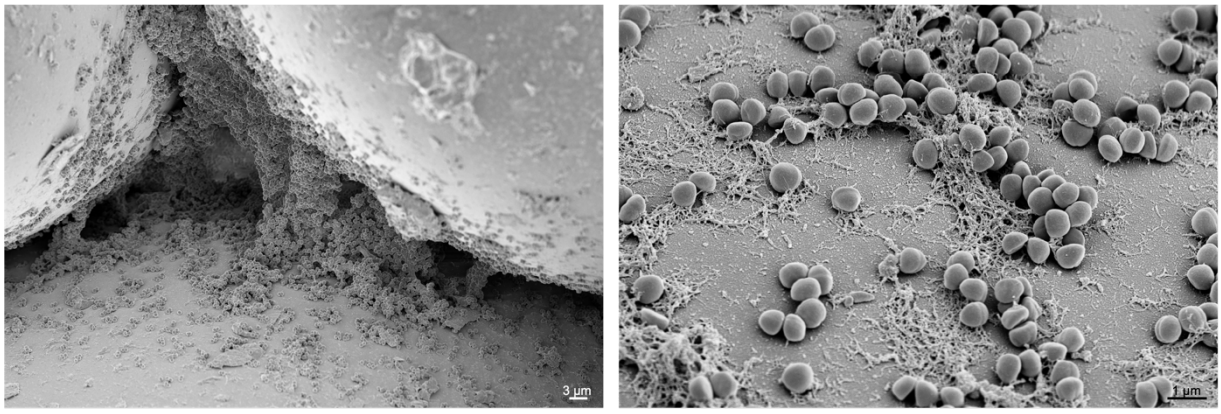

Figure 1: Scanning electron microscopy images of *Staphylococcus aureus*

Scanning electron microscopy pictures of methicillin resistant *S. aureus* (MRSA) and their extracellular matrices during biofilm formation on porous glass beads [15] at different magnifications. The left image was taken at 3000x magnification and shows a niche in the porous glass bead where the bacteria accumulate and grow. In the image on the right, a close-up of the staphylococci at a magnification of 20000x allows inspection of bacterial form, shape, position, and cell sizes (0.8 – 1.2 µm). The images were taken by Dr. Christoph Schaudinn, ZBS 4, Robert Koch Institute.

Furthermore, staphylococci are Gram-positive bacteria [6], since they possess a cell wall of 20 – 40 nm thickness [16]. The cell wall is composed of several layers of peptidoglycan (murein), which is responsible for the robustness and the shape of the cells [6]. Peptidoglycan consists of repeating N-acetylglucosamine and N-acetylmuramic acid disaccharides, which are joined via a β-1,4-glycosidic bond by transglycosylation. The stem peptide L-alanine–D-iso-glutamine–L-lysine–D-alanine–D-alanine of the N-acetylmuramic acid molecules binds to a corresponding N-acetylmuramic acid molecule of an adjacent layer. These cross-links subsequently confer considerable cell stability and contribute to the bacterium's resilience to external challenges [6, 17, 18].

The genome of *S. aureus* is circular, with an average size of about 2.85 Mbp containing approximately 2600 genes [19], including a basic set of genes shared by all *S. aureus* strains, known as the core genome [20]. These conserved genes, commonly referred to as

housekeeping genes, are involved in basic cellular processes responsible for replication, transcription, translation, recombination, and cell assembly [19, 20]. Furthermore, the profile yielded by the specific numbers assigned to allelic variants of seven defined housekeeping genes, namely carbamate kinase (*arc*C), shikimate dehydrogenase (*aro*E), glycerol kinase (*glp*F), guanylate kinase (*gmk*), phosphate acetyltransferase (*pta*), triosephosphate isomerase (*tpi*) and acetyl coenzyme A acetyltransferase (*yqi*L) is used to classify *S. aureus* isolates into specified sequence types (ST) [19, 21]. This method of identifying and naming the clonal lineages is called multilocus sequence typing (MLST) [21]. Closely related STs cluster within clonal complexes (CC) [19, 21].

1.3 Genomic alterations contribute to the evolution of *S. aureus*

S. aureus is continuously exposed to external challenges, such as contact with novel host species or changing environmental conditions, including substrate deficiency (“starvation”), an increased salt concentration, extreme temperatures, and the presence of toxins or antibiotic agents [6]. However, *S. aureus* can adapt to these changes by utilising various strategies in which mutations and mobile genetic elements are of utmost importance.

1.3.1 Mutations

S. aureus reproduces by cell division, i.e., one bacterial cell subsequently divides into two daughter cells. During this process, the DNA of the parent cell needs to be replicated, and errors such as base pair mismatches occasionally occur. DNA polymerases and various repair systems usually detect and remove these alterations during the proofreading process of the novel DNA strands. However, some variations in the genomic sequence remain which subsequently cause heritable changes in the bacteria [6, 22]. A single nucleotide change (substitution) can lead to a silent, missense, or nonsense mutation. Briefly summarised, silent mutations are characterised by the fact that the nucleotide substitution generates a new base triplet (codon) encoding the same amino acid as the replaced codon, and the resulting peptide or protein stays the same. On the contrary, a missense mutation alters the genetic code and causes a change in the base triplet, leading to a different amino acid. This subsequently alters the resulting peptide or protein and may modify or even impair partially or totally its function. A nonsense mutation originates from changes that lead to stop codons, which causes a premature end of a coding sequence. This change often results in a truncated, defective or functionless protein [22]. Furthermore, any deletion or insertion of nucleotides, which are not multiples of three, in a genome sequence is accompanied by a change of the reading frame, known as a frameshift mutation [22].

Overall, these natural and spontaneous mutations occur at a frequency of $2.0 – 2.8 \times 10^{-10}$ per nucleotide per generation in *S. aureus* [23]. However, the natural mutation frequency increases when the DNA is exposed to damaging factors, including but not limited to harmful physical or chemical influences, enhanced temperature, or UV radiation [6, 24].

1.3.2 Mobile genetic elements

Mobile genetic elements (MGEs) consist of DNA sequences, that often include genes encoding for enzymes and proteins required for their intra- and intercellular movements, as well as resistance and / or virulence factors [25]. Although MGEs frequently harbour genes that provide beneficial functions in challenging environments, these elements are not present in every cell of a particular bacterial species and therefore belong to the accessory genome [19, 20]. Nevertheless, MGEs can comprise up to 20 % of the total bacterial genome of a particular strain [26].

MGEs can be exchanged between cells by horizontal gene transfer (HGT). The three main mechanisms comprise transformation, transduction, and conjugation [22]. Transformation is characterised by incorporating freely available foreign DNA, for instance from dead cells, into the bacterial cell [22, 27]. Transduction generally describes the transfer of genetic material between different bacterial cells by bacteriophages [22] and during conjugation, two cells form a physical contact before exchanging genetic material [22, 28]. This connection allows the donor cell to transfer genetic material to the respective recipient. Of note, the transfer of genetic material is limited to one direction at a time [22, 28].

Common MGEs harboured by bacteria (examples are listed in Table 1):

- **Bacteriophages** are viruses that infect bacteria. Their genetic information is encoded in either RNA, single- or double-stranded DNA, which ranges in size from 5 to 500 kb. Virulent (lytic) phages replicate within the bacterial cell and subsequently lyse it. In contrast, non-lytic phages, known as prophages, usually integrate into and replicate with the bacterial genome. However, various environmental conditions can activate the incorporated prophage, allowing it to enter a lytic cycle [20, 25].
- **Plasmids** are circular DNA molecules with a size between 3 – 200 kb. Unlike the bacterial genome, plasmids do usually not contain essential genes [6, 20].
- **Insertion sequence (IS)-elements** commonly comprise 400 – 10000 bp [29] and contain a transposase responsible for their mobilisation. Characteristically, IS-elements are flanked by inverted repeats ranging in length between 9 to 40 bp [6, 20].

- **Transposons** can be divided into two classes. Class I transposons are flanked by IS-elements and often encode antibiotic or heavy metal resistance. Class II transposons are flanked by inverted repeats and harbour genes for a transposase, a resolvase, and antibiotic resistance [6, 20].
- **Staphylococcal Cassette Chromosomes (SCC)** will be discussed in detail in Chapter 1.4.

Table 1: Common mobile genetic elements in *S. aureus*

Mobile genetic elements (MGEs) of different sizes and natures are involved in the adaptation process of *S. aureus* to environmental challenges. Some examples of clinical importance are listed to highlight the general adaptive capacity of *S. aureus* mediated by MGEs.

Mobile genetic element	**Example**			
	Designation	**Gene**	**Mechanism of action**	**Reference**
Bacteriophage	ΦSa3	*chip, scn, sak*	may integrate into the β-hemolysin of the *S. aureus* genome via site-specific recombination, often carries host-specific variants of staphylokinase, chemotaxis inhibitory protein and the staphylococcal complement inhibitory protein, belonging to the immune evasion cluster (IEC), enabling *S. aureus* to evade the host's immune system	[30, 31]
Plasmid	pT181	*tet*K	mediates resistance against tetracyclines	[32]
Staphylococcal Cassette Chromosome	SCC*mec*	*mec*A	mediates resistance against β-lactam antibiotics	[33]
Transposon	Tn6009	*merA, B*	mediates inorganic and organic mercury resistance	[34]
Insertion sequences	IS*431*	-	frequently associated with the *mec* gene complex, is capable of mediating deletion of parts of SCC*mec* elements	[35, 36]

1.4 Resistance against β-lactam antibiotics is a prominent example of adaptation

1.4.1 A historical review of penicillin

In 1928, Alexander Fleming detected mould spores on his agar plates, which were capable of inhibiting the growth of *S. aureus* in its immediate proximity. Fleming further characterised the respective fungus as *Penicillium notatum* and realised that not the mould itself but its secreted metabolites were responsible for the observed inhibition of bacterial growth [37]. This rather accidental discovery laid the foundation for further developments regarding the medical application of penicillin as an anti-infective drug [38].

However, Fleming himself was unable to isolate penicillin in suitable quantities from the respective mould due to its chemical instability. In 1939, British scientists at Oxford University became aware of Fleming's discovery. Howard Florey, Ernst Chain, Norman Heatley, and Edward Abraham then began to work on a process that enabled the isolation of penicillin. In addition to its instability, the scientists also struggled with problems such as the slow growth of *Penicillium notatum* and its low yield of penicillin. Finally, their sequential and collaborative efforts paid off when they successfully conducted a first-in-human clinical trial [37].

After this success, Florey, Chain, and the other scientists faced the challenge of not being able to produce sufficient quantities of penicillin to reach market maturity. To solve this problem, the scientists left Oxford. They moved to the United States (U.S.), where they received assistance from the Northern Regional Research Laboratory of the U.S. Department of Agriculture in Peoria, Illinois, and various American pharmaceutical companies. This collaboration finally laid the basis for the mass production of penicillin. The scientists were able to implement novel techniques and processes to improve the performance of the drug production chain [37, 39]. This achievement saved many lives during World War II, especially those suffering from bacterial pneumonia and meningitis [38, 40].

Only two years after the market introduction of penicillin, in 1942, scientists described the first staphylococcal isolates resistant to penicillin [18, 41, 42]. In response to the subsequent spread of penicillin resistance among *S. aureus*, a more penicillinase-resistant β-lactam derivative methicillin, marketed under the name Celbenin, was developed, and introduced into clinical use in 1959 [43-45]. Nevertheless, only one year later, the first methicillin resistant *S. aureus* (MRSA) was detected in a clinical case in the United Kingdom [44].

1.4.2 β-lactam resistance mechanisms in *S. aureus*

The uptake of MGEs harbouring β-lactamase encoding genes enables *S. aureus* to inactive β-lactam antibiotics [46, 47]. These penicillinases are encoded by allelic variants of *bla*Z (termed A – D) [48], a gene that is regulated by its inducer *bla*R1 and its repressor gene *bla*I [49]. The type B β-lactamases are usually integrated into the bacterial chromosome, whereas the *bla*Z variants A, C, and D are commonly located on various plasmids [50, 51]. These plasmids often harbour further genes conferring antibiotic resistance, for example, towards erythromycin, fusidic acid, the aminoglycosides kanamycin, and gentamicin or heavy metals, such as cadmium, lead, mercury, and zinc [51].

The hydrolytic activity of the β-lactamases opens the β-lactam ring structure of the antibiotic [52]. Consequently, the altered structure of the hydrolysed β-lactams inhibits their ability to attach themselves to the active site of the penicillin-binding proteins (PBP) of *S. aureus* and abolishes their anti-infective properties [42, 46].

In response to the rapid spread of β-lactamases among *S. aureus* by MGEs [42], the “classic structure” of penicillin was further developed [43, 45]. This led to the market introduction of a penicillinase-resistant β-lactam antibiotic called methicillin [43-45]. The effect of methicillin is based on its capability to form irreversible bonds with the PBPs of *S. aureus*. Especially, binding to PBP2 prevents the natural cross-linking activity of this enzyme during cell wall assembly, which is required to maintain the integrity of the bacteria (see Chapter 1.2). As a result, *S. aureus* is no longer able to form a functional cell wall during cell division in the presence of β-lactams, a fact that subsequently leads to cell lysis and characterises the bactericidal effect of this class of antibiotics (Figure 2) [53, 54]. However, the acquisition of the Staphylococcal Cassette Chromosome (SCC)*mec* element, with its methicillin resistance-mediating gene *mec*A, provides *S. aureus* with another opportunity to circumvent the effect of β-lactam antibiotics [33]. The *mec*A gene, as well as its variants, encode an additional PBP (also known as PBP2’) [33], which substitutes the function of the native PBP2 enzyme in the presence of many β-lactam antibiotics [55]. As a result, *S. aureus* harbouring the *mec* gene are still able to form a functional cell wall in the presence of β-lactam antibiotics hence mediating resistance (reviewed in [42]).

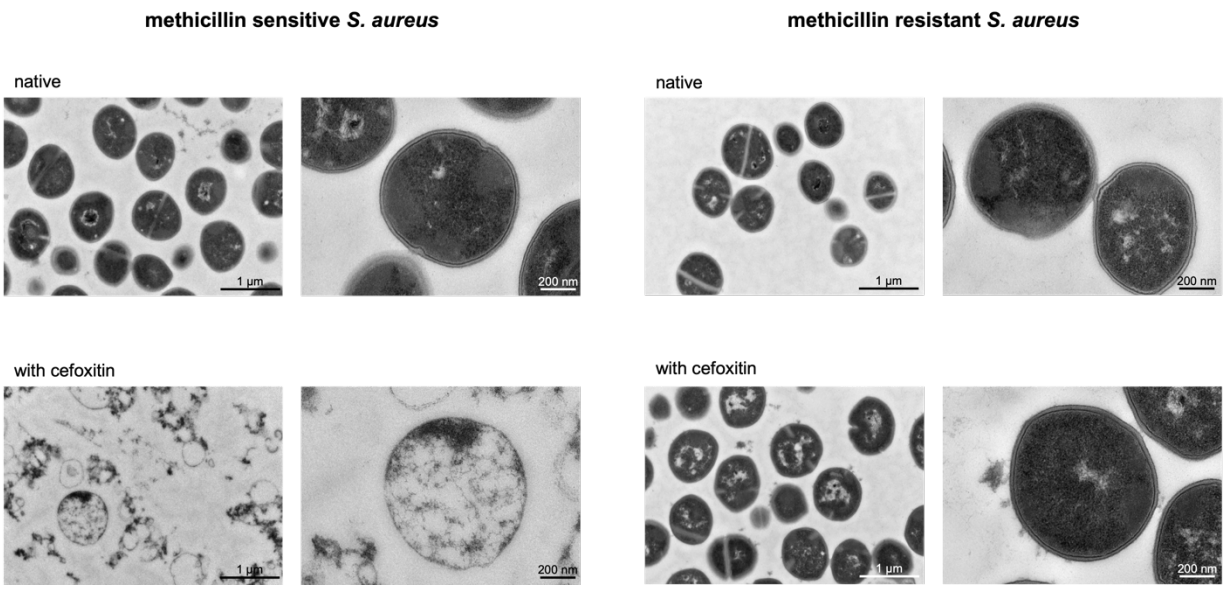

Figure 2: MSSA and MRSA in the presence and absence of cefoxitin

Transmission electron microscopy images of methicillin sensitive *S. aureus* (MSSA) and methicillin resistant *S. aureus* (MRSA) in brain heart infusion media in the presence and absence of 2 µg/ml cefoxitin (corresponds to the half minimum inhibitory concentration (MIC) according to Clinical & Laboratory Standards Institute Guidelines). Comparing the cells grown in the unchallenged media, no apparent differences in shape, size, or structure between MRSA and MSSA are detectable. However, in the presence of 2 µg/ml cefoxitin, the MRSA isolate does not show significant changes, while the MSSA sample contains mainly cell detritus. The images were taken by Tobias Hoffmann, Gudrun Holland, and Petra Kaiser, ZBS 4, Robert Koch Institute.

1.4.2.1 Currently known *mec*A homologues

Besides *mec*A, further allelic variants of the β-lactam resistance-mediating gene have been identified. Garcia-Alvarez et al. [56] described the strain *S. aureus* LGA251 isolated from a bulk milk sample, which showed resistance to oxacillin and cefoxitin. However, this strain tested negative for the *mec*A gene using standard PCR approaches [56]. After sequencing, a *mec*-like gene was identified, revealing 70 % nucleotide sequence identity with the original *mec*A. The novel gene was designated *mec*A_{LGA251} and later renamed *mec*C by the "International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements" (IWG-SCC, www.sccmec.org). Like *mec*A, *mec*C also encodes an alternative PBP (PBP2c, previously known as PBP2a_{LGA}) [33, 57, 58]. A recent study demonstrated that *mec*C-MRSA is older than previously assumed, i.e., the bacteria were already present as commensals of European hedgehogs before the introduction of antibiotics [59]. Larsen et al. [59] further suggested that the resistance in *S. aureus* arose as an adaptation process to the two β-lactam antibiotics produced by the hedgehog dermatophyte *Trichophyton erinacei*. This naturally acquired resistance likely contributed to *mec*C-MRSA having a selective advantage while exposed to antibiotics in a livestock environment, allowing them to spread to

different hosts [59]. Meanwhile, *mec*C-MRSA occur ubiquitously in humans, domestic-, zoo-, and wild animals, as well as in wastewater [60-64].

Baba et al. [65] discovered a further *mec* gene homologue in the methicillin resistant *Macrococcus* (*M.*) *caseolyticus* strain JCSC5402, which was isolated from the skin of a domestic chicken. This homologue exhibits 62 % nucleotide sequence similarity to *mec*A and is now referred to as *mec*B [66]. Routine MRSA screening has previously identified a plasmid carrying the *mec*B gene in an *S. aureus* isolate that was negative for *mec*A and *mec*C [67].

In 2017, another *mec*A homologue named *mec*D was identified in *M. caseolyticus* of bovine and canine origin [68]. At the nucleotide sequence level, *mec*D shares about 61 % sequence identity with *mec*A [68]. However, to date, the *mec*D gene has not been reported in staphylococci.

1.4.2.2 Characteristics and structure of SCC*mec* elements

The structural organisation and the genetic content of SCC*mec* elements are highly variable [69]. According to the IWG-SCC [70], SCC*mec* elements have the following characteristics in common:

- the ***mec* gene complex** consists of one methicillin resistance gene, *mec*A or *mec*C, in combination with an intact or truncated version of the regulatory genes *mec*I (repressor), *mec*R1 (sensor inducer) and transposons [70-72]
- the **cassette chromosome recombinase (*ccr*) gene complex** with the site-specific recombinase genes *ccr*A and *ccr*B or *ccr*C [70, 73]
- it integrates into the *S. aureus* genome at the 3' end (downstream) of the gene for the ribosomal RNA large subunit methyltransferase H (*rlm*H, formerly designated *orf*X) [74] referred to as the **integration site sequence (ISS)** [70]
- it is flanked by **direct repeat (DR) sequences** [70]

Furthermore, SCC*mec* elements contain so-called joining (“J”)-regions, which have been originally designated as “junkyard” regions [75, 76]. There are three J-regions, which may contain various IS, transposons, DR sequences, and antibiotic- as well as heavy metal-resistance and virulence genes [77-81].

The classification of SCC*mec* elements into 14 types described so far is based on the combination of the *ccr* gene complex and the *mec* gene complex [70, 77, 82, 83]. Depending on the arrangement of *mec*A or *mec*C and the regulatory genes, as well as the different IS, the *mec* gene complexes A, B, C1, C2 and E can be distinguished (see Table 2). The main characteristics of the *ccr* gene complex are the three phylogenetically distinct *ccr* genes (*ccr*A, *ccr*B, and *ccr*C), with DNA sequence similarities below 50 % [70]. Furthermore, each

recombinase gene can be further classified into allotypes. The *ccr* genes belong to the same allotype if a nucleotide identity of more than 85 % is given [70]. A different allotype is acknowledged when allelic identities range between 50 % and 85 % [70]. The occurrence of various allotypes resulted in the *ccr* gene complex assignments (see Table 2). This wide variation in genetic composition and the frequent occurrence of mosaic and hybrid structures explains the size difference among SCC*mec* elements ranging from 20 kb to approximately 60 kb [77, 82, 83].

Table 2: Overview of currently described SCC*mec* elements

The SCC*mec* type results from the combination of the *mec* gene complex and the *ccr* gene complex. Regarding the *mec* gene complex C1 and C2, they differ in the organisation of the two IS*431* elements, which are either arranged in the same (C1) or opposite (C2) direction [70, 82-84]. Δ*mec*R1: truncated *mec*R1

SCC*mec* type	*mec* gene complex	*mec* gene complex structure	*ccr* gene complex	*ccr* genes
I	B	IS*431*-*mec*A-Δ*mec*R1-IS*1272*	1	*ccr*A1, *ccr*B1
II	A	IS*431*-*mec*A-*mec*R1-*mec*I	2	*ccr*A2, *ccr*B2
III	A	IS*431*-*mec*A-*mec*R1-*mec*I	3	*ccr*A3, *ccr*B3
IV	B	IS*431*-*mec*A-Δ*mec*R1-IS*1272*	2	*ccr*A2, *ccr*B2
V	C2	IS*431*-*mec*A-Δ*mec*R1-IS*431*	5	*ccr*C1
VI	B	IS*431*-*mec*A-Δ*mec*R1-IS*1272*	4	*ccr*A4, *ccr*B4
VII	C1	IS*431*-*mec*A-Δ*mec*R1-IS*431*	5	*ccr*C1
VIII	A	IS*431*-*mec*A-*mec*R1-*mec*I	4	*ccr*A4, *ccr*B4
IX	C2	IS*431*-*mec*A-Δ*mec*R1-IS*431*	1	*ccr*A1, *ccr*B1
X	C1	IS*431*-*mec*A-Δ*mec*R1-IS*431*	7	*ccr*A1, *ccr*B6
XI	E	*blaZ*-*mec*C-*mec*R1-*mec*I	8	*ccr*A1, *ccr*B3
XII	C2	IS*431*-*mec*A-Δ*mec*R1-IS*431*	9	*ccr*C2
XIII	A	IS*431*-*mec*A-*mec*R1-*mec*I	9	*ccr*C2
XIV	A	IS*431*-*mec*A-*mec*R1-*mec*I	5	*ccr*C1

1.4.2.3 SCC*mec* integration and loss by large serine recombinases

Both, the genomic integration and loss of SCC*mec* elements can be mediated by cassette chromosome recombinases [33]. These enzymes belong to the group of site-specific, large serine recombinases of the integration / excision family, which are denominated after their 400 to 700 amino acid large C-terminal domain and their serine-containing active site [77,

85]. To date, three phylogenetically distinct *ccr* genes have been described, designated as *ccr*A, *ccr*B, and *ccr*C [70]. SCC*mec* elements carry either a combination of *ccr*A and *ccr*B as part of a two-gene operon or only *ccr*C [75, 77]. The *ccr* genes catalyse site-specific recombination by recognising short DNA sequences ("sites") in the DNA that is followed by subsequent cutting and ligation of the DNA [86].

Regarding the SCC*mec* integration into the *S. aureus* genome, the *ccr*-mediated recombination occurs between the bacterial attachment site *att*B at the 3' end of *rlm*H and the specific site *att*SCC on the SCC*mec* element (Figure 3). The *ccr* genes thereby recognise characteristic 15 bp DR sequences (5'-AGAGGCGTATCATAA-3' and 5'-AGAAGCATATCATAA-3') [33, 87-89]. Incorporating the SCC*mec* element into the bacterial genome and the respective rearrangement of the DNA is followed by the ligation of the strands [86]. Consequently, the SCC*mec* element is flanked by *att*L and *att*R in the (novel) MRSA genome. The SCC*mec* element may also be removed from the bacterial genome. In this case, *att*L and *att*R recombine and the original sites *att*B and *att*SCC are reconstituted [33, 84, 90].

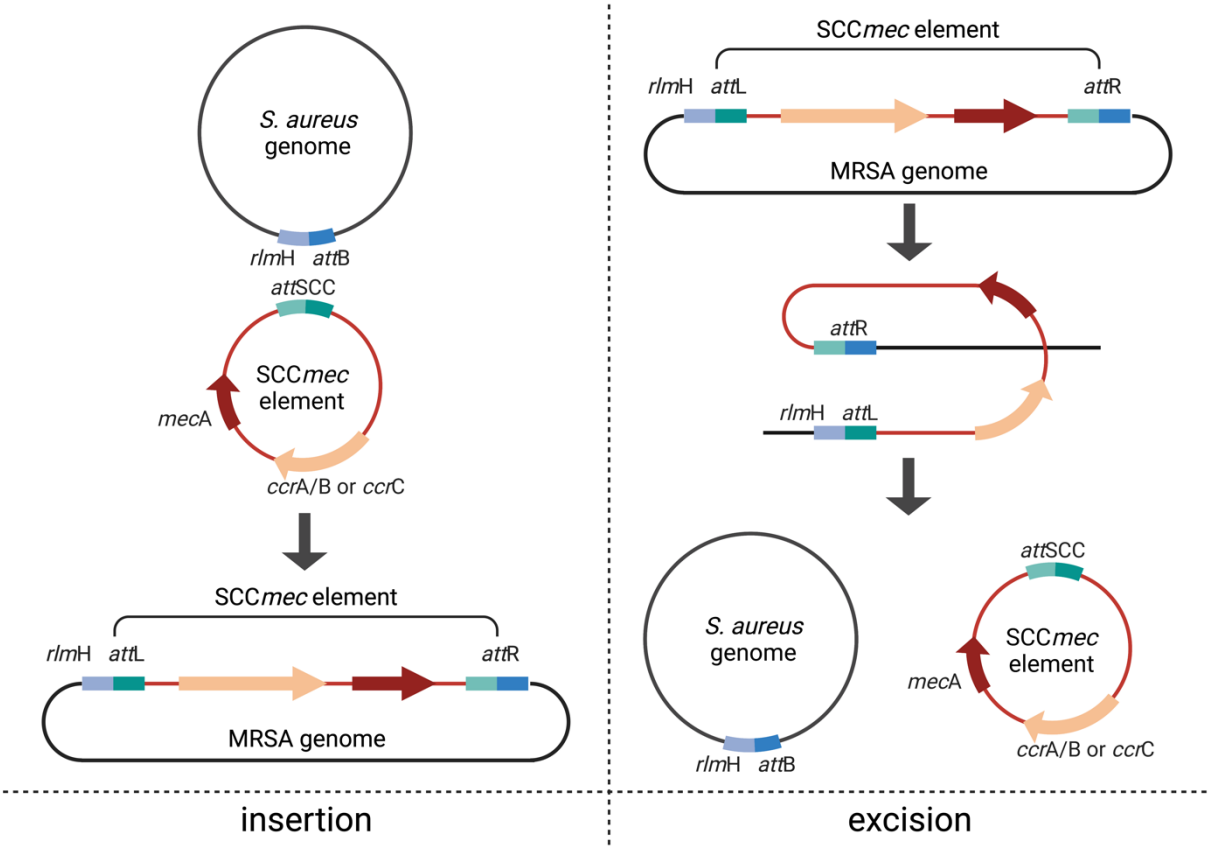

Figure 3: Recombinase-mediated integration and loss of SCC*mec* into / from the *S. aureus* genome

The cassette chromosome recombinase (*ccr*) recognises the *att*B site (bacterial attachment site [91]) at the 3' end of *rlm*H on the staphylococcal chromosome and the *att*SCC on the Staphylococcal

Cassette Chromosome (SCC)*mec* element. This triggers DNA cleavage, leading to a DNA rearrangement whereby the SCC*mec* element is incorporated into the staphylococcal chromosome, leading to methicillin resistant *S. aureus* (MRSA). During SCC*mec* excision, *att*L and *att*R recombine and the original sites *att*B and *att*SCC are reconstituted (modified from [77]). Created with BioRender.com (October 2022).

1.5 Clinical relevance of *S. aureus* and its methicillin resistant variants in human and animal medicine

1.5.1 *S. aureus* occurs as a commensal and a pathogen

The anterior nares of 20 – 80 % of the human population are colonised by *S. aureus,* including methicillin resistant variants, and are therefore regarded as the primary site of residence for *S. aureus* in humans [92-94]. Other carriage sites comprise skin, perineum, pharynx, gastrointestinal tract, and axillae (reviewed in [92, 95]). However, geographic location, age, gender, ethnicity, or body niche seems to have an impact on colonisation rates [96]. It is assumed that about 20 % of the human population is persistently colonised while 30 % carry *S. aureus* only occasionally. Nearly 50 % of the (remaining) population are considered as non-carriers [97, 98].

In addition, several risk factors increase the likelihood of being MRSA colonised, including a history of elucidated antibiotic use in the past 12 months and / or receiving ambulatory care [99]. People with a disease-related background, such as persons being human immunodeficiency virus (HIV)-positive, also have a significantly higher risk of being colonised with MRSA (16.8 %) compared to those without HIV (5.8 %) [100]. Moreover, young children and the elderly, athletes, military personnel, and prisoners are more likely to be MRSA carriers (reviewed in [95]).

S. aureus and its methicillin resistant variants are frequently reported to colonise livestock, companion, and wild animals. The nares, mouth, perineum, and abdominal area are common *S. aureus* carriage sites (reviewed in [101]). Risk factors for acquiring MRSA in companion animals include residing with a colonised human or other animals, hospitalisation, surgery, and antibiotic treatment (reviewed in [102]). Nevertheless, colonisation rates published for different animal species vary in the literature. For example, the reported rates for samples from cats range between 1.48 % and 6.63 % [103, 104]. On a completely other level are the MRSA colonisation rates commonly reported for samples of livestock animal origin, such as pigs, calves, and poultry. Several studies, for instance, demonstrated colonisation rates of 49 % or even higher in pig farming [101, 105].

Of note, *S. aureus* colonisation does not necessarily result in an infection but elevates the individual infection risk two to twelvefold [106]. The actual onset of an infection depends on the ability of *S. aureus* to overcome the skin and mucosal barriers of the body, for example, through injuries, allowing the bacteria to infiltrate and / or penetrate vulnerable tissues or even the bloodstream [107-109]. The most frequently reported pyogenic or purulent infections caused by *S. aureus* in humans include abscesses, furuncles, empyema, wound infections, otitis media, parotitis, mastitis, pneumonia, endocarditis, and sepsis [110]. This panel largely overlaps with conditions occurring in animals since wound infections, pyoderma, otitis, urinary tract infections, bacteraemia, osteomyelitis, or pneumonia are among the most frequently reported diseases caused by *S. aureus* (reviewed in [102, 111]). Due to its adherence properties to hydrophobic surfaces such as plastic and silicon, *S. aureus* is also an important cause of catheter-related infections [112-114]. Infections associated with foreign bodies and implants occur often, and *S. aureus* is responsible for approximately two-thirds of orthopaedic implant infections [113, 115].

1.5.2 Occurrence and assignment of different MRSA types in humans and animals

Shortly after the market introduction of methicillin, the first methicillin resistant *S. aureus* variants were identified in a hospital in southeast England [44]. During subsequent years, MRSA was frequently detected in samples obtained from human patients either during their hospitalisation or after visiting facilities providing healthcare in Europe and the U.S. (reviewed in [116, 117]), which is why MRSA was historically referred to as being **healthcare-associated** (HA) [118]. Risk factors for HA-MRSA include, beyond others, oral antibiotic treatment, endoscopic interventions, hospitalisation, surgery, and medical implants [119]. However, in the 1990s, MRSA cases were reported for individuals who were not exposed to any of these risk factors [117] and had no obvious connection to hospitals or nursing facilities [117, 120]. These new MRSA strains have therefore been termed **community-associated** (CA)-MRSA [117, 118]. The first CA-MRSA outbreaks were detected among children in the U.S. and indigenous communities in Western Australia. In both cases, the CA-MRSA isolates had no relationship to the predominant MRSA clonal lineages associated with hospitals at that time and in that geographic area (reviewed in [116]). The genetic backgrounds of the CA-MRSA strains revealed STs and SCC*mec* elements that were distinct from those of the existent and epidemic HA-MRSA lineages. Initially, HA-MRSA was presumed to carry SCC*mec* elements I, II, or III, whereas CA-MRSA was assigned to SCC*mec* type IV or V (reviewed in [116]).

Contrary to HA-MRSA, CA-MRSA often possessed the genes encoding for the Panton-Valentine Leukocidin (PVL), an important virulence factor, which was more or less absent in HA-MRSA strains (reviewed in [121]). Meanwhile, CA-MRSA occurs worldwide, with certain STs particularly prevalent in different regions (reviewed in [116]). Probably the best-known example in this context is the PVL-positive CA-MRSA belonging to CC8, more commonly referred to as USA300, which became the predominant CA strain in the U.S. [122]. In contrast, CA-MRSA CC80, which is also PVL-positive, is the most common strain in Europe. This lineage has probably spread from Denmark to the entire European continent via Finland, Greece, and France (reviewed in [123]). However, the original distinction between CA-MRSA and HA-MRSA becomes increasingly blurred since CA-MRSA also occurs in hospitals [124].

In response to the rising MRSA numbers and the spread of MRSA in the healthcare system, an MRSA “search and destroy” policy was introduced in the Netherlands and the Scandinavian countries as early as 1988, with the aim of preventing further transmission of MRSA [125]. Shortly after its introduction, this policy proved to be successful. While Denmark had an MRSA frequency in samples isolated from hospitalised patients of 15 % between 1967 and 1971, it decreased to 0.2 % in the 1980s [126]. According to a report by the European Centre for Disease Prevention and Control from 2019, the proportion of MRSA in samples of invasive infections from the European population has been constant or even declined, presumably due to improved infection prevention and more prudent use of antibiotics [127]. However, a north-to-south gradient is still evident in Europe, with lower MRSA rates reported from the United Kingdom, Germany, Austria or Slovenia (5 % – < 10 %) compared to France, Spain (both 15 % – < 25 %), Italy or Portugal (both 25 % – < 50 %) [127].

In 2004, the first transmission of MRSA between humans and livestock animals was described in the Netherlands. During a routine screening for MRSA before major surgery, a 6-months-old girl tested positive for MRSA. The entire family, including the girl, was not exposed to any known risk factors for MRSA colonisation at that time. In addition, MRSA was detected in samples obtained from the child’s parents and in swabs taken from pigs on their farm. Shortly thereafter, MRSA was isolated from other pig farmers, a veterinarian, the veterinarian’s son, and the son’s nurse. These isolates all belonged to three different *spa* types, but pattern analysis using pulsed-field gel electrophoresis after DNA restriction with endonuclease *Sma*I failed [128].

Further characterisation of these isolates identified a so far undescribed clonal complex, CC398 [129]. Due to the close contact between humans and animals on the pig farm, CC398 isolates were frequently transmitted between the livestock animals, farmers, and

veterinarians [130-132]. For this reason, isolates belonging to CC398 have been defined as **livestock-associated** (LA)-MRSA [118, 133]. However, the transmission of MRSA CC398 between humans is also possible, as demonstrated, for example, by a hospital outbreak in 2007 [134]. Research results indicated that MRSA belonging to CC398 are probably descendants from a human methicillin susceptible *S. aureus* (MSSA) lineage, which acquired methicillin resistance during host transition from humans to pigs [135]. MRSA CC398 predominantly carry SCC*mec*V or, more rarely, SCC*mec*IV and usually lack the genes encoding PVL [136]. In addition to the acquired methicillin resistance, LA-MRSA CC398 often harbour further genes conferring resistance to antibiotics such as tetracycline and less frequently against macrolides, lincosamides, aminoglycosides, trimethoprim, and fluoroquinolones (reviewed in [121]). Furthermore, the occurrence and spread of LA-MRSA CC398 are not limited to humans and pigs. This lineage has been reported to be associated with veal calves, poultry, horses, dogs, rats, and other animals (reviewed in [121]). However, other “non-CC398” MRSA isolates have also emerged and spread in livestock animals.

The first commonly known case of transmission between humans and animals dates back to 1987. In a rehabilitation ward, three patients were colonised or infected with MRSA. To identify the source, hospital staff, the physiotherapist, his dog and a cat that regularly visited the ward were screened. Besides five nurses and the physiotherapist, the cat was heavily contaminated with MRSA on its paws and back fur. Presumably, the cat acquired MRSA from the skin scales of a heavily colonised patient and transmitted MRSA directly to other patients. The nurses who petted the cat and did not wash their hands afterwards also transmitted MRSA further and thus contributed to the outbreak [137].

Since then, multiple reports on MRSA transmission events between humans and animals have been published. A dog, for example, acquired MRSA through close physical contact with its owner, a 76-year-old man suffering from diabetes mellitus, chronic lymphocytic leukaemia, and chronic obstructive pulmonary disease [138]. The man had been repeatedly hospitalised because of cellulitis. Shortly after the man’s dog was subjected to orthopaedic surgery, the dog developed a surgical site infection and molecular evidence linked the MRSA directly to the strain frequently isolated from samples from the dog’s owner [138].

As mentioned before, MRSA CC398 is a predominating strain regarding transmission in livestock and farm environments, typically involving farmers, veterinarians, pigs and cattle [130-132, 139]. Another example is the transmission of MRSA CC5 from humans to poultry, whereby MRSA are a major cause of infectious diseases and subsequent losses in chickens [140, 141]. These examples demonstrate that the human-to-animal and vice versa transmission makes *S. aureus* ubiquitous (reviewed in [102]).

1.6 Aims of the thesis

S. aureus is a transmissible pathogen, which is therefore frequently isolated from humans, companion animals including cats, dogs, and horses, livestock animals such as pigs, cattle, chickens, and wild animals like hedgehogs or even aquatic animals e.g., dolphins [59, 63, 142-144].

Over the past decades, MRSA have acquired resistance towards further classes of antibiotics [145-149]. Therefore, it is not surprising that the World Health Organization (WHO) considers MRSA a major threat to human health and has subsequently placed MRSA on the global priority list of antibiotic-resistant bacteria requiring the development of new antibiotics [150]. However, exhibiting antibiotic resistance is not the only reason for MRSA to remain on the panel of concerning pathogens. A vast repertoire of traits harnessed by *S. aureus* allow the bacteria to adapt when challenged by novel environmental conditions or hosts. Gaining further insights into these adaptive strategies is essential, especially regarding novel treatment options. Against this background, the aims of this cumulative thesis were:

1. to examine how changes of the major regulatory quorum sensing system promote niche adaptation of *mec*C-harbouring MRSA across multiple host species
2. to investigate the genetic structure and putative role of the MGE conferring methicillin resistance in pig-farm-associated MRSA

2 Publications

2.1 Publication I

Silence as a way of niche adaptation: *mec*C-MRSA with variations in the accessory gene regulator (*agr*) functionality express kaleidoscopic phenotypes

Charlotte Huber, Ivonne Stamm, Wilma Ziebuhr, Gabriella Marincola, Markus Bischoff, Birgit Strommenger, Greta Jaschkowitz, Tessa Marciniak, Christiane Cuny, Wolfgang Witte, Joerg Doellinger, Christoph Schaudinn, Andrea Thürmer, Lennard Epping, Torsten Semmler, Antina Lübke-Becker, Lothar H. Wieler & Birgit Walther

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Author contribution:
C.H. and B.W. designed the project. **C.H.**, B.W., C.S., W.Z. and J.D. conceived and designed the experiments. I.S., B.S., C.C. and W.W. collected and screened the isolates. A.T. sequenced the isolates and T.S. and L.E. analyzed the phylogenetic relationship based on WGS data. **C.H.**, G.J., T.M., C.S., A.L-B. and J.D. performed the laboratory experiments. **C.H.**, B.W., W.Z., G.M., M.B., J.D., C.S., L.E., T.S., A. L-B. and L.H.W. analyzed the data. **C.H.**, W.Z., M.B., J.D., C.S., T.S., L.H.W. and B.W. wrote the manuscript.

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Silence as a way of niche adaptation: *mec*C-MRSA with variations in the accessory gene regulator (*agr*) functionality express kaleidoscopic phenotypes

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Functionality of the accessory gene regulator (*agr*) quorum sensing system is an important factor promoting either acute or chronic infections by the notorious opportunistic human and veterinary pathogen *Staphylococcus aureus*. Spontaneous alterations of the *agr* system are known to frequently occur in human healthcare-associated *S. aureus* lineages. However, data on *agr* integrity and function are sparse regarding other major clonal lineages. Here we report on the *agr* system functionality and activity level in *mec*C-carrying methicillin resistant *S. aureus* (MRSA) of various animal origins (n = 33) obtained in Europe as well as in closely related human isolates (n = 12). Whole genome analysis assigned all isolates to four clonal complexes (CC) with distinct *agr* types (CC599 *agr* I, CC49 *agr* II, CC130 *agr* III and CC1943 *agr* IV). Agr functionality was assessed by a combination of phenotypic assays and proteome analysis. In each CC, isolates with varying *agr* activity levels were detected, including the presence of completely non-functional variants. Genomic comparison of the *agr* I–IV encoding regions associated these phenotypic differences with variations in the *agr*A and *agr*C genes. The genomic changes were detected independently in divergent lineages, suggesting that *agr* variation might foster viability and adaptation of emerging MRSA lineages to distinct ecological niches.

Methicillin-resistant *Staphylococcus aureus* (MRSA) is among the leading causes of opportunistic infectious diseases in human and veterinary medicine worldwide[1,2]. Mobile genetic elements (MGEs) originally denominated as staphylococcal cassette chromosome *mec* (SCC*mec*) cause horizontal spread of methicillin resistance among staphylococci[3], with either *mec*A or *mec*C[4,5] as the resistance-mediating gene. So far, thirteen distinct SCC*mec* elements have been described, including the *mec*C-harbouring element SCC*mec* XI[5,6]. When these MRSA were first isolated from specimens of human and ruminant hosts back in 2011[7–9], detection and verification of *mec*C was challenging due to the limited sequence similarity to *mec*A and its common association with a moderate or even low minimal inhibitory concentration (MIC) for oxacillin[5,9–11]. Since then, *mec*C-MRSA were not only

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found in samples from humans and dairy cattle[5,12], but also in domestic-, zoo-, and wild animals as well as in wastewater[8,10,13–15]. Human cases of infection associated with *mec*C-MRSA were reported from the United Kingdom, Germany, Austria, Spain and many other European countries[7–9,16] suggesting that *mec*C-harbouring isolates have become ubiquitous[10,14].

Mechanisms enabling opportunistic bacteria to cross species barriers, to infect a new host or to enhance their viability in the environment are still poorly understood. Changes expected to be involved in (niche) adaptation processes of *S. aureus* encompass (among others) alterations of metabolic pathways[17], escape of host defence mechanisms[1], biofilm formation[18], iron acquisition abilities[19], generation of pseudogenes[20,21] or nucleotide changes altering promoter structures[22]. In addition, production of cytotoxins[23,24] and other virulence factors as well as their regulation circuits might have a strong influence on host adaptation[23,25,26]. Acquisition of specific virulence factors from a host-specific gene pool[1,27] by horizontal gene transfer as well as core genome diversification contributes to broadening the host range of *S. aureus*[27], leading to the definition of extended host spectrum genotypes[28].

In *S. aureus*, the accessory gene regulator (*agr*) locus represents a quorum sensing system that orchestrates the switch from expressing of surface associated factors (needed for initial attachment) to the expression of exotoxins at high cell density via the effector molecule RNAIII[29]. Notably, *agr* activity appears to be essential for skin and soft tissue infections[30]. Previous research demonstrated the important role of *agr* for virulence factor transcription in animal models of acute infections[31–33], while *agr* defective mutants seemed to be frequently associated with chronic diseases[34–39], persistent bacteremia[40] and cystic fibrosis[41], indicating adaptation of *S. aureus* to the infected host[42].

The staphylococcal *agr* locus is an autoinducing control unit that combines bacterial quorum sensing with a classical bacterial two-component regulatory system (TCRS) and the employment of a regulatory RNA (i.e. RNAIII) as effector molecule[29]. The locus consists of two divergently arranged transcription units, which are transcribed from promoters P2 and P3, respectively. The P2-derived RNAII harbours the *agrBDCA* operon, while P3 drives transcription of RNAIII. In *S. aureus*, the cell–cell communication (i.e. quorum sensing) depends mainly on autoinducing, short peptides (AIPs) encoded by *agr*D, which are exported by the transmembrane endopeptidase (AgrB) and finally trimmed by a type I extracellular signal peptidase (SspB; reviewed in[43]). At sufficient extracellular AIP concentrations, the peptides binds to the sensor histidine kinases (AgrC) which then phosphorylates the response regulator AgrA (AgrA-P)[43,44]. While a weak baseline transcription from the P2 and P3 promoters is detectable even in the absence of AgrA-P[43,44], the activated transcription factor strongly (auto)induces expression of both *agrBDCA* and RNAIII[44]. In addition, AgrA-P directly leads to transcription of phenol-soluble modulin (PSM) genes. The majority of *agr*-regulated genes, however, is controlled via RNAIII which influences their transcription and translation[29,43,45]. RNAIII represents a dual-function regulatory RNA that harbours, in addition to its base-pairing functions, a small open reading frame (*hld*) encoding staphylococcal delta-haemolysin (Hld). The *hld* Shine-Dalgarno site was shown to be accessible to ribosomes, indicating that *hld* represents a translatable unit on the RNAIII molecule[46], which in turn makes Hld detection a suitable proxy to determine RNAIII expression and Agr activity in general.

S. aureus virulence often depends on the secretion of large amounts of toxins, including exotoxins with superantigenic functions, i.e., the staphylococcal enterotoxins (SEA, SEB, etc.) and toxic shock syndrome toxin (TSST-1)[47]. Expression of ordinary virulence determinants such as proteases, lipases or nucleases, are promoted by an activated *agr* system, whereby expression of surface binding proteins is downregulated. Nonetheless, this generalized summary still includes exceptions and lineage-specific differences[48]. In addition, primary transcription regulation of virulence factors not encoded by the core genome, e.g. genes associated with the enterotoxin gene cluster (*egc*) harbouring staphylococcal enterotoxins (SE), appears to be less depending on increased *agr* activation[49].

Biofilm formation is another important feature in *S. aureus* pathogenesis which is influenced by *agr*. The effect of a cellular increase of the *agr* transcript RNAIII, however, is not entirely clear. While previous studies demonstrated enhanced biofilm formation in *agr* deficient strains[42,50], recent reports, focusing on non-lab-adapted strains, found lineage-specific differences[51], or a variable role of the *agr* system[38,52].

Occurrence of independent genomic changes in divergent clonal lineages is generally considered to reflect the adaptation power of bacteria to changing environments[53]. Aim of the study presented here was to analyse the genome structures of emerging *mec*C-MRSA isolates of animal and human origin with the overarching goal to identify genomic patterns that might be associated with the adaptation of such strains to novel hosts and ecological niches. While differences in exotoxin and virulence factor endowments of the isolates were mainly associated with distinct clonal complexes (CC), we found independent genomic variations of the *agr* locus across different *mec*C-MRSA clonal lineages. These changes were associated with varying *agr* activity patterns and kaleidoscopic phenotypes regarding haemolysis and biofilm architecture, suggesting a role of the *agr* system in niche adaptation of the isolates.

Materials and methods

Sample collection and pre-screening. *Staphylococcus aureus* isolates obtained from samples of various animal species were collected from January 2014 to December 2016 by IDEXX Laboratories in Ludwigsburg, Germany. Inclusion criteria for isolates were (1) a methicillin resistant phenotype based on growth in the presence of 6 µg/ml cefoxitin according to the manufacturer's VITEK 2 Advanced Expert System (Nürtingen, Germany) instruction together with (2) oxacillin MICs < 4 µg/ml, as reported for *mec*C-MRSA before[14]. Isolates from seven different European countries were included. Species identity and methicillin resistance mediated by *mec*C was confirmed by PCR as described elsewhere[54]. Antimicrobial susceptibility testing (AST) was carried out using the VITEK 2 system (BioMérieux, Germany) according to the standards given by CLSI VET01-A4

ID	Original-ID	Sample source	Host	Country	MLST	*spa*	Oxacillin MIC µg/ml
IMT31818	VB971510	Nose	Dog	Germany	ST599	t278	1
IMT31819	VB971922.1	Uterus	Horse	Germany	ST130	t843	0.5
IMT32509	VB985303	Wound	Cat	Germany	ST599	t278	1
IMT32510	VB992333	Ear	Cat	Poland	ST130	t843	1
IMT32513	VB998882.1	Ear	Cat	Germany	ST599	t16473	1
IMT32929	VB962079.2	Wound	Cat	Netherlands	ST130	t1519	0.5
IMT34478	VB994753.1	Nose	Cat	Germany	ST130	t843	2
IMT34479	VB962798.2	AC	Dog	Germany	ST130	t9165	0.5
IMT34480	VB962790	Ear	Rabbit	Germany	ST130	t9165	1
IMT34485	VB982561.3	Wound	Dog	Germany	ST2361	t10855	1
IMT34488	VB989315	Skin	Cat	Germany	ST130	t843	2
IMT34489	VB993969	Wound	Cat	Germany	ST130	t843	0.5
IMT34491	VB963607.2	Wound	Cat	Germany	ST130	t843	2
IMT36943	VB973587.1	Ear	Cat	Germany	ST599	t278	2
IMT36945	VB972803.2	Frontal sinus	Cat	Italy	ST49	n.a	0.5
IMT36946	VB981317	Wound	Cat	Germany	ST130	t843	2
IMT36947	VB986304	Wound	Cat	Switzerland	ST599	t5930	1
IMT36948	VB987750	TC	Cat	Germany	ST599	t5930	1
IMT36950	VB960116	Skin	Cat	Germany	ST130	t843	2
IMT36952	VB978601	Wound	Cat	Germany	ST2361	t3391	0.5
IMT38113	VB902052	Nose	Cat	Germany	ST2361	t3391	2
IMT38115	VB968721.3	Abscess	Cat	Sweden	ST130	t373	2
IMT38116	VB949481.1	Nose	Hedgehog	Germany	ST599	t278	2
IMT38119	VB972539	Skin	Cat	Germany	ST1764	t524	0.5
IMT39816	PF169945.2	Tissue	Cat	France	ST1245	n.a	0.5
IMT39819	VB945444	Faeces	Sheep	Germany	ST130	t843	2
IMT39820	VB911819.2	Wound	Hedgehog	Germany	ST2361	t3391	2
IMT39824	VB950088.2	Nose	Cat	Germany	ST599	n.a	0.5
IMT39825	VB957945	Nose	Dog	Germany	ST599	t12332	2
IMT40504	BF136317	Nose	Cat	France	ST1943	t8835	1
IMT40506	VB966538	Wound	Cat	Germany	ST130	t843	2
IMT40507	VB966063.1	Wound	Cat	Germany	ST2361	t2345	2
IMT41554	VB999839.3	Wound	Cat	Germany	ST130	t1736	2
RKI5962	14-03729	Ulcus cruris	Human	Germany	ST599	t12332	0.5
RKI5963	13-00970	Wound	Human	Germany	ST599	t5930	0.5
RKI5964	16-02552	Wound	Human	Germany	ST599	t9925	≤0.25
RKI5965	14-02098	Abscess	Human	Germany	ST1245	t13902	0.5
RKI5966	15-00967	Wound	Human	Germany	ST130	t14848	0.5
RKI5967	16-01171	Wound	Human	Germany	ST130	t15938	0.5
RKI5968	10-00991	Wound	Human	Germany	ST130	t1736	0.5
RKI5969	13-03754	Wound	Human	Germany	ST2361	t3391	1
RKI5970	18-00258	Infection	Human	Germany	ST2361	t3391	1
RKI5971	18-00326-1	UTI	Human	Germany	ST2361	t3391	1
RKI5972	19-00523	Unknown	Human	Germany	ST49	t208	1
RKI5973	19-00418	Abscess	Human	Germany	ST2361	t2345	0.5

Table 1. Isolate characteristics of 45 *mec*C-MRSA. Isolate characteristics of 45 *mec*C-MRSA showing low or moderate oxacillin MICs while being able to grow in the presence of 6 µg/ml cefoxitin. ID, isolate identification number (this study); Original-ID, original isolate identification number; MLST, multilocus sequence type; *spa*, spa type based on allelic variants of the gene encoding protein A; AC, abdominal cavity; TC, thoracic cavity; UTI, urinary tract infection; n.a., not assigned.

and M100-S24[55,56]. For comparative analysis, additional 12 *mec*C-positive isolates of human origin belonging to corresponding clonal backgrounds collected by the national reference laboratory for staphylococci at the Robert Koch Institute in Germany were included (Table 1).

Whole genome sequencing and genomic analysis of *mec*C-MRSA. MRSA isolates positive for *mec*C were whole-genome sequenced (WGS) using Illumina MiSeq 300 bp paired-end sequencing with an

obtained coverage > 90X. Raw reads were used for de novo assembly into contiguous sequences (contigs) and subsequently into scaffolds using SPAdes v3.12[57]. Assembled draft genomes of the isolates were annotated using Prokka[58]. WGS data were used for genotypic characterization including the determination of the sequence type (ST) (MLST v2.0)[59]. Genomic sites of interest and genes encoding for major regulators including the accessory gene regulator (*agr*) system were investigated using Geneious 11.1.5 (Biomatters Ltd., Australia)[60]. Genomic data were further analysed with ResFinder-2.2 (threshold: 90% ID, 80% minimum length), VirulenceFinder-1.6 (threshold: 95% ID, 80% minimum length) and *spa*Typer[59,61–63].

In order to compare the genomes at high resolution, we used the maximum common genome (MCG) that is defined by those orthologous genes present in all genomes[64]. The coding sequences were clustered based on the parameters of nucleotide sequence similarity (≥ 90%) and gene coverage (≥ 90%). The MCG was defined as those genes that were present in each genome and fulfilled the threshold parameters, yielding 2,094 genes. Allelic variants of these genes were subsequently extracted from all genomes by a blast-based approach, then aligned individually for each gene and concatenated, resulting in an alignment of 1.95 Mbp for these isolates. The alignment was used to generate a maximum likelihood phylogenetic tree using RAxML 8.2.9[65] which was visualized together with the distribution of the accessory gene content using phandango[66].

Phenotypical assessment of accessory gene regulator (*agr*) activity. In this study, complementary phenotypical methods including colony spreading and haemolysin production were employed to investigate the activity of the *agr* system. Synergistic production of different haemolysins (SPDH) on columbia agar plates (Oxoid, Germany) supplemented with 5% sheep blood (SBA) was investigated by cross-streaking tested bacteria perpendicularly to laboratory strain *S. aureus* RN4220 as described before[1]. RN4220 is characterized by a strong β-haemolysin production while α-haemolysin secretion is missing and PSM α peptides are produced only at non-considerable amounts[35,67,68]. Of note, RN4220 was reported to produce the α-haemolysin encoding mRNA (*hla*), indicating that *hla* transcription cannot necessarily be correlated with α-haemolysin production and/or secretion in *S. aureus*[69]. In addition, the capacity to cause synergistic haemolysis with β-haemolysin is to a large extend due to AgrA-induced expression of PSMs[67], while β-haemolysin inhibits α-haemolysins' effect on SBA[35]. After overnight incubation of the plates at 37 °C, the haemolysis zones were examined and pictures were taken.

A modified CAMP test for verification of β-haemolysin production was performed using *Streptococcus agalactiae* (ATCC12386) and the β-haemolysin producing *S. aureus* strain ATCC25923 (positive control) as described recently[1]. Briefly, staphylococci of interest were streaked perpendicularly to CAMP-factor producing ATCC12386. A positive interaction of CAMP-factor from Group B streptococci with β-haemolysin (phospholipase C) of *S. aureus* is characterized by completely lysed sheep blood erythrocytes forming a clear semilunar-shaped area[70].

We also performed a colony spreading assay on soft agar described by Kaito et al.[71], since an activated state of the *agr* system[72] as well as AgrA-depending production PSM α3 are necessary for *S. aureus* to slide on wet surfaces[73]. Beyond that, a mutation in the membrane-bound transpeptidase sortase A (SrtA), or for instance the lack of its substrates fibronectin binding protein A and B (FnBPA, FnBPB), clumping factor A and B (ClfA, ClfB) enhances spreading[74]. Briefly, 20 ml tryptic soy soft agar plates (0.24%) were used to investigate spreading activity of all MRSA. After overnight incubation of the plates at 37 °C, the spreading zones were examined, and pictures were taken.

The standard laboratory strains USA300 (FPR3757), the USA300 *agr*A::Tn mutant (NE1532) and RN4220 were included in all phenotype assays as controls for wild type (wt) *agr* functionality, a loss-of-function- and a functionally impaired *agr* system, respectively[69]. In lab strain RN4220, a frameshift mutation in the *agr*A-coding region adds three amino acids to the C-terminus of AgrA, a variation known to cause a considerable delay in RNAIII transcription compared to the wild type[70]. To verify production of TSST, the TST-RPLA Kit TD940 (Thermo Fisher Scientific, USA) was used according to the manufacturer's instructions.

All surveys were repeated thrice for each of the isolates including the reference strains.

Proteomic analysis of α-, β- and δ-haemolysin production in *mec*C-MRSA. Protein abundance values for Hla/Hlb/Hld from whole bacterial cell preparations were measured to assess RNAIII transcription capabilities by Hld production and Hla and Hlb (pre-) proteins of the bacteria and their general ability to produce them.

Sample preparation by easy extraction and digestion (SPEED). Samples were prepared in triplicates using SPEED as previously described[75]. In brief, cells were re-suspended in trifluoroacetic acid (TFA) (Uvasol for spectroscopy, Merck, Darmstadt, Germany) (sample/TFA 1:4 (v/v)) and incubated at 70 °C for 3 min. Samples were neutralized with 2 M TrisBase using 10 × volume of TFA and further incubated at 95 °C for 5 min after adding Tris(2-carboxyethyl)phosphine (TCEP) to a final concentration of 10 mM and 2-Chloroacetamide (CAA) to a final concentration of 40 mM. Protein concentrations were determined by turbidity measurements at 360 nm, adjusted to 0.25 µg/µl using a 10:1 (v/v) mixture of 2 M TrisBase and TFA and then diluted 1:5 with water. Digestion was carried out for 20 h at 37 °C using Trypsin (Promega, Fitchburg, WI, USA) at a protein/enzyme ratio of 50:1. Resulting peptides were desalted using StageTips C18[76].

Liquid chromatography and mass spectrometry. Peptides were analyzed on an EASY-nanoLC 1,200 (Thermo Fisher Scientific, Bremen, Germany) coupled online to a Q Exactive™ Plus mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). 1 µg peptides were loaded on an Acclaim PepMap trap column (20 mm × 75 µm i.d., 100 Å, C18, 3 µm; Thermo Fisher Scientific, Bremen, Germany) and were subsequently separated on a 200 cm µPAC column (PharmaFluidics, Ghent, Belgium). The flow rate was set to 800 nl/min and a stepped 40 min gradient was applied: 3–10% B in 4 min, 10–33% B in 17 min, 33–49% B in 4 min, 49–80% B

in 7.5 min and 80% B for 7.5 min. Solvent A was 0.1% (v/v) formic acid (FA) in water, solvent B consisted of 80% (v/v) acetonitrile in 0.1% (v/v) FA.

The Q Exactive Plus was operated in data-independent (DIA) manner in the m/z range of 350–1,150. Full scan spectra were recorded with a resolution of 70,000 using an automatic gain control (AGC) target value of 3×10^6 with a maximum injection time of 100 ms. The Full scans were followed by 53 DIA scans of dynamic window widths using an overlap of 0.5 Th (Supplemental Table 1). DIA spectra were recorded at a resolution of 17,500@200 m/z using an AGC target value of 3×10^6 with a maximum injection time of 55 ms and a first fixed mass of 200 Th normalized collision energy (NCE) was set to 25% and default charge state was set to 3.

Mass spectrometric data analysis. The mass spectra were searched in DIA-NN (Version 1.7.6)[77] using the deep-learning based spectra and RT prediction for sequences from the complete proteome of *S. aureus* strain NCTC 8,325 (UP000008816, 2,889 sequences, downloaded 4/10/18) and sequences of selected genes (*hla*, *hlb* and *hld*) obtained from whole genome sequencing. Spectra were searched with a tolerance of 10 ppm in MS^1 and 20 ppm in MS^2 mode, strict trypsin specificity (KR not P) and allowing up to one missed cleavage site. Cysteine carbamidomethylation and N-terminal methionine excision were set as modifications. Peptide length was restricted to 7–30 amino acids. The m/z ranges were 350–1,150 for full scans and 200–1,800 for DIA scans. A false discovery rate of 1% was applied for precursor and protein identifications.

Biofilm formation assay on polystyrene tissue culture plates. Biofilm formation to inert artificial surfaces was tested in 96-well polystyrene tissue culture plates (Greiner Bio-One, Cellstar, F-form) as described previously[78]. Briefly, *S. epidermidis* RP62A and *S. carnosus* TM300 were used as positive and negative controls, respectively. Bacterial overnight cultures in triplicates were grown in Trypticase Soy Broth (TSB; Becton Dickinson) which contains, according to the standard composition, 2.5 g/l glucose. Cultures were diluted in fresh TSB to an OD_{600} of 0.05 and 200 µl filled in each well (four wells per biological replicate) and incubated under static condition at 30 °C for 18 h. Supernatant was discarded and adherent cells were washed twice with 1 × PBS buffer before the remaining cells were heat-fixed at 65 °C for 1 h. Plates were then stained with 10 mg/ml crystal violet for 2 min, washed twice with double-distilled water before proceeding with measuring the absorbance at OD_{492} by an ELISA plate reader (Multiskan Ascent).

Imaging of the biofilm architecture by confocal laser scanning microscopy (CLSM). In order to study the putative effect of different *agr* non-wt variants on growth/biofilm characteristics on glass surfaces, overnight cultures of closely related isolates were subjected to comparative analysis: *S. aureus* isolates belonging to CC130 IMT38119 (*agr* III wt), RKI5966 (*agr* III non-wt AgrC variant) and IMT31819 (*agr* III non-wt AgrA variant) were diluted to 10^5 bacteria per ml. One milliliter diluted suspension was used to inoculate the wells of a 24-well plate with glass bottom (µ-Plate 24 Well Black, ibidi GmbH, Germany), which was then cultivated for 20 h at 37 °C with 150 rpm on an orbital shaker. Afterwards, samples were photographed (Lumix GM1, Panasonic, Japan) on a light table, stained with LIVE/DEAD (LIVE/DEAD Cell Viability Assay, ThermoFisher Scientific, Germany) according to the manufacturer's instructions and imaged with a confocal laser scanning microscope (LSM780, Carl Zeiss AG, Germany) using the Plan-Apochromat 20 × /0.8 objective.

When necessary, images were cropped, adjusted for optimal brightness and contrast (applied to the whole image) using Adobe Photoshop CS6 (Adobe Systems, San Jose, CA, USA).

Database accession numbers. Genomic sequencing data used are available for download from the National Center for Biotechnology Information (NCBI) under BioProject accessions PRJNA588740. Accession numbers of whole genomes sequences are provided in Supplemental Table 2. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (https://proteomecentral.proteomexchange.org) via the PRIDE partner repository with the dataset identifier PXD016486.

Results

General features of the *mec*C-MRSA strain collection. A total of 33 *mec*C-MRSA were obtained from seven different European countries and six different animal species for whole genome sequencing (WGS). Isolates from cats dominated the collection (24/33), followed by those obtained from dogs (4/33) and other companion-, wild- and livestock animals (Table 1). We also included 12 isolates from human patients belonging to matching genotype lineages (Table 1, Fig. 1) for further phenotype assays and subsequent comparative genome analysis. All 45 isolates displayed low or moderate oxacillin MICs < 4 µg/ml, while being able to grow in the presence of 6 µg/ml cefoxitin (Table 1), which is typical for *mec*C-MRSA[14,79]. AST results revealed additional resistance to aminoglycosides (gentamicin, kanamycin) for isolate RKI5962 only. A positive latex agglutination test verified production of the TSST toxin for all isolates harbouring a *tst*-bov variant[80]. Interestingly, we noticed considerable phenotypic differences regarding haemolysis on SBA between the isolates, even among those sharing the same phylogenetic background. This prompted further investigations into putative genomic changes which might account for these variations.

Phylogenetic relationship and distribution of virulence-associated factors among *mec*C-MSRA. WGS of the isolates revealed that the 45 *mec*C-positive MRSA belonged to four CCs: CC599 (12), CC130 (21), CC1943 (10) and CC49 (2) (Table 1, Fig. 1). Pairwise SNP-distances between the core genomes were calculated for all MRSA isolates (Supplemental Table 3), showing a very close phylogenetic relationship of those genomes belonging to the same CC. While the differences within one clonal complex ranged between 100

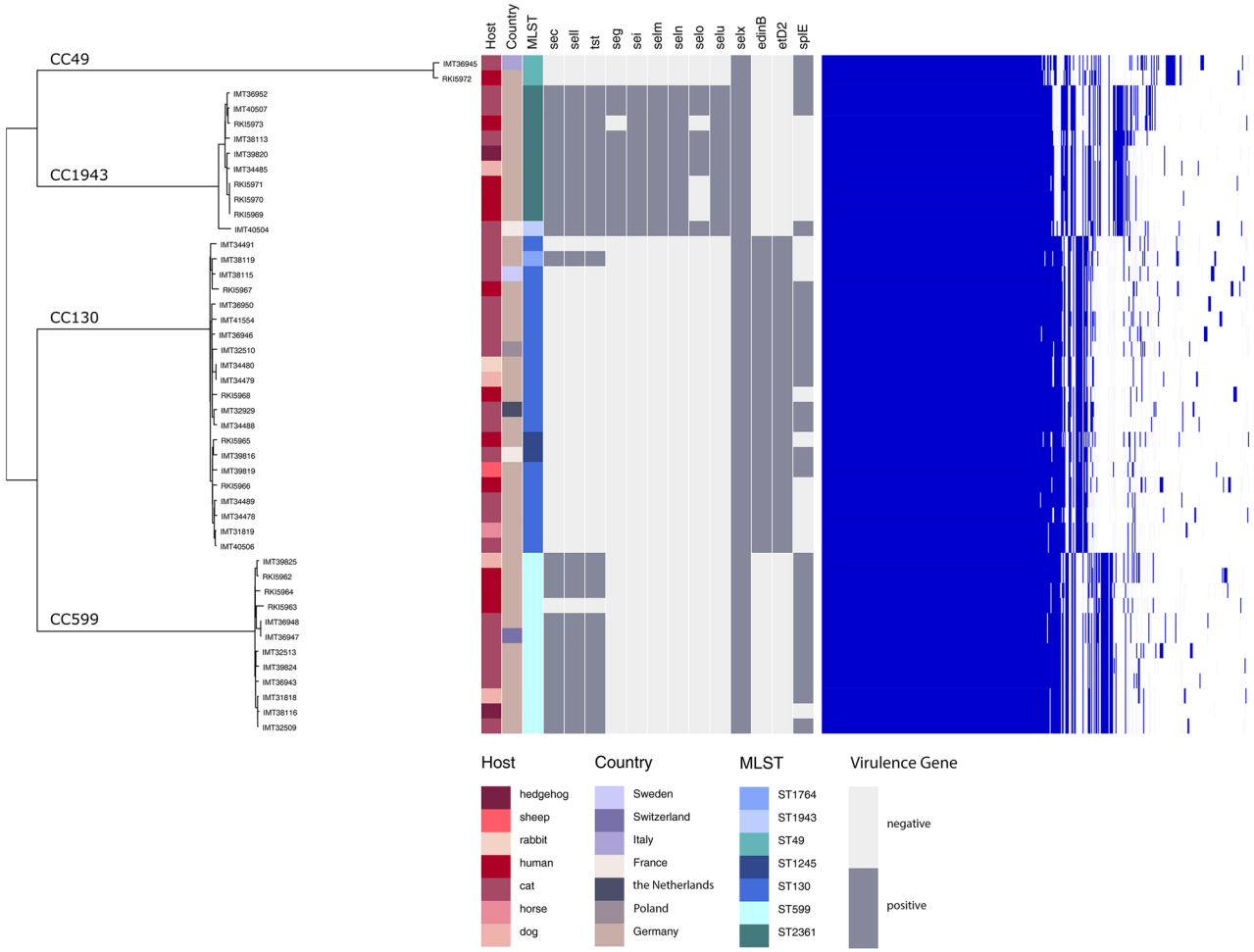


Figure 1. The core genome phylogeny based on the Maximum Common Genome. The core genome phylogeny based on the Maximum Common Genome comprising 2,094 orthologous genes present in all isolates show four distinct clusters, whereby the genetic diversity within the clusters is rather low. Furthermore, the isolates metadata show no significant association with the core genome clusters. The 2,003 accessory genes show a distribution pattern that is highly correlated with the core genome clusters (right side), suggesting a lineage-specific gene content. Genes for aureolysin (*aur*), leucotoxins D and E (*luk*D, *luk*E), gamma-haemolysin component A–C (*hlg*A, *hlg*B, *hlg*C) and proteases SpIA or SpIB are present in all isolates. All isolates belonging to ST-1943 as well as some CC130 and CC599 were positive for different variants of the Staphylococcal pathogenicity island (SaPI) harbouring a toxic shock toxin encoding gene (*tst*), which were variants of *tst*-bov[80]. Moreover, 48.5% of the *mec*C-positive isolates harboured staphylococcal enterotoxins (SE). The protease SpIE can just be found in 23/33 isolates and is not associated with any sequence type. The epidermal cell differentiation inhibitor B (*edin*B) cannot be determined in the isolates of ST-1943, ST-2361, ST-49 and ST-599.

and 500 SNPs, the genomes belonging to different CCs differed by more than 10,000 SNPs, resulting in a clear clustering of MRSA from the same CC (Fig. 1). Metadata such as geographic origin, disease or animal species showed no significant association with the core genome clustering, but a lineage-specific association of the variable gene content was obvious (Fig. 1).

Nevertheless, a core set[81] of *S. aureus* virulence factors was present in all isolates, including aureolysin (*aur*), bi-component leukotoxin (*luk*D/E), γ-haemolysin components A–C (*hlg*A, *hlg*B, *hlg*C) and genes encoding for the serine proteases SpIA and SpIB. Presence of the epidermal cell differentiation inhibitor B (*edin*B) and exfoliative-like toxin D2[82] was associated with CC130. When considering factors promoting biofilm production in *S. aureus*, all isolates harbour complete *ica* loci (*ica*R/*ica*ADBC) but not the biofilm-associated surface protein *bap*. For iron acquisition, all isolates harboured amongst others the iron-regulated surface determinant gene cluster *isd*ABCDEF.

While only one isolate lacked the gene encoding α-haemolysin (*hla*) completely, three isolates belonging to CC130 carried *hla* variations which resulted in aa sequence alterations and two further isolates harboured insertions (details are provided in Supplemental Table 4). Bacteriophages converting the β-haemolysin gene (*hlb*) were identified in four isolates (for details see Supplemental Table 4). Some of the *mec*C-MRSA, especially those belonging to CC599 and CC1943 harboured variants of previously described staphylococcal pathogenicity islands, including SaPI*bov*[80] encoding SEC, a TSST*bov* variant and SEIL (Fig. 1). Isolates belonging to CC1943

were positive for an *egc* cluster variant (*seg*, *sei*, *selm*, *seln*, *selo*, *selu*), while all isolates were positive for *sel*X (Fig. 1). Furthermore, all isolates were positive for intact and identical genes encoding phenol-soluble modulins (PSM α1 to PSM α4 and PSM β1 and PSM β2), and differences in the promoter regions of these operons seemed to particularly mirror the genomic lineage (data not shown).

_mec_C-MRSA belonging to CC599, CC49, CC130 and CC 1943 harbour _agr_ variations. According to the typing scheme used by Shopsin et al.[83], CC599 MRSA were assigned to the *agr* type I, CC49 to type II, CC130 to type III and CC1943 to type IV. Of the isolates harbouring the type I *agr* system, two isolates showed aa changes for AgrA and all AgrB aa sequences showed the variation A182T when compared to the *S. aureus* strain N315 sequences (Supplemental Table 4), while the respective aa sequences for AgrC and AgrD were identical (Fig. 2). All isolates belonging to CC599 and CC49 showed a nucleotide insertion (T) at bp 55 within the region encoding RNAIII, resulting in 514 + 1 bp length and an additional uracil (U) in the hairpin 2, according to the secondary structure model of RNAIII proposed by Benito and colleagues[46]. Since this insertion was found in all isolates associated with the *agr* types I and II in this study, this variation was considered as the wild type (wt) sequence here. Only two isolates belonged to the *agr* type II, and variation among them was detected in terms of a premature stop codon created by an insertion in the AgrA encoding gene in isolate IMT36945 (Fig. 2). Most variants were detected among the aa sequences for AgrA (one insertion, two changes) and AgrC (four changes) of the CC130 isolates harbouring the type III *agr* system (Fig. 2, and Supplemental Table 4). For the CC1943 isolates (type IV *agr*), only IMT36952 showed an aa sequence variation in AgrA (Fig. 2). The nucleotide sequence regions encoding for the 26-aa δ-haemolysin integrated in RNAIII and the promoter sequences for RNAII (P2) and RNAIII (P3) were conserved in all 45 genomes investigated here (Fig. 2a–d).

Assessment of genes associated with virulence factor transcription in _mec_C-MRSA. Only in isolate IMT41554 (CC130), a C to T change within the − 35 promoter region previously identified as the AgrA binding region of the *psm* α operon[84] was detected at position − 27. As the phenotype did not deviate from closely related isolates, this single nucleotide change is unlikely to affect the AgrA-binding abilities upstream of *psm* α1–4 in IMT41554. Yet, many two component regulatory systems (TCRS) and nucleic acid-binding proteins seem to modulate *S. aureus* virulence factor expression, especially of exotoxins with haemolytic activity[85]. Since TCRS variations might influence the particular phenotype appearance of *S. aureus*, the isolate collection was screened for obvious deletions, insertions or changes generating for instance amino acid (aa) substitutions or premature stop codons. Among the TCRS included in the analysis were those either positively or negatively influenced by *agr* such as the staphylococcal accessory regulator nucleic acid-binding protein (SarA) and the regulator of toxins (Rot). Overall, amino acid changes (referred to the respective reference sequence) in global regulators were rare and mostly lineage-specific (Supplemental Table 5), indicating a limited role of these variations for the phenotype differences observed for each CC.

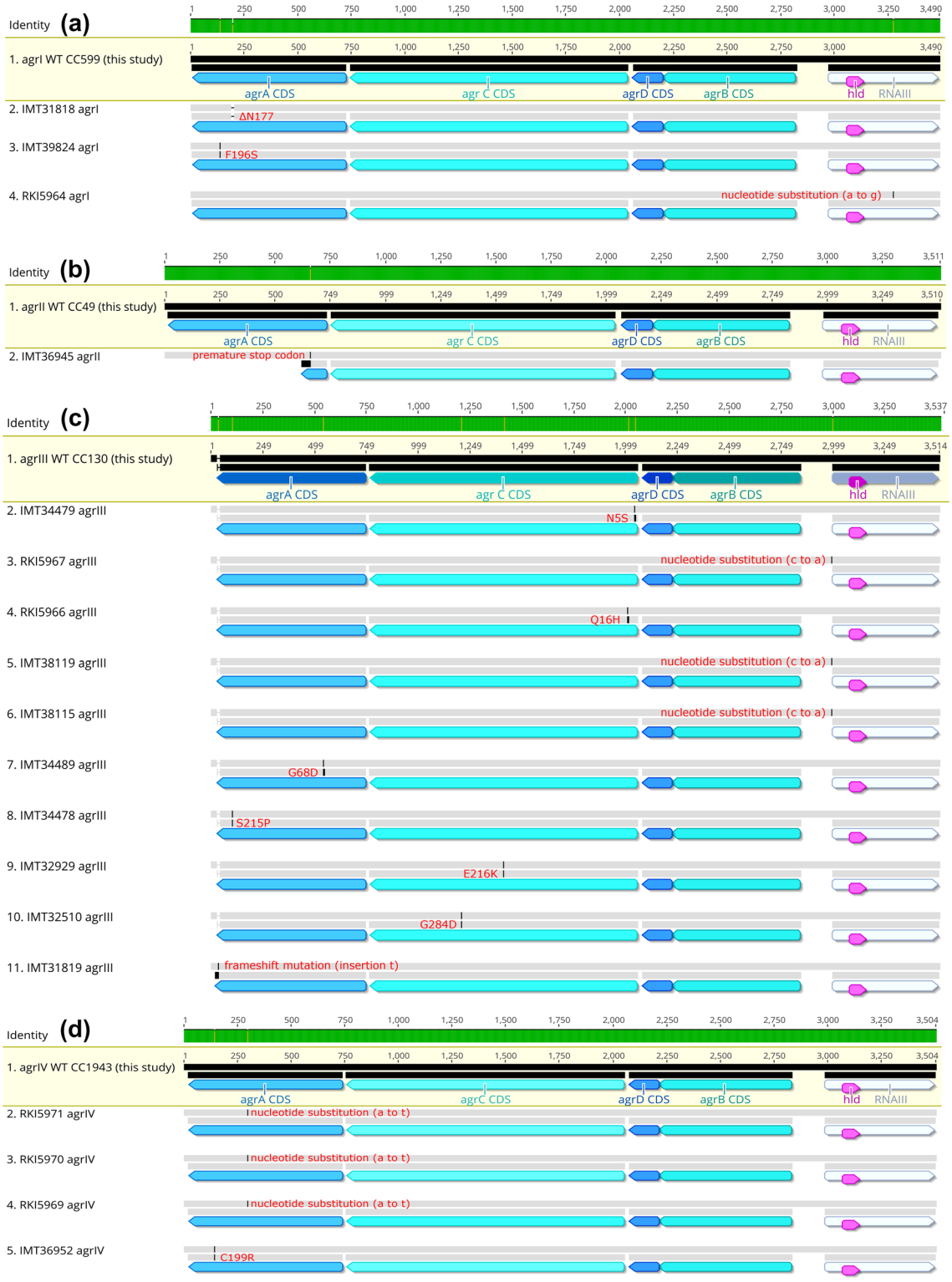
Haemolysis and colony spreading of _mec_C-MRSA carrying _agr_ variations. While analysing the *mec*C-MRSA belonging to four different phylogenetic lineages, we noticed a broad range of different phenotypes with respect to haemolysis on SBA plates.

Initially, haemolytic activities were assessed using a SPDH cross-streaking test utilizing strain RN4220 (Fig. 3). Here, only 30 of 45 isolates showed the typical haemolysis pattern for α-, β- and δ-haemolysin production (Fig. 4, Supplemental Figures 1 and 2), suggesting that some of the isolates might harbour genomic alterations affecting haemolytic activities (Table 2). We then used the CAMP test which indicates secretion of β-haemolysin by *S. aureus* through a characteristic arrow-shaped synergistic haemolysis zone on SBA. 10/45 isolates and two of the reference strains failed to produce the corresponding phenomenon (Table 2, Supplemental Figure 1), with four isolates (and two reference strains) harbouring a phage disrupting the *hlb* gene (Supplemental Table 4). The remaining six isolates lacked the *hlb*-converting phage but showed instead AgrA variations that clearly deviated from the wildtype (Fig. 2, Supplemental Table 4).

Since functionality of the *agr* system is also reflected by the ability of *S. aureus* to slide over wet surfaces[72], we performed a colony spreading assay on semisolid agar plates. As a result, 38/45 isolates showed the characteristics of "sliding bacteria", which are exemplarily shown in Fig. 5. A comprehensive summary of all phenotype characteristics of the isolate collection and the reference strains included is presented in Table 2.

Agr activity assessment by measuring δ-haemolysin production in _mec_C-MRSA using proteomics. The protein abundance of Hld (δ-haemolysin) was used to assess RNAIII transcription capabilities of *S. aureus* isolates, as the corresponding coding gene (*hld*) is integrated in the RNAIII transcript. Hld abundance was measured using whole cell proteomics to cover the actual expression level. Therefore, the assessment of direct whole-cell δ-haemolysin production appears as a suitable method to predict RNAIII transcription, as long as occurring sequence variations do not affect the *hld* encoding gene or the promoter regions in in the *agr* operon (Figs. 2 and 4a).

The protein abundance levels for δ-haemolysin measured by mass spectrometry are shown in Supplemental Table 6, and putative CC-specific differences were noticed (Fig. 4a). Isolates sharing the CC1943 background and lacking *agr* alterations, for example, showed higher overall δ-haemolysin abundance values than those belonging to CC130 or CC8 (Supplemental Table 6, Fig. 4a). Based on the δ-haemolysin detection level, we categorized the individual isolates' *agr* activity as follows: 0, lack of detectable *agr* activity; +, weak *agr* activity and ++, strong *agr* activity (Fig. 4a). The reference strains included showed Hld abundances which clearly correlate with their previously reported *agr* functionality: For RN4220, a delay towards production of Hld due to an *agr*A mutation is known[86]. This alteration is also mirrored by a decreased protein abundance (Fig. 4a) and was consequently


Identity
(a)
1. agrI WT CC599 (this study)
agrA CDS
agr C CDS
agrD CDS
agrB CDS
hld
RNAIII
2. IMT31818 agrI
ΔN177
3. IMT39824 agrI
F196S
4. RKI5964 agrI
nucleotide substitution (a to g)
Identity
(b)
1. agrII WT CC49 (this study)
agrA CDS
agr C CDS
agrD CDS
agrB CDS
hld
RNAIII
2. IMT36945 agrII
premature stop codon
Identity
(c)
1. agrIII WT CC130 (this study)
agrA CDS
agr C CDS
agrD CDS
agrB CDS
hld
RNAIII
2. IMT34479 agrIII
N5S
3. RKI5967 agrIII
nucleotide substitution (c to a)
4. RKI5966 agrIII
Q16H
5. IMT38119 agrIII
nucleotide substitution (c to a)
6. IMT38115 agrIII
nucleotide substitution (c to a)
7. IMT34489 agrIII
G68D
8. IMT34478 agrIII
S215P
9. IMT32929 agrIII
E216K
10. IMT32510 agrIII
G284D
11. IMT31819 agrIII
frameshift mutation (insertion t)
Identity
(d)
1. agrIV WT CC1943 (this study)
agrA CDS
agrC CDS
agrD CDS
agrB CDS
hld
RNAIII
2. RKI5971 agrIV
nucleotide substitution (a to t)
3. RKI5970 agrIV
nucleotide substitution (a to t)
4. RKI5969 agrIV
nucleotide substitution (a to t)
5. IMT36952 agrIV
C199R

◂**Figure 2.** Sequence alignments of *agr* regions in *mec*C-MRSA. Sequence alignments for (**a**) CC599 (*agr* I), (**b**) CC49 (*agr* II), (**c**) CC130 (*agr* III) and (**d**) CC1943 (*agr* IV) isolates. First row shaded in yellow indicates the wild type (wt) exemplarily shown for all isolates sharing 100% coverage and 100% nucleotide and amino acid sequence identity. For each of the non-wt isolates on the display, changes within the upper gray line indicates a nucleotide sequence alteration while changes in the second line indicates amino acid sequence alteration. For all details and the reference sequences used for each CC see Supplemental Table 4. (**a**) First row, wt *agr* I in CC599 shared by IMT32509, IMT32513, IMT36943, IMT 36,947, IMT36948, IMT38116, IMT39825, RKI5962, RKI5963 and RKI5964. Second row, IMT31818 has a triplet nucleotid deletion resulting in ΔN177 in AgrA; third row, IMT39824 shows a non synonymous substitution (from A to G in position 289) leading to F196S in AgrA. (**b**) First row, wt *agr* II in CC49 represented by RKI5972 while the second row shows IMT36945 with an insertion creating a premature stop codon in AgrA. (**c**) First row, wt *agr* III in CC130 shared by IMT34480, IMT34488, IMT34491, IMT36946, IMT36950, IMT38115, IMT38119, IMT39816, IMT39819, IMT40506, IMT41554, RKI5965, RKI5967 and RKI5968. Rows 2, 4, 9 and 10 show amino acid variations in AgrC for IMT34479 (N5S), RKI5966 (Q16H), IMT32929 (E216K) and IMT32510 (G284D) generated by corresponding non-synonymous substitutions. RKI5967 (row 3), IMT38119 (row 5) and IMT38115 (row 6), harbour a nucleotide substitution (**c** to **a**) at position -2 upstream the RNAIII sequence start, respectively. Rows 7, 8 and 11 harbour variants of AgrA for IMT34489 (G68D), IMT34478 (S215P) caused by non-synonymous substitutions and IMT31819 shows an insertion at position 711 bp in *agr*A causing an alternate stop codon. (**d**) First row, WT *agr* IV in CC1943 shared by RKI5973, IMT34485, IMT40507, IMT40504, IMT38113. Second to fourth row (upper gray line) show a synonymous substitution (a to t at position 442) in *agr*A for RKI5971, RKI5970 and RKI5969 while the fifth row indicates a further variation of *agr*A (C199R) generated by a non-synonymous substitution.

considered as "weak" *agr* activity. Moreover, the USA300 *agr*A::Tn strain (NE1532) completely lacked Hld detection, as expected. Thus, detection of Hld production together with a genomic inspection of the *agr* encoding region is indeed suitable to predict RNAIII transcription and *agr* functionality.

Comparison of phenotype assay results and haemolysin abundances. We then plotted the proteomic data against the results obtained from the phenotype assays described above.

The δ-haemolysin protein abundance and haemolysis phenotype associated with the isolates and reference strains are shown in Fig. 4a. Strikingly, all isolates (and reference strains) showing a strong reduction or lack of δ-haemolysin production were found to carry non-wt variants of either AgrA or AgrC (Fig. 4a). In addition, these AgrA or AgrC non-wt variants were associated with non-wt haemolysis patterns, too (Table 2, Supplemental Table 4, Fig. 4a).

The results for β-haemolysin (Hlb) protein abundances and the isolates' capability to produce a synergistic haemolysis (CAMP phenomenon) are presented in Supplemental Figure 2 and Table 2, with isolates harbouring phage-disrupted *hlb* genes and *agr* alterations being indicated. Interestingly, all isolates with non-wt AgrA variants also failed to produce the CAMP phenomenon, even when whole-cell β-haemolysin abundance levels (e.g. for IMT36945) were obviously sufficient to induce the phenotype in matching *agr* wt isolates (e.g. RKI5972). Two further isolates harbouring non-wt variants of AgrC (IMT32510 and IMT32929) produced only a weak CAMP phenomenon (blue dots in Supplemental Figure 2). Thus, a functionally active *agr* system seems to be necessary for *S. aureus* to produce the exhibit CAMP phenomenon on SBA.

When comparing the results of whole-cell α-haemolysin detection with the haemolysis phenotypes obtained by the SPDH test we noted considerable discrepancies. Thus, we clearly found Hla protein production in some of the isolates harbouring AgrA and AgrC variations, but the strains did not exhibit an α-haemolysin phenotype on SBA (Fig. 3 and Supplemental Figure 1). Vice versa, in five isolates showing α-, β-, δ-haemolysin activity on SBA (blue dots in Supplemental Figure 1), no Hla protein levels were detectable. This could for instance be a result from repeated mis-judgement of the phenotype, from significant differences for Hla abundances between proteome- and secretome or the detection limit of the mass spectrometry. Of note, the α-haemolysis phenotype was absent in all isolates harbouring AgrA or AgrC variants (Supplemental Figure 1), again indicating that a functional *agr* system is required for inducing an α-haemolysis zone on SBA.

Finally, colony spreading on wet surfaces is known to be directly induced by AgrA-P. As shown in Table 2, all isolates lacking this feature displayed non-wt variants of AgrA or AgrC, while some of the isolates with AgrA or AgrC variants were still capable to spread (Figs. 4b,5).

***Agr* variation is not associated with differences in biofilm formation on polystyrene tissue culture plates.** All 45 *mec*C-MRSA isolates were tested for their ability to form stable biofilms on the inert surfaces of polystyrene tissue culture plates in a standard crystal violet-staining assay. The test allows for the detection and quantification of sturdy biofilms that are firmly attached to artificial surfaces and which are not removable by washing. With respect to the 12 isolates belonging to CC599, only two (IMT36947 and IMT36948) formed a visible biofilm (value-1-biofilm) in the crystal violet assay (Table 2, detailed information in Supplemental Table 7). Among the 21 CC130 isolates, 12 were biofilm negative (value-0-biofilm), eight displayed moderate biofilm levels (value-1-biofilm) and one isolate produced a strong biofilm (value-2-biofilm). In addition, two out of ten CC1943 *mec*C-MRSA were strong biofilm producers (value-2-biofilms), while the remaining isolates lacked this ability completely. Summarizing the results of Table 2 and Supplemental Table 3, neither the *agr* type nor the non-wt variants of AgrA or AgrC were attributable to the ability to form a stable, non-removable biofilm on inert polystyrene surfaces.

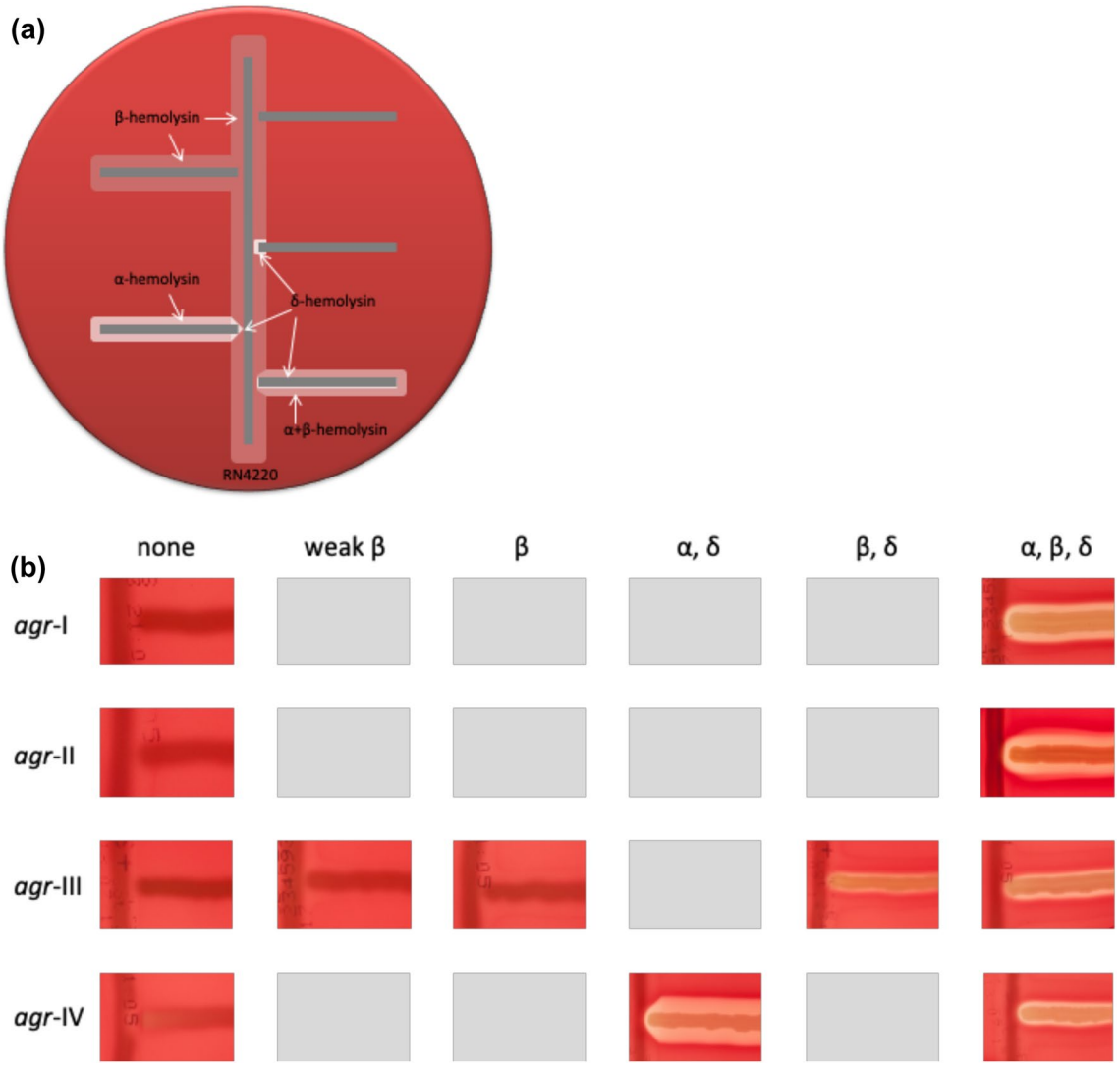


Figure 3. Haemolytic activities of *S. aureus* isolates on sheep blood agar (SBA) plates. (**a**) Scheme for assessment of haemolytic activity based on Geisinger et al.[118]. The isolates were tested by *cross-streaking perpendicularly* to *S. aureus RN4220* on sheep blood agar (SBA) plates. The turbid zone induced by β-haemolysin production of RN4220 enhanced lysis by δ-haemolysin and PSMs (clear zone at the intersection) and inhibited α-haemolysin (V-shaped zone at the intersection). (**b**) Haemolytic activity of *mec*C-positive *S. aureus* belonging to different *agr* types on SBA plates. The lack of a corresponding phenotype in the isolate collection is indicated by a grey rectangle. One exemplarily image was used to illustrate the differences, respectively.

AgrA and AgrC variations influence biofilm architecture. While the crystal violet-staining assay exclusively detects robust biofilms that remain permanently attached to surfaces upon washing, CLSM imaging allows for monitoring the biofilm development in situ. By this approach, even delicate interactions of bacterial communities on surfaces can be visualized. In order to elucidate the potential impact of *agr* functionality on biofilm architecture, we selected three representative isolates of the CC130 carrying different genomic variants of *agr* (marked by * in Table 2 and Supplemental Table 4) and analyzed them by CLSM imaging. Although the selected isolates were biofilm-negative in the crystal violet-staining assay on polystyrene, they clearly displayed an in situ biofilm on glass slides during CLSM monitoring, with remarkable differences between the isolates. Thus, the biofilm of the Hld-producing isolate IMT38119 harbouring the CC130-wt *agr* system, formed a flat, dense biofilm that covered almost the entire area of the well (Fig. 6a,b). In contrast, isolate RKI5966 (a non-wt AgrC-variant lacking Hld production) built a dense, local aggregate on the slide (Fig. 6c), with the biofilm mass growing much higher than that of *agr*-WT isolate IMT38119 (i.e. 307 µm vs. 174 µm) (Fig. 6b,d). Similarly, isolate IMT31819, a non-wt AgrA variant lacking Hld production, also formed a local aggregate (Fig. 6e) and displayed a tall biofilm mass whose architecture, however, appeared less dense than those of the other two isolates tested (Fig. 6f). The combined data suggest that the *agr* system may rather influence the biofilm architecture than the overall biofilm-forming capacity of *S. aureus*.

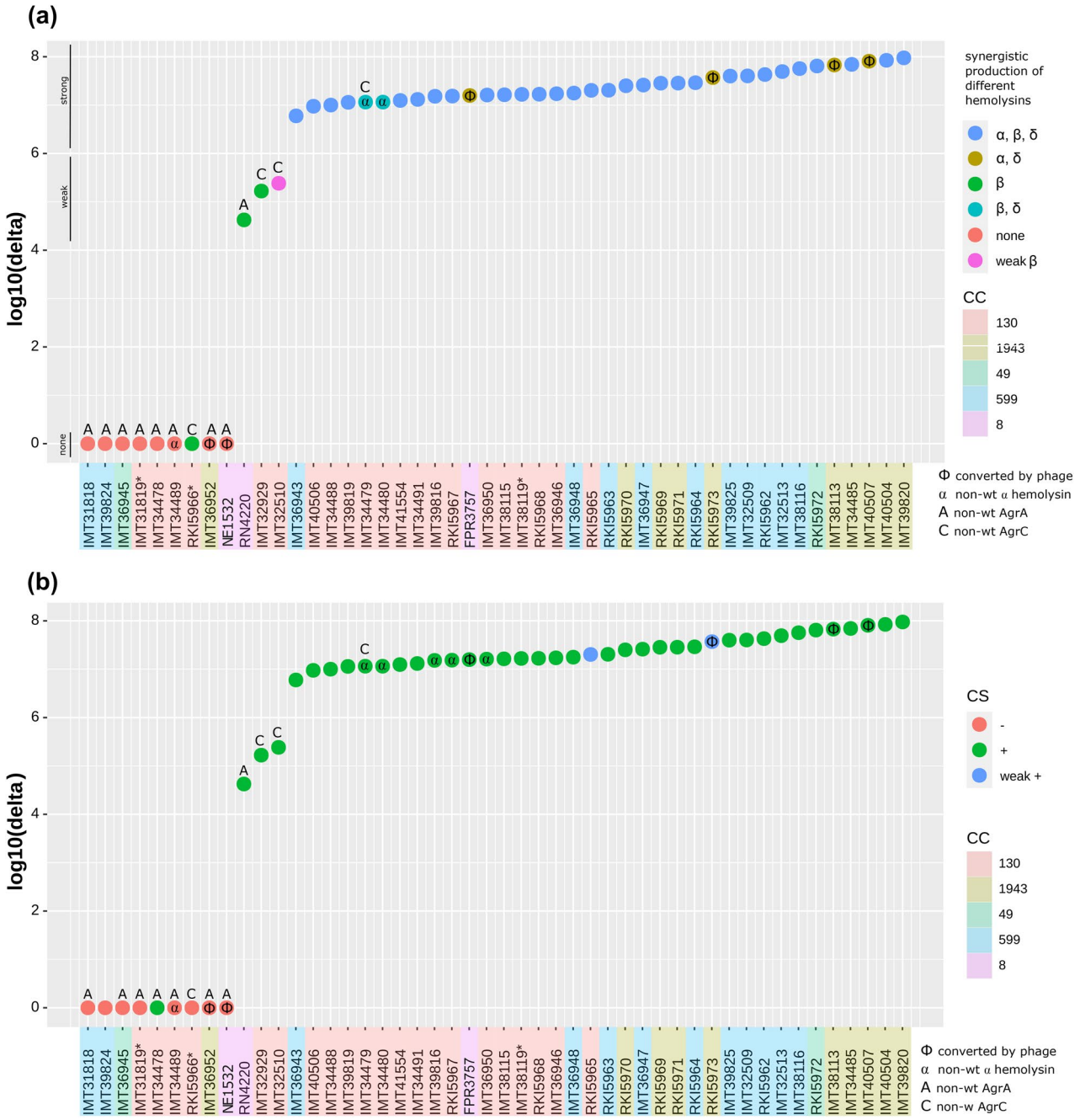


Figure 4. Illustration showing protein abundance values, relevant genomic variation and phenotype results of *mec*C-MRSA. (**a**) δ-haemolysin (Hld) protein abundance and the synergistic production of different haemolysins (SPDH test). (**b**) δ-haemolysin (Hld) protein abundance and the isolates' capability for colony spreading.

Discussion

Agr variants fine-tune virulence levels in *mec*C-MRSA belonging to CC130, CC1943, CC599 and CC49. In the past decade, MRSA have established both as commensals and as infectious agents in animals at alarming rates[87,88]. In addition to classical *mec*A-carrying MRSA clonal lineages, emerging *mec*C-MRSA further add to this problem by affecting various animal species as well as humans, suggesting a broad host range of such strains[8,14,16]. The *mec*C-MRSA isolates analysed in our study were found to belong to clonal complexes CC130, CC1943, CC599 and CC49, confirming the widespread occurrence of these lineages, at least in Europe[8, 14,16]. Each of the four CCs harboured distinct isolates with altered *agr* loci, resulting in reduced or even total loss of *agr* activity. By employing mass spectrometry, we determined Hld production which served as a proxy for RNAIII transcription in our experiments. Agr functionality was further assessed by testing haemolysis on SBA and a colony spreading assay on semisolid agar whose accuracy for predicting Agr activity was shown before[35,71]. Based on the δ-haemolysin amounts detected, the 45 isolates (and three reference strains) were assigned to dis-

ID	CC	*agr*	CS	Haemolysis on SBA		BF	*agr* acitivity level (predicted)
				CAMP	SPDH		
IMT31818	599	I	–	–	None	0	0
IMT32509	599	I	+	+	α, β, δ	0	++
IMT32513	599	I	+	+	α, β, δ	0	++
IMT36943	599	I	+	+	α, β, δ	0	++
IMT36947	599	I	+	+	α, β, δ	1	++
IMT36948	599	I	+	+	α, β, δ	1	++
IMT38116	599	I	+	+	α, β, δ	0	++
IMT39824	599	I	–	–	None	0	0
IMT39825	599	I	+	+	α, β, δ	0	++
RKI5962	599	I	+	+	α, β, δ	0	++
RKI5963	599	I	+	+	α, β, δ	0	++
RKI5964	599	I	+	+	α, β, δ	0	++
IMT36945	49	II	–	–	None	0	0
RKI5972	49	II	+	+	α, β, δ	0	++
IMT31819*	130	III	–	–	None	0	0
IMT32510	130	III	+	Weak +	Weak β	0	++
IMT32929	130	III	+	Weak +	β	1	++
IMT34478	130	III	+	–	None	1	0
IMT34479	130	III	+	+	β, δ	1	++
IMT34480	130	III	+	+	β, δ	1	++
IMT34488	130	III	+	+	α, β, δ	0	++
IMT34489	130	III	–	–	None	0	0
IMT34491	130	III	+	+	α, β, δ	0	++
IMT36946	130	III	+	+	α, β, δ	0	++
IMT36950	130	III	+	+	α, β, δ	0	++
IMT38115	130	III	+	+	α, β, δ	0	++
IMT38119*	130	III	+	+	α, β, δ	0	++
IMT39816	130	III	+	+	α, β, δ	0	++
IMT39819	130	III	+	+	α, β, δ	2	++
IMT40506	130	III	+	+	α, β, δ	1	++
IMT41554	130	III	+	+	α, β, δ	1	++
RKI5965	130	III	Weak +	+	α, β, δ	1	++
RKI5966*	130	III	–	+	β	0	0
RKI5967	130	III	+	+	α, β, δ	0	++
RKI5968	130	III	+	+	α, β, δ	1	++
IMT34485	1943	IV	+	+	α, β, δ	0	++
IMT36952	1943	IV	–	–	None	2	0
IMT38113	1943	IV	+	–	α, δ	0	++
IMT39820	1943	IV	+	+	α, β, δ	0	++
IMT40504	1943	IV	+	+	α, β, δ	0	++
IMT40507	1943	IV	+	–	α, δ	0	++
RKI5969	1943	IV	+	+	α, β, δ	0	++
RKI5970	1943	IV	+	+	α, β, δ	0	++
RKI5971	1943	IV	+	+	α, β, δ	0	++
RKI5973	1943	IV	Weak +	–	α, δ	2	++

Table 2. Phenotypic characterisics of *mec*C-MRSA. Genomic variation within the *agr* encoding region is indicated by use of bold ID letters (for details see Fig. 2). The isolates further investigated with respect to biofilm structures using CLSM are marked with *. Agr activity prediction according to Hld values presented in Fig. 4a (0, +, ++). ID, isolate number; CC, clonal complex; *agr*, accessory gene regulatory type; CS, colony spreading (phenotypical verification for *agr* functionality); CAMP, phenotypical verification for β-haemolysin production; SPDH, test results for synergistic production of different heamolysins using RN4220 (phenotypical verification of α-, β- and δ-haemolysin production); BF, biofilm formation (mean absorbance 492 nm; values: 0, < 0.2811; 1, 0.2811 < x < 1.0; 2, > 1.0); +, positive; –, negative.

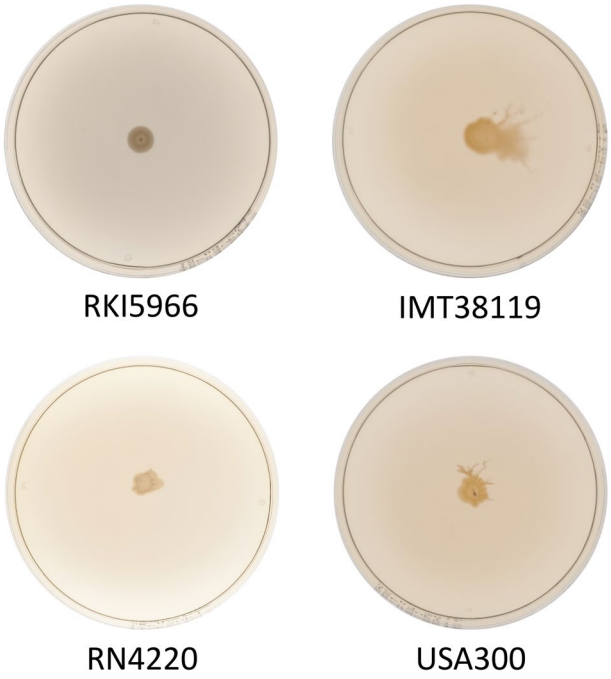


Figure 5. Colony spreading assay results for *mec*C-*S. aureus* with different *agr* functionalities on soft agar plates. A TSA soft agar plate (0.24%) was inoculated with 2 µl overnight culture of *S. aureus*. Examples shown here include isolate RKI5966 associated with a weak *agr* functionality, which was not able to spread on semisolid agar plates, while isolate IMT38119 (strong *agr* functionality) showed spreading. As controls we have employed the standard laboratory strains RN4220 (weak *agr* functionality) and USA300 (strong *agr* functionality).

tinct *agr* activity groups (i.e. 0, +, ++) which matched well with synergistic haemolysis on SBA (Fig. 4a) and the results of the colony spreading assay (Fig. 4b).

Isolates showing amino acid changes in the C-terminal DNA-binding domain of the AgrA response regulator[89] such as IMT31818 (ΔN177), IMT39824 (F196S), IMT34478 (S215P) and IMT36952 (C199R) did not produce δ-haemolysin, indicating that these aberrations silenced the *agr* system. However, IMT34478 was still capable of colony spreading, suggesting residual or *agr*-independent PSM production by a mechanism that still needs to be established.

With respect to non-wt AgrC variants, RKI5966 (Q16H) showed a variation in the transmembrane 1 domain of the protein, while the aa exchange E216K in IMT32929 is located in the C-terminal dimerization/histidine phosphotransfer subdomain of the protein[90]. These changes are prone to impair AgrC-mediated AIP sensing and signal transduction[90], and are therefore likely to cause the *agr*-negative phenotype observed in the isolates.

Further, comparative analysis of Hld production values with the CAMP test results confirmed that an active *agr* system is necessary to induce the lunar-shaped synergistic haemolysis on SBA, with Hld and/or PSMs known to contribute to the phenotype[67]. With respect to β-haemolysin production, *agr* was shown to have a major impact on *hlb* transcription[91]. However, this association is obviously not straightforward and applicable for all strains. Thus, high levels of Hlb were also detected in *S. aureus* isolates displaying low *agr* activity, such as RN4220[86], which is in good agreement with the Hlb detection results obtained in our isolates (Supplemental Figure 2). Moreover, our data indicate that at least a weak *agr* activity is necessary to induce a Hlb-mediated β-haemolysis phenotype on SBA, even when the intracellular Hlb levels were apparently sufficient to induce haemolysis in corresponding *agr* wt-isolates (Supplemental Figure 2).

Production of many secreted enzymes involved in lipid and protein degradation and haemolysins are influenced or even directly controlled by *agr*. The main effector of this locus, RNAIII, is known to promote α-haemolysin expression on the transcriptional and post-transcriptional level[92]. As expected, Hla production was not detected in isolates lacking the gene (IMT34489) and in those carrying *hla* frameshift mutations (IMT34479 and IMT34480). Surprisingly, Hla abundance values measured in whole cells of isolates with intact *hla* genes did not correlate with the *agr* activity levels (Supplemental Figure 1). Also, for some of the isolates with AgrA/AgrC aberrations and negative α-haemolysis on SBA (IMT31818, IMT32510, IMT32929, IMT36945, IMT39824, RKI5966, NE1532, and RN4220) we noticed intracellular Hla protein amounts that were comparable to that of *agr*-wt strains showing a haemolytic phenotype (Supplemental Figure 1). A study by Montgomery et al. confirmed notable *agr*-independent transcription of Hla in an *agr*-deficient strain (USA300 lineage), while the protein was not detected in the corresponding culture supernants[69], which is commonly explained by the promoting role of RNAIII for *hla* transcript translation[92]. Since our Hlb and Hla protein abundances were measured from overnight-grown cells after washing with PBS, our results indicate that some *agr*-activity is needed for toxin release. Interestingly, extracellular vesicles released from cells of the USA300 lineage were found to contain Hla[93]. In that particular study, PSMα was identified to promote biogenesis of extracellular vesicles filled with proteins by

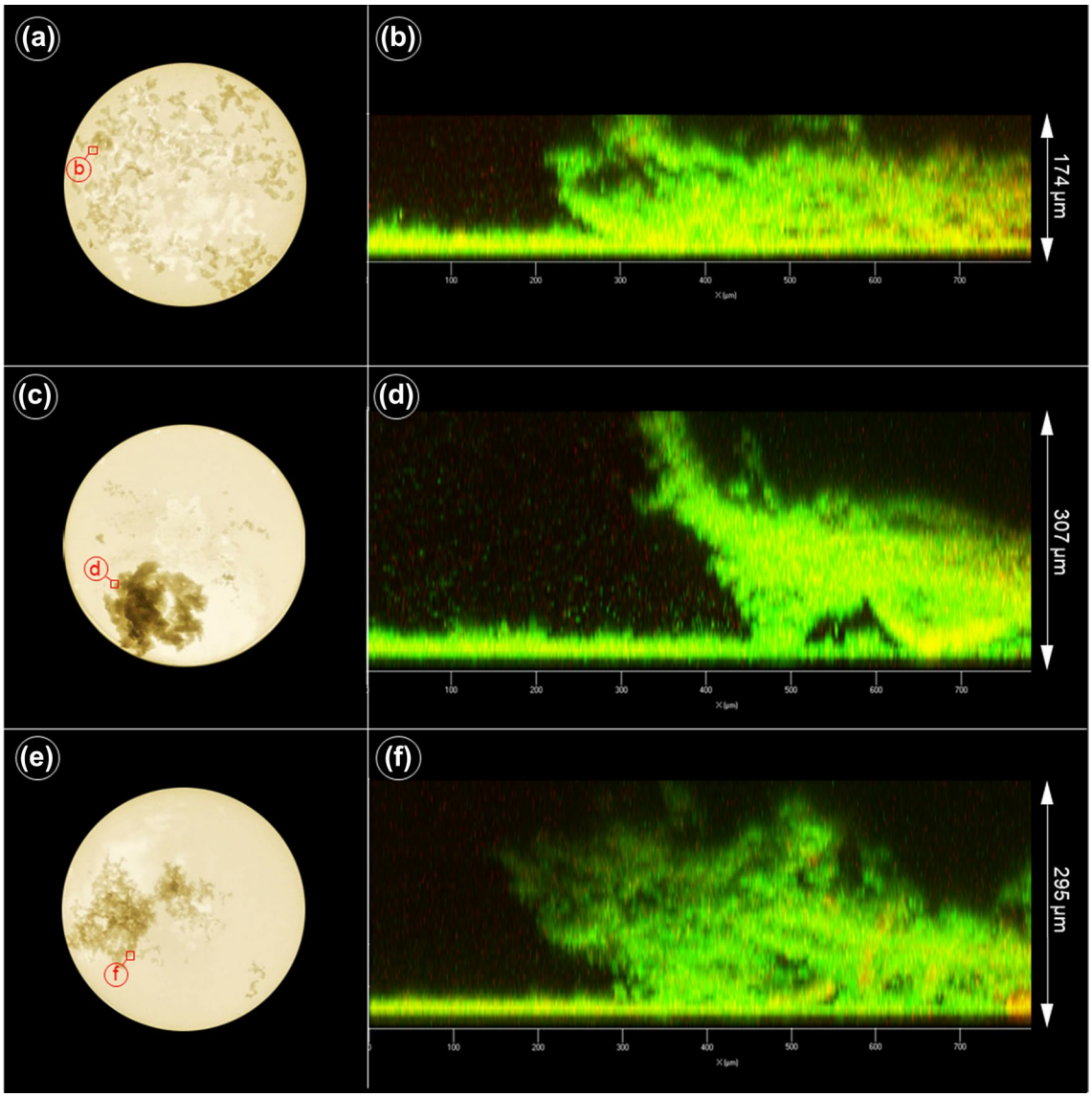


Figure 6. *Agr* activity and biofilm formation differences among *mec*C-MRSA belonging to clonal complex 130. (**a**) Macroscopic camera image of an *S. aureus* isolate (*mec*C-MRSA) harbouring the *agr* III wild type (wt) variant (IMT38119) grown in a 24-well plate and (**b**) the confocal laser scanning picture showing the biofilm profile at the indicated spot (red square). (**c**) Isolate harbouring a *agr* III variant (non-wt *agr*C variant) lacking *agr* activity (RKI5966) and (**d**) its corresponding biofilm. (**e**) Isolate lacking *agr* activity (IMT31819) and (**f**) its corresponding biofilm.

destabilisation of the cytoplasmic membrane[93]. Thus, is tempting to speculate that baseline *agr*-independent *hla* transcription and translation might occur in the variants, leading to intracellular accumulation of α-haemolysin. Lacking PSM activity (due to AgrA/C variations) might prevent release of Hla in the supernatant, resulting in the haemolysis-negative phenotypes observed. More experimental work, however, is needed to substantiate this hypothesis in the future.

Agr variants influence biofilm structure and density. Biofilm formation is a key factor in pathogenesis of persistent staphylococcal infections[94], and downregulation of *agr* is supposed to facilitate biofilm development in staphylococci[50,95–97] with strain-specific differences occurring particularly among clinical isolates[98]. When testing the *mec*C-MRSA isolates in a standard crystal violet-staining biofilm assay, we did not find an association between *agr* functionality and stable biofilm formation in polystyrene tissue culture plates (Table 2), which is in good agreement with previous findings in clinical MRSA clonal lineages[38]. Generally, we found only a few biofilm-forming isolates by this assay, although biofilm-associated genes were present and intact in all strains. In comparison to *S. epidermidis*, *S. aureus* is known to show a much weaker biofilm detection performance in the standard biofilm assay. Rather than suggesting a lower overall biofilm-forming capacity of *S. aureus*, the phenomenon may reflect a general mechanically instable contact of biofilm-associated *S. aureus* cells to inert surfaces, leading to removal of loosely attached biofilm structures upon washing of the plates. Indeed,

when using CLSM imaging, which does not involve washing of the cover slips, we detected visible biofilms, at least in the three CC130 isolates analysed (Fig. 6). Interestingly, strains possessing different *agr* activity levels displayed differences regarding biofilm thickness and architecture. Thus, while the WT-*agr* isolate formed a well-organized biofilm (Fig. 6b), the two *agr*-deficient variants displayed a much higher biofilm mass which, however, appeared less-structured (Fig. 6d,f). This might be due to the lack of *agr*-dependent production of PSMs which were previously shown to play an eminent role in functional biofilm architecture[99,100]. *S. aureus* biofilm formation is a complex and highly dynamic process which comprises, according to a recently newly defined five-stage model, attachment, multiplication, exodus, maturation and dispersal of the biofilm[101]. Apart from its function in maturation and dispersal (involving *agr*-controlled proteases and PSMs), *agr* also plays a significant role during initial attachment by facilitating (in the early growth stage) expression of cell wall-anchored proteins that mediate host matrix protein binding as well as contact to abiotic surfaces[102]. We currently speculate that the loose biofilm structure in the CC130 *agr*-variants might be associated with a diminished initial attachment of the bacteria to the surface and/or to each other. However, it is conceivable that other processes known to shape the biofilm architecture such as programmed autolysis and eDNA release[103] might be (indirectly) affected by the Agr variations as well. But, clearly more experimental work is needed to substantiate this hypothesis.

Adaptation strategies of *mec*C-MRSA might involve *agr* defectiveness and carriage of *agr*-independent virulence factors. Evolution and changes of bacterial virulence is highly dynamic and difficult to predict[104,105]. Attenuated virulence favouring host colonization and/or persistence of infection (e.g. small colony variants) is a common concept in bacterial evolution[106–108]. The *mec*C-MRSA lineages CC130, CC49, CC1943 and CC599 were obviously not among those CC's frequently reported for nasal colonization in humans and animals such as CC22 and CC398[109–112]. Consequently, other strategies might promote their viability or even spread among mammalian hosts and the environment. On one hand, the disability to produce the *agr*D-encoded autoinducer peptide (AIP) may prevent costly competition of invading strains with an "incompatible" *agr* system harboured by other *S. aureus* lineages or even other staphylococcal species in a particular host[113]. One the other hand, presence of a compatible AIP system allows the cells to take advantage of the *agr*-induced factors produced by co-habiting staphylococci[29]. A highly instructive study, using a wax moth larva virulence model, revealed that a functional *agr* system is necessary for a cooperative and beneficial "behaviour" of the local population, while *agr* defective mutants exploit ("cheat") on the cells with a functional *agr* system, allowing them to prevail when grown in mixed populations with cooperators[113]. Based on earlier published results of growth competition experiments[113,114], an increased viability of *agr* defective *S. aureus* mutants among resident staphylococci of different host species seems to be likely. This view is further supported by a recent study, showing that distinct external stress conditions drive the selection of Agr quorum-sensing mutants that may confer a fitness advantage to the *S. aureus* population[115].

Unspecific and unregulated T-cell stimulators (superantigens) such as enterotoxins, enterotoxin-like proteins, and the toxic shock syndrome toxin contribute to host cell damages inducible by *S. aureus*. Combinations of genes encoding these superantigens were identified in varying frequencies for all four lineages reported on here (Fig. 1). With respect to enterotoxins[116], Enterotoxin C (SEC), which is commonly located on a SaPI (reviewed in[49]), was identified in isolates belonging to CC130, CC599 and CC1943. However, SEC production requires *agr*-depending *rot* degradation[117], which is defective in some of the isolates reported on here. While classical enterotoxins such as SEA-C are regulated by the *agr* system, the "novel" enterotoxins and variants of the toxic shock syndrome toxin seem to be *agr* independent (reviewed in[49]). In line with this, we have shown expression of the protein encoded by *tst*-bov in isolates showing lacking detectable *agr* activity (Fig. 1, Table 2), exemplified by isolates IMT31818 and IMT39824. The variants of tst-*bov* were harboured by *S. aureus* pathogenicity islands showing mosaic structures of known and novel SaPIs, an observation which has been reported (e.g. for human clinical isolates) before[118].

Conclusion

Comparative genomics of the *agr* encoding region allow identification of variants deviating from the wildtype in *mec*C-MRSA belonging to CC130, CC599, CC49 and CC1943, and subsequent proteomics revealed the capability of each altered *agr* system to transcribe RNAIII, which was directly mirrored by the corresponding Hld protein values. Our research indicates that *mec*C-MRSA with *agr* variations are defective for *agr*-depending quorum sensing, harbour additional *agr*-independent virulence factors and exhibit varying biofilm properties as a likely part of their survival strategy. In bacteria, adaptation to a changing environment is often associated with the selection for mutations in (virulence factor) genes that become dispensable or disadvantageous in the novel niche (reviewed in[104]). This concept can obviously be extended to global regulators such as the *agr* quorum-sensing system as well, resulting in pleiotropic effects and the generation of phenotypic heterogeneity that might further support the establishment of emerging clonal lineages in new hosts and niches.

Data availability

Genomic sequencing data used are available for download from the National Center for Biotechnology Information (NCBI) under BioProject accessions PRJNA588740. Accession numbers of whole genomes sequences are provided in Supplemental Table 2. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (https://proteomecentral.proteomexchange.org) via the PRIDE partner repository with the dataset identifier PXD016486.

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Author contributions

C.H. and B.W. designed the project. C.H., B.W., C.S., W.Z. and J.D. conceived and designed the experiments. I.S., B.S., C.C. and W.W. collected and screened the isolates. A.T. sequenced the isolates and T.S. and L.E. analysed the phylogenetic relationship based on WGS data. C.H., G.J., T.M., C.S., A.L-B. and J.D. performed the laboratory experiments. C.H., B.W., W.Z., G.M., M.B., J.D., C.S., L.E., T.S., A. L-B. and L.H.W. analysed the data. C.H., W.Z., M.B., J.D., C.S., T.S., L.H.W. and B.W. wrote the manuscript. All authors have read and approved the final draft of the manuscript.

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Competing interests

The authors declare no competing interests.

Additional information

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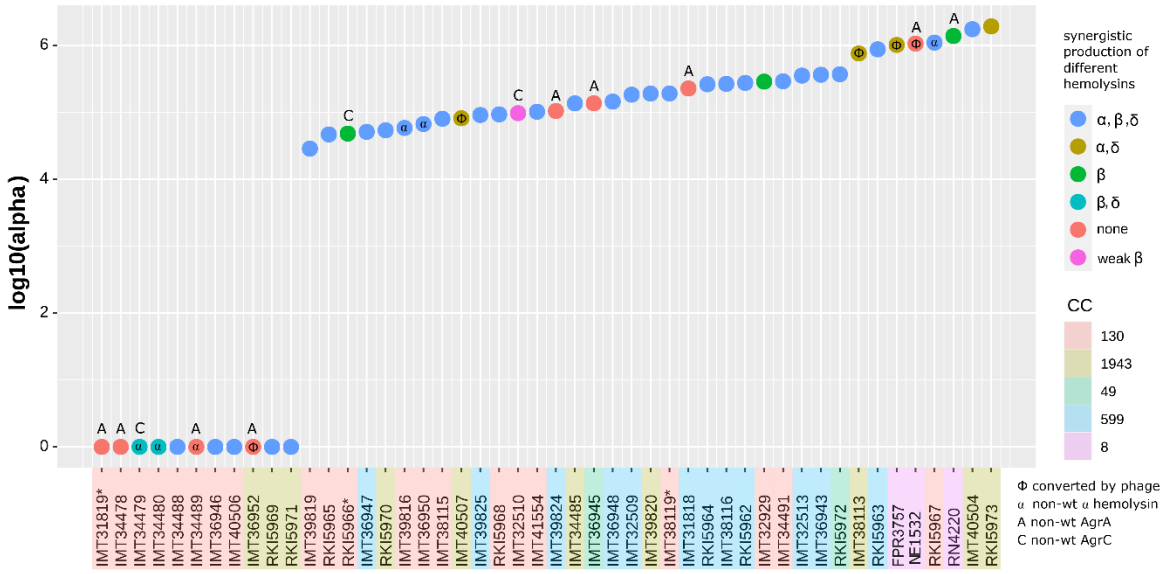
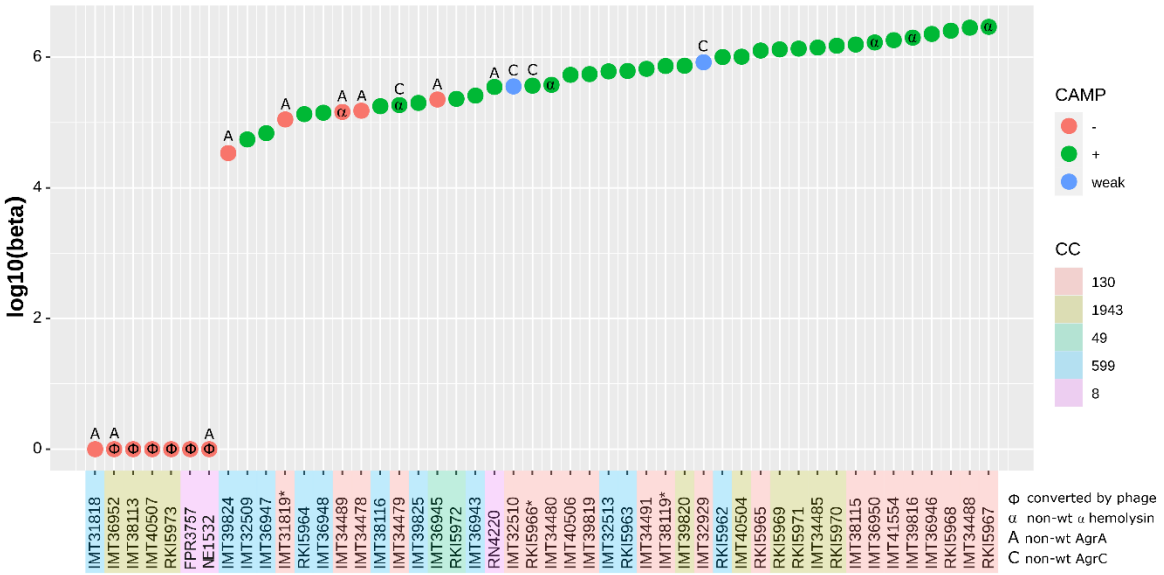
Supplementary Information

Supplemental Figure 1: Synergistic production of different haemolysins and alpha haemolysin (Hla) protein abundance levels



Supplemental Figure 2: CAMP phenomenon and β-haemolysin (Hlb) protein abundances



Supplemental Table 1: Dynamic window sizing for mass spectrometry

Mass spectrometry		
start [m/z]	end [m/z]	width [m/z]
349,5	382,5	33
381,5	406,5	25
405,5	422,5	17
421,5	437,5	16
436,5	449,5	13
448,5	460,5	12
459,5	469,5	10
468,5	478,5	10
477,5	487,5	10
486,5	496,5	10
495,5	505,5	10
504,5	514,5	10
513,5	522,5	9
521,5	531,5	10
530,5	540,5	10
539,5	548,5	9
547,5	555,5	8
554,5	563,5	9
562,5	572,5	10
571,5	581,5	10
580,5	590,5	10
589,5	599,5	10
598,5	608,5	10
607,5	617,5	10
616,5	626,5	10
625,5	635,5	10
634,5	644,5	10
643,5	652,5	9
651,5	661,5	10
660,5	671,5	11
670,5	680,5	10
679,5	690,5	11
689,5	700,5	11
699,5	710,5	11
709,5	721,5	12
720,5	733,5	13
732,5	745,5	13
744,5	758,5	14
757,5	770,5	13
769,5	782,5	13
781,5	795,5	14
794,5	811	16
810	827	17
825,5	843,5	18
842,5	860,5	18
859,5	880,5	21
879,5	902,5	23
901,5	926,5	25
925,5	954,5	29
953,5	986,5	33
985,5	1023,5	38
1022,5	1070,5	48
1069,5	1150,5	81

Supplemental Table 2: Genome Accession Numbers

BioProject accessions PRJNA588740

Genome accession numbers	
sample-ID	**accession**
IMT31818	SRR10426558
IMT31819	SRR10426557
IMT32509	SRR10426546
IMT32510	SRR10426535
IMT32513	SRR10426524
IMT32929	SRR10426518
IMT34478	SRR10426517
IMT34479	SRR10426516
IMT34480	SRR10426515
IMT34485	SRR10426514
IMT34488	SRR10426556
IMT34489	SRR10426555
IMT34491	SRR10426554
IMT36943	SRR10426553
IMT36945	SRR10426552
IMT36946	SRR10426551
IMT36947	SRR10426550
IMT36948	SRR10426549
IMT36950	SRR10426548
IMT36952	SRR10426547
IMT38113	SRR10426545
IMT38115	SRR10426544
IMT38116	SRR10426543
IMT38119	SRR10426542
IMT39816	SRR10426541
IMT39819	SRR10426540
IMT39820	SRR10426539
IMT39824	SRR10426538
IMT39825	SRR10426537
IMT40504	SRR10426536
IMT40506	SRR10426534
IMT40507	SRR10426533
IMT41554	SRR10426532
RKI5962	SRR10426531
RKI5963	SRR10426530
RKI5964	SRR10426529
RKI5965	SRR10426528
RKI5966	SRR10426527
RKI5967	SRR10426526
RKI5968	SRR10426525
RKI5969	SRR10426523
RKI5970	SRR10426522
RKI5971	SRR10426521
RKI5972	SRR10426520
RKI5973	SRR10426519

Supplemental Table 3: Pairwise SNP-distances between the core genomes were calculated for all MRSA isolates

IMI32929	IMI34488	RKI5965	IMI39816	IMI39819	RKI5966	IMI34489	IMI34478	IMI31819	IMI40506	IMI39825	RKI5962	RKI5964	RKI5963	IMI36948	IMI36947	IMI32513	IMI39824	IMI36943	IMI31818	IMI38116	IMI32509
15161	15158	15161	15172	15137	15133	15151	15141	15138	15140	15341	15331	15374	15411	15385	15385	15330	15346	15326	15343	15354	15334
15130	15127	15130	15141	15106	15102	15120	15110	15107	15109	15312	15302	15345	15382	15356	15356	15301	15317	15297	15314	15325	15305
10509	10506	10509	10514	10485	10476	10495	10487	10482	10484	10934	10922	10965	11006	10976	10976	10923	10935	10917	10936	10947	10927
10502	10497	10502	10507	10478	10469	10488	10480	10475	10477	10927	10915	10958	10999	10969	10969	10916	10928	10910	10929	10940	10920
10521	10518	10521	10526	10497	10488	10507	10499	10494	10496	10946	10934	10977	11018	10988	10988	10935	10947	10929	10948	10959	10939
10514	10511	10514	10519	10490	10481	10500	10492	10487	10489	10940	10928	10971	11012	10982	10982	10929	10941	10923	10942	10953	10933
10512	10509	10512	10517	10488	10479	10498	10490	10485	10487	10939	10927	10970	11011	10981	10981	10928	10940	10922	10941	10952	10932
10502	10499	10502	10507	10478	10469	10488	10480	10475	10477	10929	10917	10960	11001	10971	10971	10918	10930	10912	10931	10942	10922
10511	10508	10511	10516	10487	10478	10497	10489	10484	10486	10937	10925	10968	11009	10979	10979	10926	10938	10920	10939	10950	10930
10511	10508	10511	10516	10487	10478	10497	10489	10484	10486	10937	10925	10968	11009	10979	10979	10926	10938	10920	10939	10950	10930
10510	10507	10510	10515	10486	10477	10496	10488	10483	10485	10936	10924	10967	11008	10978	10978	10925	10937	10919	10938	10949	10929
10548	10545	10550	10555	10526	10517	10536	10528	10523	10525	10974	10962	11005	11046	11016	11016	10963	10975	10957	10976	10987	10967
349	346	347	354	323	315	335	329	322	324	10008	9995	10039	10081	10050	10050	9993	10009	9991	10010	10021	10001
354	351	354	361	332	322	342	336	329	331	10015	10002	10046	10088	10057	10057	10000	10016	9998	10017	10028	10008
310	307	308	315	286	276	296	290	283	285	9971	9958	10002	10044	10013	10013	9956	9972	9954	9973	9984	9964
405	402	403	409	381	371	391	385	378	380	10057	10044	10088	10129	10099	10099	10042	10058	10040	10059	10070	10050
225	222	297	304	275	265	285	279	272	274	9976	9963	10007	10049	10018	10018	9961	9977	9959	9976	9987	9967
250	247	322	329	300	290	310	304	297	299	9998	9985	10029	10071	10040	10040	9983	9999	9981	9998	10009	9989
204	201	282	289	260	250	270	264	257	259	9964	9951	9995	10037	10006	10006	9949	9965	9947	9964	9975	9955
229	226	317	324	295	285	305	299	292	294	9994	9981	10025	10067	10036	10036	9979	9995	9977	9994	10005	9985
234	231	321	328	300	290	308	304	297	299	10003	9990	10034	10076	10045	10045	9988	10004	9986	10003	10014	9994
237	234	324	331	303	293	311	307	300	302	10006	9993	10037	10079	10048	10048	9991	10007	9989	10006	10017	9997
238	235	334	341	312	302	322	316	309	311	10009	9996	10040	10082	10051	10051	9994	10010	9992	10009	10020	10000
	145	342	349	320	310	330	324	317	319	10019	10006	10050	10092	10061	10061	10004	10020	10002	10019	10030	10010
145		338	346	317	307	327	321	314	316	10016	10003	10047	10089	10058	10058	10001	10017	9999	10016	10027	10007
342	338		183	204	200	220	214	207	209	10017	10004	10048	10090	10059	10059	10002	10018	10000	10017	10028	10008
349	346	183		211	207	227	221	214	216	10022	10009	10053	10095	10064	10064	10007	10023	10005	10022	10033	10013
320	317	204	211		178	198	192	185	187	9994	9981	10025	10067	10036	10036	9979	9995	9977	9994	10005	9985
310	307	200	207	178		178	172	165	167	9987	9974	10018	10060	10029	10029	9972	9988	9970	9987	9998	9978
330	327	220	227	198	178		94	101	103	10005	9992	10036	10078	10047	10047	9990	10006	9988	10005	10016	9996
324	321	214	221	192	172	94		95	97	9997	9984	10028	10070	10039	10039	9982	9998	9980	9997	10008	9988
317	314	207	214	185	165	101	95		40	9994	9981	10025	10067	10036	10036	9979	9995	9977	9994	10005	9985
319	316	209	216	187	167	103	97	40		9996	9983	10027	10069	10038	10038	9981	9997	9979	9996	10007	9987
10019	10016	10017	10022	9994	9987	10005	9997	9994	9996		74	221	269	234	234	171	185	165	186	197	177
10006	10003	10004	10009	9981	9974	9992	9984	9981	9983	74		209	257	222	222	159	173	153	174	185	165
10050	10047	10048	10053	10025	10018	10036	10028	10025	10027	221	209		302	267	267	210	224	204	225	236	216
10092	10089	10090	10095	10067	10060	10078	10070	10067	10069	269	257	302		267	267	258	272	252	273	284	264
10061	10058	10059	10064	10036	10029	10047	10039	10036	10038	234	222	267	267		0	223	237	217	238	249	229
10061	10058	10059	10064	10036	10029	10047	10039	10036	10038	234	222	267	267	0		223	237	217	238	249	229
10004	10001	10002	10007	9979	9972	9990	9982	9979	9981	171	159	210	258	223	223		124	122	143	154	134
10020	10017	10018	10023	9995	9988	10006	9998	9995	9997	185	173	224	272	237	237	124		136	157	168	148
10002	9999	10000	10005	9977	9970	9988	9980	9977	9979	165	153	204	252	217	217	122	136		133	144	124
10019	10016	10017	10022	9994	9987	10005	9997	9994	9996	186	174	225	273	238	238	143	157	133		57	37
10030	10027	10028	10033	10005	9998	10016	10008	10005	10007	197	185	236	284	249	249	154	168	144	57		46
10010	10007	10008	10013	9985	9978	9996	9988	9985	9987	177	165	216	264	229	229	134	148	124	37	46	

Supplemental Table 4: Amino acid variation in the *agr* system of *mec*C MRSA

	Amino acid sequences (predicted)								
		Hemolysins				Accessory gene regulatory system			
ID	CC	alpha	beta	delta	*agr*	AgrA	AgrB	AgrC	AgrD
IMT31818	599	WT	WT	WT	I	ΔN177	A182T	WT	WT
IMT32509	599	WT	WT	WT	I	WT	A182T	WT	WT
IMT32513	599	WT	WT	WT	I	WT	A182T	WT	WT
IMT36943	599	WT	WT	WT	I	WT	A182T	WT	WT
IMT36947	599	WT	WT	WT	I	WT	A182T	WT	WT
IMT36948	599	WT	WT	WT	I	WT	A182T	WT	WT
IMT38116	599	WT	WT	WT	I	WT	A182T	WT	WT
IMT39824	599	WT	WT	WT	I	F196S	A182T	WT	WT
IMT39825	599	WT	WT	WT	I	WT	A182T	WT	WT
RKI5962	599	WT	WT	WT	I	WT	A182T	WT	WT
RKI5963	599	WT	WT	WT	I	WT	A182T	WT	WT
RKI5964	599	WT	WT	WT	I	WT	A182T	WT	WT
IMT36945	49	WT	WT	WT	II	short	Y76C	WT	WT
RKI5972	49	WT	WT	WT	II	WT	Y76C	WT	WT
IMT31819*	130	WT	WT	WT	III	IS	WT	WT	WT
IMT32510	130	WT	WT	WT	III	WT	WT	G284D	WT
IMT32929	130	WT	WT	WT	III	WT	WT	E216K	WT
IMT34478	130	WT	WT	WT	III	S215P	WT	WT	WT
IMT34479	130	α IS	WT	WT	III	WT	WT	N5S	WT
IMT34480	130	α IS	WT	WT	III	WT	WT	WT	WT
IMT34488	130	WT	WT	WT	III	WT	WT	WT	WT
IMT34489	130	Δ	WT	WT	III	G68D	WT	WT	WT
IMT34491	130	WT	WT	WT	III	WT	WT	WT	WT
IMT36946	130	WT	WT	WT	III	WT	WT	WT	WT

IMT36950	130	T12I	WT	WT	III	WT	WT	WT	WT
IMT38115	130	WT	WT	WT	III	WT	WT	WT	WT
IMT38119*	130	WT	WT	WT	III	WT	WT	WT	WT
IMT39816	130	K189E	WT	WT	III	WT	WT	WT	WT
IMT39819	130	WT	WT	WT	III	WT	WT	WT	WT
IMT40506	130	WT	WT	WT	III	WT	WT	WT	WT
IMT41554	130	WT	WT	WT	III	WT	WT	WT	WT
RKI5965	130	WT	WT	WT	III	WT	WT	WT	WT
RKI5966*	130	WT	WT	WT	III	WT	WT	Q16H	WT
RKI5967	130	D211G	WT	WT	III	WT	WT	WT	WT
RKI5968	130	WT	WT	WT	III	WT	WT	WT	WT
IMT34485	1943	WT	WT	WT	IV	WT	WT	I64S	WT
IMT36952	1943	WT	P	WT	IV	C199R	WT	I64S	WT
IMT38113	1943	WT	P	WT	IV	WT	WT	I64S	WT
IMT39820	1943	WT	WT	WT	IV	WT	WT	I64S	WT
IMT40504	1943	WT	A68V	WT	IV	WT	WT	I64S	WT
IMT40507	1943	WT	P	WT	IV	WT	WT	I64S	WT
RKI5969	1943	WT	WT	WT	IV	WT	WT	I64S	WT
RKI5970	1943	WT	WT	WT	IV	WT	WT	I64S	WT
RKI5971	1943	WT	WT	WT	IV	WT	WT	I64S	WT
RKI5973	1943	WT	P	WT	IV	WT	WT	I64S	WT

Amino acid sequences for alpha, beta and delta haemolysins and the *agr* system of 45 *mec*C-MRSA. Position of amino acid changes with respect to the wild type (WT) reference sequence (below) are shown.
Abbreviations: ID, isolate number; CC, clonal complex; *agr*, accessory gene regulatory type; Δ, deletion; IS, insertion; *, isolates further investigated with respect to biofilm structures using CLSM.

NCBI Reference sequences (aa) and strain IDs									
		AGR I		AGR II		AGR III		AGR IV	
		accession	strain	accession	strain	accession	strain	accession	strain
Agr	AgrA	P0A0I6.1	MW2	P0A0I6.1	MW2	P0A0I6.1	MW2	P0A0I6.1	MW2
	AgrB	QBY49017.1	O268	CZQ66246.1	1943STDY5698362	AUG74347.1	O46	SAO20788.1	n.a.
	AgrC	EVG59461.1	OCMM6067	KIT97067.1	SA-120	KIT81064.1	SA-006 (N5S)	ABB17521.1	H560
	AgrD	YP_500744.1	NCTC8325	ABQ49857.1	JH9	EFH25310.1	ATCC 51811	AGU55642.1	6850
Hemolysins	alpha	EFK81723.1	TCH70						
	beta	AAB32218.1	126/89						
	delta	Q2FWM8	NCTC8325						
Abbreviations: n.a. not available; accession, NCBI accession number for amino acid sequence; strain, wild type/reference strain									

Supplemental Table 5: Amino acid variations in global regulators (Protein ID / Strain ID)

	SaeS	SaeR	SrrA	SrrB	ArlR	ArlS	SarA	SarR	Rot	MgrA	SigB
	Histidine protein kinase	Response regulator	Transcrip-tional regulatory protein	Sensor protein	Response regulator	Signal transduc-tion histidine-protein kinase	Transcrip-tional regulator	HTH-type transcrip-tional regulator	repressor of toxins	HTH-type transcrip-tional regulator	RNA poly-merase sigma factor
Reference	Q7A6V4 / N315	Q7A6V3 / N315	Q7A5H6 / N315	Q8NWF3 / MW2	Q9KJN4 / NCTC 8325	WP_06413 3950.1	Q7A732 / N315	Q2FEJ8-1 / USA300	EEW47452. 1	Q7A6X2 / N315	P95844
IMT31818	WT	WT	WT	A322T; V434I (CC599)	WT	WT	WT	WT	WT	WT	WT
IMT32509	WT	WT	WT	A322T; V434I (CC599)	WT	WT	WT	WT	WT	WT	WT
IMT32513	WT	WT	WT	A322T; V434I (CC599)	WT	WT	WT	WT	WT	WT	WT
IMT36943	WT	WT	WT	A322T; V434I (CC599)	WT	WT	WT	WT	WT	WT	WT
IMT36947	WT	WT	WT	A322T; V434I (CC599)	WT	WT	WT	WT	WT	WT	WT
IMT36948	WT	WT	WT	A322T; V434I (CC599)	WT	WT	WT	WT	WT	WT	WT
IMT38116	WT	WT	WT	A322T; V434I (CC599)	WT	WT	WT	WT	WT	WT	WT
IMT39824	WT	WT	WT	A322T; V434I (CC599)	WT	WT	WT	WT	WT	WT	WT
IMT39825	WT	WT	WT	A322T; V434I (CC599)	WT	WT	WT	WT	WT	WT	WT
RKI5962	WT	WT	WT	A322T; V434I (CC599)	WT	WT	WT	WT	WT	WT	WT

RKI5963	WT	WT	WT	A322T; V434I (CC599)	WT	WT	WT	WT	WT	WT	WT
RKI5964	WT	WT	WT	A322T; V434I (CC599)	WT	WT	WT	WT	WT	WT	WT
Reference	Q7A6V4 / N315	Q7A6V3 / N315	Q7A5H6 / N315	Q8NWF3 / MW2	Q9KJN4 / NCTC 8325	WP_06413 3950.1	Q7A732 / N315	Q2FEJ8-1 / USA300	EEW47452. 1	Q7A6X2 / N315	P95844
IMT36945	WT	WT	WT	V434I; D509A	WT	WT	WT	WT	WT	WT	WT
RKI5972	WT	WT	WT	V434I; D509A	WT	WT	WT	WT	WT	WT	WT
Reference	Q7A6V4 / N315	Q7A6V3 / N315	Q7A5H6 / N315	Q8NWF3 / MW2	Q9KJN4 / NCTC 8325	WP_06413 3950.1	Q7A732 / N315	Q2FEJ8-1 / USA300	EEW47452. 1	Q7A6X2 / N315	P95844
IMT31819	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
IMT32510	WT	E225G	WT	WT	WT	WT	WT	WT	WT	WT	WT
IMT32929	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	A253V
IMT34478	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
IMT34479	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
IMT34480	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
IMT34488	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
IMT34489	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
IMT34491	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
IMT36946	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
IMT36950	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
IMT38115	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
IMT38119	WT	WT	WT	T123S	WT	WT	WT	WT	R59I	WT	WT
IMT39816	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
IMT39819	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
IMT40506	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
IMT41554	WT	WT	WT	insertion	WT	WT	WT	WT	WT	WT	WT
RKI5965	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT

RKI5966	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
RKI5967	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
RKI5968	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
Reference	Q7A6V4 / N315	Q7A6V3 / N315	Q7A5H6 / N315	Q8NWF3 / MW2	Q9KJN4 / NCTC 8325	WP_10320 5040.1	Q7A732 / N315	Q2FEJ8-1 / USA300	EEW47452. 1	Q7A6X2 / N315	P95844
IMT34485	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
IMT36952	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
IMT38113	D269G	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
IMT39820	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
IMT40504	P193L	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
IMT40507	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
RKI5969	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
RKI5970	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
RKI5971	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
RKI5973	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	I214N

WT, wild type

Supplemental Table 6: alpha-, beta- and delta-haemolysin abundance levels

	Hemolysin		
ID	**alpha**	**beta**	**delta**
IMT31818	226466	0	0
IMT32509	182889	55119	40203500
IMT32513	352463	606824	49393600
IMT36943	362910	258289	5991790
IMT36947	50962	68713	25962700
IMT36948	144179	141258	17740600
IMT38116	265550	177798	56901700
IMT39824	104197	34039	0
IMT39825	90945	199562	39853100
RKI5962	274323	1006250	42899800
RKI5963	871106	615917	20333500
RKI5964	261854	134389	29062200
IMT36945	136132	225265	0
RKI5972	369213	230575	64359000
IMT31819*	0	111460	0
IMT32510	96885	357332	242092
IMT32929	286669	835736	167153
IMT34478	0	151672	0
IMT34479	0	184999	11537100
IMT34480	0	377950	11540400
IMT34488	0	2829180	10020100
IMT34489	0	145289	0
IMT34491	289696	663535	13129800
IMT36946	0	2268950	17193500
IMT36950	66586	1679970	16183400
IMT38115	79961	1564050	16393100
IMT38119*	190628	734137	16701500
IMT39816	58233	1993900	15230400
IMT39819	28569	551468	11417000
IMT40506	0	536742	9470570
IMT41554	101750	1824170	12473100
RKI5965	46568	1264390	20195000
RKI5966*	47915	366644	0
RKI5967	1097210	2914410	15388400
RKI5968	93002	2544930	16805900
IMT34485	135750	1403930	69541000
IMT36952	0	0	0
IMT38113	755597	0	67475600
IMT39820	190022	740548	94823900
IMT40504	1740330	1017250	84429200

IMT40507	81362	0	80901100
RKI5969	0	1317800	28418500
RKI5970	53724	1498920	25153000
RKI5971	0	1351630	28530200
RKI5973	1917410	0	37149400
RN4220	1379010	350906	42317
FPR3757	1012840	0	15792500
NE1532	1060150	0	0

* selected for CLSM

Supplemental Table 7: Original data from the biofilm crystal violet-staining assay

Biofilm crystal violet assay (original data)

	1st biological replicate				2nd biological replicate				3rd biological replicate				
ID	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**mean absorbance 492nm**
IMT31818	0,29	0,35	0,25	0,26	0,21	0,26	0,21	0,19	0,17	0,25	0,22	0,18	0,2369
IMT31819	0,14	0,15	0,13	0,16	0,19	0,24	0,18	0,16	0,16	0,16	0,14	0,19	0,1647
IMT32509	0,11	0,11	0,12	0,11	0,11	0,12	0,11	0,11	0,12	0,11	0,10	0,12	0,1115
IMT32510	0,21	0,23	0,29	0,20	0,22	0,19	0,20	0,22	0,21	0,33	0,22	0,20	0,2275
IMT32513	0,16	0,22	0,27	0,21	0,32	0,29	0,26	0,28	0,22	0,25	0,20	0,23	0,2428
IMT32929	0,52	0,54	0,45	0,71	0,50	0,47	0,49	0,45	0,75	0,75	0,72	0,83	0,5969
IMT34478	0,76	0,87	0,73	0,84	0,96	0,87	0,82	0,66	1,11	0,94	0,84	0,66	0,8366
IMT34479	0,28	0,34	0,48	0,42	0,30	0,30	0,27	0,26	0,23	0,26	0,23	0,22	0,2982
IMT34480	0,23	0,27	0,26	0,21	0,32	0,37	0,37	0,23	0,33	0,34	0,27	0,39	0,2978
IMT34485	0,15	0,09	0,08	0,09	0,11	0,09	0,09	0,10	0,10	0,09	0,09	0,08	0,0970
IMT34488	0,26	0,30	0,25	0,28	0,21	0,23	0,18	0,19	0,17	0,21	0,17	0,20	0,2181
IMT34489	0,25	0,23	0,25	0,24	0,18	0,19	0,23	0,24	0,22	0,24	0,23	0,19	0,2238
IMT34491	0,18	0,20	0,19	0,20	0,19	0,21	0,19	0,17	0,16	0,20	0,17	0,20	0,1893
IMT36943	0,19	0,28	0,24	0,24	0,16	0,21	0,20	0,19	0,16	0,22	0,22	0,20	0,2068
IMT36945	0,17	0,18	0,18	0,20	0,17	0,21	0,17	0,21	0,18	0,19	0,18	0,20	0,1857
IMT36946	0,19	0,12	0,14	0,10	0,13	0,11	0,11	0,11	0,14	0,08	0,11	0,11	0,1216
IMT36947	0,46	0,36	0,40	0,38	0,42	0,46	0,42	0,53	0,19	0,26	0,23	0,24	0,3621
IMT36948	0,21	0,23	0,23	0,39	0,42	0,53	0,37	0,46	0,24	0,22	0,23	0,25	0,3142
IMT36950	0,16	0,17	0,25	0,23	0,13	0,13	0,16	0,16	0,14	0,16	0,14	0,21	0,1693
IMT36952	1,00	1,19	1,18	1,08	1,08	1,26	1,40	1,19	1,35	1,56	1,57	1,21	1,2555
IMT38113	0,10	0,10	0,09	0,10	0,09	0,09	0,09	0,09	0,12	0,09	0,09	0,12	0,0973
IMT38115	0,20	0,17	0,18	0,25	0,16	0,25	0,22	0,24	0,18	0,36	0,35	0,33	0,2407
IMT38116	0,08	0,11	0,09	0,11	0,13	0,12	0,10	0,14	0,10	0,09	0,10	0,13	0,1093
IMT38119	0,20	0,20	0,22	0,26	0,18	0,19	0,18	0,19	0,29	0,33	0,43	0,32	0,2473
IMT39816	0,18	0,23	0,24	0,28	0,23	0,25	0,22	0,22	0,23	0,23	0,35	0,36	0,2503

IMT39819	1,77	1,49	1,67	1,75	2,37	2,46	2,36	2,37	1,99	1,46	2,39	1,71	1,9811
IMT39820	0,15	0,18	0,17	0,14	0,11	0,15	0,15	0,14	0,20	0,19	0,18	0,20	0,1636
IMT39824	0,26	0,27	0,30	0,28	0,14	0,16	0,15	0,19	0,15	0,14	0,14	0,15	0,1932
IMT39825	0,10	0,09	0,08	0,09	0,15	0,14	0,11	0,11	0,10	0,11	0,12	0,11	0,1089
IMT40504	0,25	0,35	0,25	0,29	0,19	0,20	0,21	0,20	0,30	0,32	0,35	0,39	0,2730
IMT40506	0,62	0,64	0,66	0,64	0,65	0,65	0,44	0,38	0,34	0,39	0,27	0,34	0,5013
IMT40507	0,14	0,22	0,17	0,18	0,14	0,14	0,11	0,14	0,08	0,09	0,12	0,11	0,1363
IMT41554	0,27	0,45	0,39	0,29	0,26	0,20	0,21	0,18	0,26	0,35	0,42	0,20	0,2898
RKI5962	0,25	0,30	0,29	0,26	0,21	0,25	0,22	0,32	0,30	0,23	0,30	0,25	0,2652
RKI5963	0,24	0,20	0,22	0,22	0,16	0,18	0,20	0,20	0,16	0,17	0,23	0,24	0,2011
RKI5964	0,22	0,20	0,19	0,22	0,17	0,11	0,11	0,11	0,20	0,19	0,13	0,21	0,1713
RKI5965	0,40	0,26	0,34	0,22	0,41	0,39	0,30	0,28	0,34	0,44	0,39	0,39	0,3448
RKI5966	0,18	0,21	0,24	0,15	0,14	0,26	0,14	0,16	0,14	0,26	0,14	0,16	0,1828
RKI5967	0,15	0,18	0,17	0,17	0,17	0,17	0,13	0,16	0,17	0,17	0,14	0,16	0,1614
RKI5968	0,48	0,66	0,64	0,66	0,39	0,41	0,54	0,52	0,39	0,41	0,53	0,52	0,5127
RKI5969	0,14	0,14	0,15	0,15	0,19	0,17	0,17	0,18	0,19	0,17	0,17	0,18	0,1666
RKI5970	0,20	0,17	0,18	0,17	0,18	0,18	0,18	0,18	0,18	0,19	0,18	0,18	0,1804
RKI5971	0,14	0,15	0,14	0,17	0,16	0,17	0,17	0,16	0,16	0,18	0,18	0,17	0,1632
RKI5972	0,10	0,09	0,11	0,09	0,09	0,10	0,10	0,09	0,09	0,10	0,09	0,09	0,0960
RKI5973	2,30	2,16	2,21	2,06	1,96	2,39	1,98	2,16	1,96	2,39	1,98	2,16	2,1418
RP62A	4,08	4,02	2,69	2,05	2,02	2,27	2,03	2,02	2,03	4,33	3,92	3,91	
	3,94	2,78	3,40	3,54	3,39	3,27	4,30	4,28	4,17	4,04	4,80	4,80	
	3,90	4,10			3,66	3,74			3,81	3,95			3,5071
TM300	0,10	0,13	0,06	0,08	0,07	0,05	0,08	0,08	0,05	0,05	0,07	0,09	
	0,08	0,07	0,09	0,08	0,07	0,05	0,10	0,12	0,18	0,11	0,11	0,13	
	0,14	0,15			0,13	0,09			0,12	0,09			0,0937

Measured optical density OD492 of all tested samples (three biological replicates). *S. epidermidis* RP62A and *S. carnosus* TM300 were used as positive and negative controls, respectively. A sample will be classified as biofilm positive if its measured optical density is at least three times the optical density of the negative control TM300.

2.2 Publication II

How to survive pig farming: Mechanism of SCC*mec* element deletion and metabolic stress adaptation in livestock-associated MRSA

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CH, AL-B, LW, LE, SW, and BW contributed to conception and design of the study. **CH**, JB, JA, and BW performed laboratory experiments and analysis. Genomics and transcriptomics were carried out by LE, SW, AT, and TS. **CH**, MB, MH, AB, WZ, and BW analysed the results. **CH** and BW wrote the first draft of the manuscript. LE, SW, WZ, and MB wrote sections of the manuscript. All authors contributed to the article and approved the submitted version.

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How to survive pig farming: Mechanism of SCC*mec* element deletion and metabolic stress adaptation in livestock-associated MRSA

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Previous research on methicillin susceptible *Staphylococcus aureus* (MSSA) belonging to livestock-associated (LA-) sequence type (ST) 398, isolated from pigs and their local surroundings, indicated that differences between these MSSA and their methicillin resistant predecessors (MRSA) are often limited to the absence of the staphylococcal cassette chromosome *mec* (SCC*mec*) and few single nucleotide polymorphisms. So far, our understanding on how LA-MRSA endure the environmental conditions associated with pig-farming as well as the putative impact of this particular environment on the mobilisation of SCC*mec* elements is limited. Thus, we performed in-depth genomic and transcriptomic analyses using the LA-MRSA ST398 strain IMT38951 and its methicillin susceptible descendant. We identified a mosaic-structured SCC*mec* region including a putative replicative SCC*mec*Vc which is absent from the MSSA chromosome through homologous recombination. Based on our data, such events occur between short repetitive sequences identified within and adjacent to two distinct alleles of the large cassette recombinase genes C (*ccr*C). We further evaluated the global transcriptomic response of MRSA ST398 to particular pig-farm associated conditions, i.e., contact with host proteins (porcine serum) and a high ammonia concentration. Differential expression of global regulators involved in stress response control were identified, i.e., ammonia-induced alternative sigma factor B-depending activation of genes for the alkaline shock protein 23, the heat shock response and the accessory gene regulator (*agr*)-controlled transcription of virulence factors. Exposure to serum transiently induced the transcription of distinct virulence factor encoding genes. Transcription of genes reported for mediating the loss of methicillin resistance, especially *ccr*C, was not significantly

different compared to the unchallenged controls. We concluded that, from an evolutionary perspective, bacteria may save energy by incidentally dismissing a fully replicative SCC*mec* element in contrast to the induction of *ccr* genes on a population scale. Since the genomic SCC*mec* integration site is a hot-spot of recombination, occasional losses of elements of 16 kb size may restore capacities for the uptake of foreign genetic material. Subsequent spread of resistance, on the other hand, might depend on the autonomous replication machinery of the deleted SCC*mec* elements that probably enhance chances for reintegration of SCC*mec* into susceptible genomes by mere multiplication.

KEYWORDS

methicillin resistant *Staphylococcus aureus*, livestock associated, SCC*mec*, transcriptome analysis, recombination, deletion, ammonium, manure (litter)

Introduction

Since the mid-2000s, sequence type (ST)398 and its closely related descendants represent the predominant clonal complex (CC) of livestock-associated methicillin resistant *Staphylococcus aureus* (LA-MRSA) throughout Europe (Butaye et al., 2016). ST398 LA-MRSA most likely originate from a lineage of human methicillin susceptible *S. aureus* (MSSA) and have since adapted to livestock (particularly to pig farming) *via* acquisition of resistance genes against tetracycline and beta-lactam antibiotics (Price et al., 2012). ST398 LA-MRSA are also capable of causing infections in humans (Williamson et al., 2014; Sieber et al., 2019; Lu et al., 2021), a problem that is of particular concern across regions with high pig farming density (Köck et al., 2009; Vanderhaeghen et al., 2010; Gómez et al., 2020). Indeed, some MRSA are prone to cross species barriers (Sheppard et al., 2018) and are capable of rapid adaptation to different habitats and environmental conditions (Larsen et al., 2022). Adaptation processes were mainly studied on the genomic level and include either changes of the core genome (Huber et al., 2020) or an uptake of mobile genetic elements (MGEs; Mccarthy and Lindsay, 2013; Walther et al., 2018; Sieber et al., 2019; Huber et al., 2020; Leinweber et al., 2021).

Genome alterations also affect the staphylococcal cassette chromosome *mec* (SCC*mec*) including its *mec* complex which confers broad-spectrum β-lactam resistance in *S. aureus* and whose spontaneous loss was reported for ST398 isolates (Chlebowicz et al., 2010; Gómez et al., 2020). SCC*mec* elements represent large genomic islands of various sizes and compositions, which are inserted into the highly conserved region between the *rlm*H (previously *orfX*) and *dus* (previously *orf*Y) genes of staphylococcal chromosomes (Semmler et al., 2016). Loss of SCC*mec* elements can either result from homologous recombination (HR) events (involving the recombination apparatus of the cell) or through precise excision of the element from its integration site. The latter process is driven by the SCC*mec*-encoded site-specific serine recombinases (i.e., *ccr*A/B or *ccr*C) which also mediate integration of the element into the *rlm*H gene (Hanssen and Ericson Sollid, 2006; Chlebowicz et al., 2010; Wang and Archer, 2010; Liu et al., 2017; Gómez et al., 2020).

Interestingly, loss of SCC*mec* is thought to provide an advantage for *S. aureus* in challenging environments, for example when exposed to antibiotics (Noto et al., 2008) and/or the host immune system (Read et al., 2018; Tickler et al., 2020); and in LA-MRSA, the specific environmental conditions surrounding pig farming were proposed as factors that might facilitate deletion of the element (Nübel et al., 2010; Vandendriessche et al., 2014).

In industrial pig farming, the toxic gas ammonium hydroxide (NH_3) is released from manure during (bacterial) deamination of proteins fed to the animals. The gas subsequently accumulates in the air of the barn and, due to its high solubility in fluids, it is harmful to airway mucosal surfaces of pig farm workers and exposed pigs (Urbain et al., 1996; Wang et al., 2020) Thus, it is unavoidable that MRSA colonizing moist mucosal surfaces of the anterior nostrils of pigs are exposed to increased concentrations of ammonia. Therefore, exposure to high concentrations of ammonia is a condition commonly encountered by LA-MRSA ST398, and a recent study demonstrated that these bacteria indeed survive under such conditions (Astrup et al., 2021).

Regarding cytosolic pH, ammonia is the preferred nitrogen source of most microorganisms, and once in the cell, is directed to glutamine synthesis (Gruswitz et al., 2007). However, information on ammonia-induced (metabolic) gene expression patterns in LA-MRSA are currently scarce. Aside from exposure to the harsh barn environment, LA-MRSA remain associated with pigs as their primary host, which illustrates another condition the bacteria are required to adapt to.

Taking these different environmental conditions into account, the aims of this study were: (i) to reconstruct the region downstream of *rlm*H in an ST398 MRSA and its methicillin susceptible descendant (MSSA) in order to reveal the mechanisms involved in the loss of SCC*mec*; (ii) to evaluate the global transcriptomic response of MRSA ST398 exposed to particular LA environmental conditions, i.e., contact with host proteins (porcine

serum) and enhanced ammonia concentrations, with a special focus on (iii) transcription levels of genes previously reported as being associated with the acquisition or loss of SCC*mec* elements.

Materials and methods

Bacterial strains

Strain MRSA IMT38951, isolated from a nose swab of a pig in 2016, and its isogenic MSSA descendant IMT38951_42 (Busche et al., 2018) were used throughout this study. The MSSA isolate was discovered during serial-dilutions of overnight grown broth cultures [Mueller-Hinton (MH) bouillon (Becton Dickinson, Heidelberg, Germany)] of IMT38951. Presence and absence of methicillin resistance was determined according to CLSI standards (Clinical and Laboratory Standards Institute, 2013, 2014) and verified by PCR (Merlino et al., 2002). MSSA IMT38951_42 was enclosed to comparatively analyze the chromosomal integration site downstream of *rlm*H (Boundy et al., 2013), as described previously (Chlebowicz et al., 2010; Wang et al., 2017; Wu et al., 2018; Gómez et al., 2020).

DNA isolation, whole-genome sequencing and assembly

Genomic bacterial DNA was extracted using the QIAamp DNA Mini Kit (QIAGEN, Venlo, Netherlands) according to the manufacturer's instructions. Isolates were pre-incubated with 0.1 mg/ml Lysostaphin and 0.1 mg/ml Proteinase K (both from Sigma-Aldrich, St. Louis, Missouri, United States). NanoDrop Spectrophotometer (Thermo Fisher Scientific, United States) as well as the Qubit 2.0 Fluorometer (Invitrogen, United States) were used to evaluate the DNA quality and quantity, respectively.

Whole-genome sequencing (WGS) was performed using an Illumina MiSeq which resulted in 300 bp paired-end reads with an obtained coverage >90X. Furthermore, size selection was conducted on the DNA using a 10 K Blue Pippin kit for additional sequencing with the Single Molecule Real-Time (SMRT) Sequencing Technology on a PacBio RS II by GATC Biotech AG (Konstanz, Germany). PacBio raw data was assembled by Flye v2.8.2-b1689 (Kolmogorov et al., 2019). Adapter-trimmed Illumina short reads were used for assembly correction utilizing the polishing procedure of unicycler v0.4.8 (Wick et al., 2017). *De novo* assembled genomes were annotated using Prokka v1.14.6 (Seemann, 2014). Both genomes were comparatively investigated with respect to single nucleotide polymorphisms (SNPs), insertions and deletions by read mapping utilizing botwie2 v2.3.0 (Langmead and Salzberg, 2012). Putative sequence variants were defined as divergent nucleotides with a minimum coverage of 10X and an allele frequency of >80%. ResFinder-4.1 (Zankari et al., 2012; Bortolaia et al., 2020) was used to identify resistance genes, and further genomic sites of interest were investigated using Geneious 11.1.5 (Biomatters Ltd., Australia; Kearse et al., 2012). PHASTER (Arndt et al., 2016) was utilized to identify putative (pro-) phages across both genomes.

Selection of RNA Seq samples

Cells of IMT38951 were grown in 60 ml MH II and incubated overnight at 37°C and 250 rpm and, on the following day, diluted to an OD_{600} of 0.1 in 100 ml of fresh MH II and further incubated at 37°C and 250 rpm.

Based on previous research (Anderson et al., 2010; Malachowa et al., 2011; Cardile et al., 2014; Pinon et al., 2015) we used an ammonia concentration of 0.3% (Th. Geyer, Renningen, Germany), 10% porcine serum (Innovative Research Inc., Peary Court Novi, Michigan, United States) as well as a combination of both in order to mimic environmental conditions associated with pig-farming. Sampling time points were selected based on results of Stojanov et al. (2015) on SCC*mec* excision and the major regulative activity of the accessory gene regulator (*agr*)-system (Wang and Muir, 2016; Huber et al., 2020). At an OD_{600} of 0.4, the broths were supplemented with either porcine serum (10%), ammonia (0.3%), or both. To ensure the viability of the bacteria in the supplemented media, the viable plate count was determined every 15 min (0–90 min after media supplementation). Media from controls were not supplemented.

A previously published protocol (Lavoie and Summers, 2018) was used to select the samples for RNA sequencing to guarantee overall comparability. Briefly, for each of the experiments, seven biological replicates were initially grown alongside seven corresponding controls. The OD_{600} was determined for each set of samples and controls (Supplementary Table 1). Then, three out of the seven samples that exhibited the most similar OD_{600} values per growth condition and time point (10 and 60 min after media supplementation) were selected for subsequent RNA isolation ($n = 18$ samples). In addition, the three most similar control samples per growth condition and time point were selected as well ($n = 18$; written in bold in Supplementary Table 1). Each aliquot was centrifuged at 20,000 g and 4°C for 3 min. The supernatant was aspirated, and the pellet was immediately frozen at −80°C following previously published protocols (Mäder et al., 2016; Lavoie and Summers, 2018).

RNA sequencing and differential transcriptomic analysis

In total, 36 cell pellets (18 samples and 18 associated controls) were shipped to LGC Genomics GmbH (Berlin, Germany), where the RNA was isolated using the RNASnap method (Stead et al., 2012). RNA sequencing was performed on an Illumina NextSeq 500/550 V2, resulting in one channel paired-end reads. Details of the company's standard protocols for quality control, RNA extraction and rRNA depletion using Ribo-Zero (Epicentre

Biozym, Hessisch Oldendorf, Germany) are available online.[1] cDNA synthesis, library generation, indexing and cluster generation were performed using Illumina technology (TruSeq RNA Sample Preparation Kit v2).

SCORE v1.0.2 (Wolf et al., 2020) was used to perform differential RNA sequencing analysis by comparing the transcriptomic profiles of selected challenges (ammonia, porcine serum or the combination of them) with control samples, resulting in the identification of differentially expressed genes (DEGs) across the sample set. Initial analysis steps including read preprocessing (trimming of low-quality ends with Phred score < 20), mapping to the genomic reference, transcript quantification (genes below 10 counts were discarded), statistical identification of differentially expressed genes between sample groups (corrected $p \leq 0.05$), overrepresentation analysis of associated Gene Ontologies (GO) and visualization of affected Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were performed within SCORE. An additional fold change filter (log2FC > 1) was applied to ensure biological relevancy of the DEGs (Slany et al., 2017). Genes not fulfilling these criteria were counted as not being differentially expressed between conditions. The resulting expression tables were subsequently merged and additionally characterized using CD-Search v3.18 (Marchler-Bauer and Bryant, 2004) and eggNOG-mapper v2.0.5 (Huerta-Cepas et al., 2019). Principal component analysis (PCA) was performed based on normalized transcripts per million (TPM) expression values to assess inter-sample distances (Scholes and Lewis, 2020).

Results

Reconstruction of the SCC*mec* integration site downstream of *rlm*H revealed the loss of a complete SCC*mec*Vc element harbouring a replication machinery

To allow determination of the precise genomic sites flanking the loss of a *mec*A-containing sequence in MSSA IMT38951_42, a finished whole-genome reference sequence was required. PacBio long reads were utilized to assemble the draft genomes using Flye, resulting in a coverage of >335-times and > 331-times. Short-read polishing of the genomes was performed, providing an additional 161X and 187X sequence coverage to the reconstructed genomes of IMT38951 (MRSA) and IMT38951_42, respectively (Figure 1).

Further comparative WGS analysis revealed an overall genome size of 3,022,864 bases for IMT38951 and 3,011,968 bases for IMT38951_42. The former contains 2,896 coding sequences (CDS), 19 rRNA and 61 tRNAs, while the latter harbors 30 CDS less according to the annotation. Initial strain typing of IMT38951 and IMT38951_42 assigned both isolates to LA-ST398 and *spa* type t12359. In addition to the *mec*A gene present in IMT38951, both genomes harbour a similar set of further resistance genes [*aad*D, *fex*A, *bla*Z, *dfr*G, *tet*(K), *tet*(M), *lnu*(B) and *lsa*(E); Figure 1]. Prediction of prophages by PHASTER based on sequence similarity-based searches revealed three hits for complete phages, PHAGE_Staphy_47_NC_007054 (72.2 kb in size), PHAGE_Staphy_phiJB_NC_028669 (60 kb) and PHAGE_Staphy_StauST398_3_NC_021332 (66.9 kb) and two further incomplete phages of the lengths 16.6 kb (PHAGE_Staphy_PT1028_NC_007045) and 41.2 kb (PHAGE_Staphy_47_NC_007054), respectively (Figure 1). Both strains harbour a small plasmid of 5,359 bp size that resembles the *Staphylococcus hyicus* plasmid pKKS966 (99.82% identity, 73% coverage GenBank: FN677368.1) carrying the trimethoprim resistance gene *dfr*K (Kadlec et al., 2012).

Pairwise distance and relatedness (Figure 1) was calculated, revealing a total difference of 10 SNPs (excluding phage regions) and the lack of a 16 kb sequence downstream of the SCC*mec* integration site (*rlm*H) in IMT38951_42 (Figure 2). A closer inspection of the region revealed the loss of a complete SCC*mec*Vc element (Figure 2) comprising the *ccr*C8 type recombinase, the C2 *mec* complex and a recently described putative operon containing an A-family DNA polymerase (CCPol) together with a small protein lacking conserved domains ("middle protein," MP) as well as a putative helicase (Cch2; referred to as "CCPol-MP-Cch2" I & II in Figures 2, 3), as recently described by Bebel et al. (2020). The region downstream of *rlm*H in IMT38951 includes a notable repertoire of homologues sequences, i.e., two distinct but closely related *ccr*C variants and two nearly homologues intergenic spacer regions of 224 bp length, four transposases (*tnp*) of insertion sequence (IS) element 431 (IS431) and two variants of the replication machinery CCPol-MP-Cch2 (Figure 2; Supplementary Table 2) (region *rlm*H – *dus*, highlighted in green). The data suggest that the deletion of SCC*mec*Vc occurred during HR between either *ccr*C8 and *ccr*C1 (first 48 bp are 100% identical) or the terminal 38 bp (identity: 100%) of the two 224 bp-comprising intergenic spacer regions upstream of the *ccr*C variants, respectively (Figure 3; Supplementary Figure 1). Both loci harbour short repetitive sequences ("TAAAA," A & B and C & D in Figure 3) that provide at least two distinct possibilities for HR without any further sequence alterations apart from the loss of the SCC*mec* element within this region. Both intergenic spacer sequences present in the MRSA variant harbor the original (identical) promoter region of *ccr*C variants, including the 19 bp SOS box described by Liu et al. (Liu et al., 2017; Supplementary Figure 1). Interestingly, the deleted SCC*mec*Vc element still harbours its putative autonomous replication apparatus, since all loci involved (direct repeats, inverted repeats, origin of replication, CCPol-MP-Cch2 II) remain completely unaffected by the proposed recombination event (Supplementary Figure 1).

A *ccr*-mediated integration and excision, on the other hand, is known to change the number of 15 bp DR in the region downstream of *rlm*H (Semmler et al., 2016). Here, the total number and location of the two distinct 15 bp repeats present in

1 https://www.biosearchtech.com/services/

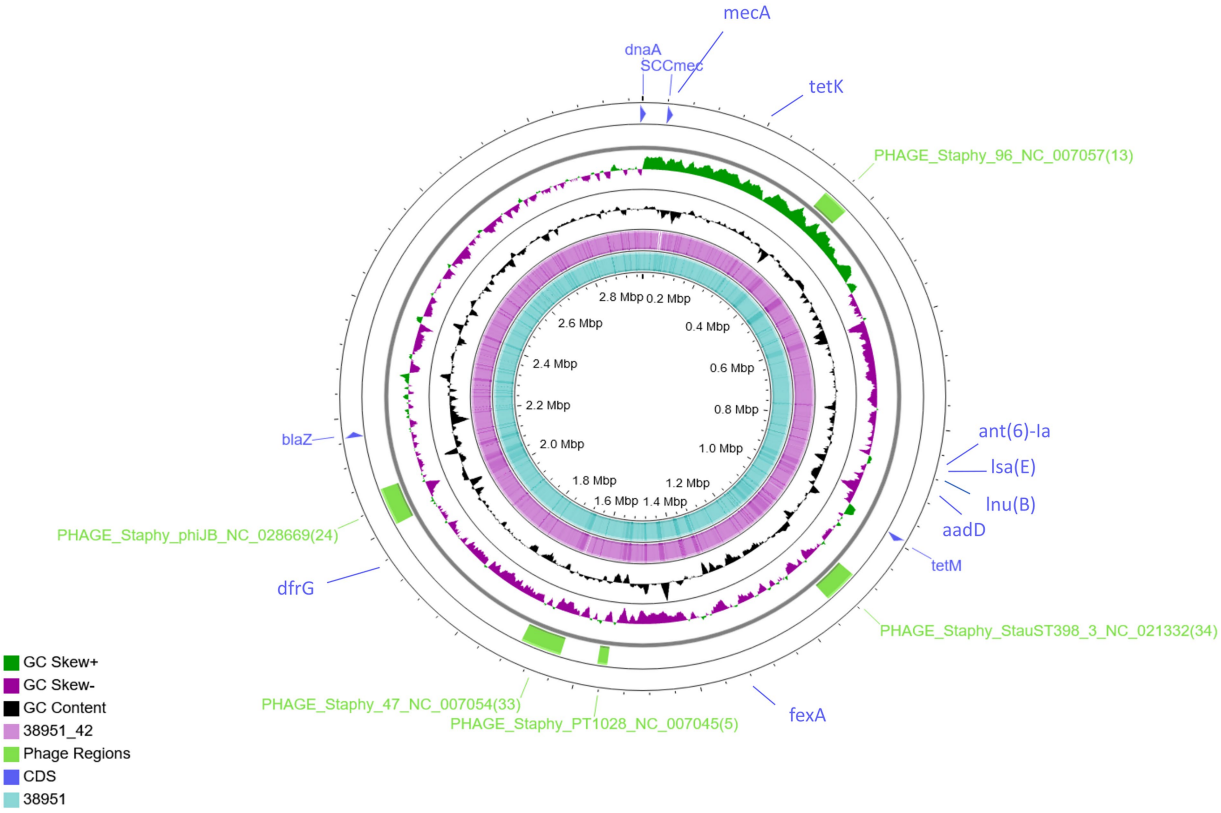

FIGURE 1
Genome-wide comparison of MRSA ST398 (IMT38951) and its descendant MSSA (IMT38951_42). A combinatorial approach including long reads (PacBio) and short reads (Illumina sequencing) allowed precise reconstruction of both genomes. Five regions harbouring phages or phage-associated genes identified using PHASTER are indicated in green. Location of resistance genes identified using ResFinder 2.1 and the SCC*mec* intergration site are indicated in blue. Comparative genomics determined a difference of 10 single nucleotide polymorphisms in total (excluding phage regions).

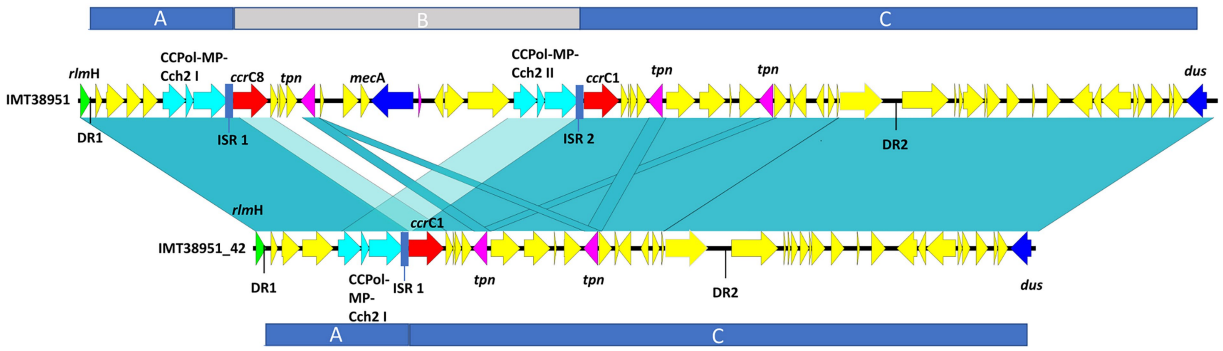

FIGURE 2
Sequence comparison of IMT38951 and IMT38951_42 from *rlm*H (previously: *orf*X) to *dus* (previously *orf*Y). Repetitive elements and homologues regions challenged the precise reconstruction of MRSA IMT38951 and its isogenic descendant lacking SCC*mec*Vc (region B). Since sequence identities of genes and intergenic spacer regions present in this region range from 98/99% (light blue-green) to 100% (blue-green), including sequences and genes that flank (regions A and C) the missing SCC*mec* element in IMT38951_42. DR, direct repeat of 15 bp length; CCPol-MP-Cch2, operon containing an A-family DNA polymerase (CCPol) together with a small protein lacking conserved domains ("middle protein," MP) as well as a putative helicase (Cch2) (Bebel et al., 2020); *ccr*C, chromosomal cassette recombinase C; *tpn*, transposase associated with insertion element (IS) 431; ISR, intergenic spacer region of 224 bp length.

the MSSA descendant is unchanged compared to its MRSA predecessor (Figure 2), arguing against recombinase driven loss of SCC*mec*Vc.

Further sequence comparison with the type V SCC*mec* (GenBank: AB512767) genes and intergenic regions of a strain (TSGH17) belonging to another livestock-associated genetic

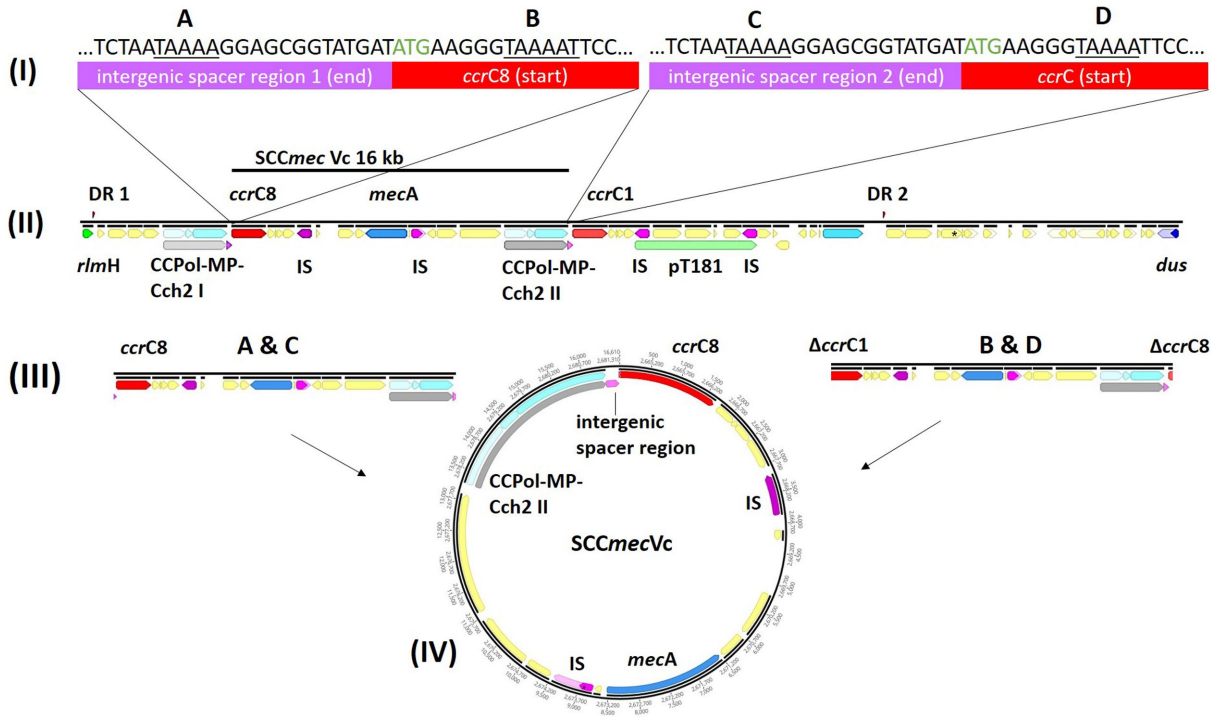

FIGURE 3
Tracking the loss of SCC*mec*Vc. MRSA ST398 IMT38951: Illustration of the SCC*mec* integration site downstream of *rlm*H. **(A)** Comparison of the terminal sequences of the intergenic sequences spacer region 1 (I) and 2 (III) and the initial basepairs of *ccr*C8 (II) and *ccr*C1 (IV) (start codon: green) revealed two possibilities of homologues recombination after deletion of SCC*mec:* A & C, intergenic regions 1 and 2; and B & D, *ccr*C8 and *ccr*C1. **(B)** The region between *rlm*H (previously "*orfX*") and *dus* (previously *orfY*) carries various repetitive DNA sequences. **(C)** Both recombination events would allow deletion of an identical and complete SCC*mec*Vc element. **(D)** Circular SCC*mec* element with putative functional recombinase *ccr*C8 and a CCPol-MP-Cch2 II complex (Bebel et al., 2020) conferring its autonomous replication capabilities. *ccr*, chromosomal cassette recombinase; IS, insertion sequence; DR, directs repeats associated with *ccr*-mediated integration and excision of SCC*mec* elements (Semmler et al., 2016).

background (ST59), used by Bebel et al. (2020) to describe the CCPol-MP complex and their Cch2 helicases, revealed a sequence identity of 94.9% and 80 SNPs, two larger insertions/deletions of 40 bp and 1,275 bases with 4 single insertions/deletions proving continuous evolvement of the region downstream of *rlm*H.

Exposure of ST398 to porcine serum and ammonia alters activity of regulatory pathways and genes involved in environmental adaptation

The transcriptomic profiles of IMT38951 triplicate samples challenged by environment-mimicking conditions such as an increased ammonia concentration (pH 9.0), porcine serum (pH 7.3) and a combination of them (pH 8.9) were compared to corresponding unchallenged control samples (pH 7.2) after 10 and 60 min of exposure. Cell viability and growth kinetics were assessed from immediate exposure to 90 min afterwards (Supplementary Figure 2). In addition, exposure to each of the environment mimicking conditions seemed to require a characteristic cellular response, as demonstrated by the principal component analysis carried out to ensure limited variation between individual samples (Supplementary Figure 3). A global and summarized overview on gene expression in transcripts per million (TPM) and DEGs (corrected $p \leq 0.05$), is provided in Supplementary Table 2. DEGs were divided into upregulated ($\log2FC > 1$) and downregulated genes ($\log2FC < -1$), revealing characteristic transcription patterns for each sampling time point and growth condition (global overview provided in Figure 4). Based on the heatmap revealing the highest differentially expressed genes across all sample conditions investigated (Figure 5), the most important mechanisms and pathways involved in transcriptomic responses have been further evaluated.

Global regulators

Global regulators that might be affected by the growth conditions were the class I heat shock responses comprising the HrcA regulon (*hrcA-grpE-dnaK-dnaJ* and *groESL*) that is embedded within the CtsR regulon (Michel et al., 2006) which was mostly induced, especially in the presence of ammonia (Table 1).

The center of the regulatory network in *S. aureus* consists of quorum sensing through the accessory gene regulator (*agr*)

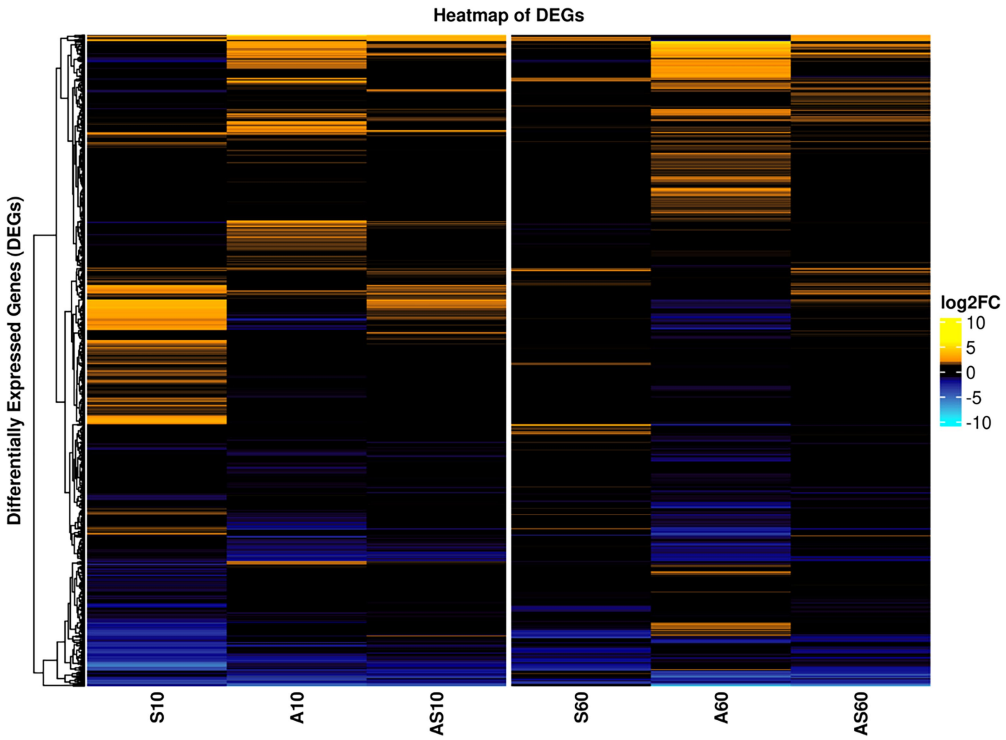

FIGURE 4
Global overview on differentially expressed genes for samples and conditions after 10 and 60min of exposure. Pattern analysis of differentially expressed genes (DEGs) are visualized based on their log2 fold-change (log2FC) compared to the corresponding control samples, ranging from up- (yellow) to downregulated (blue). Each column corresponds to a distinct growth condition (S=serum, A=ammonia, AS=ammonia & serum) and time point (10 min and 60 min of exposure). Genes are clustered based on their respective expression across the tested conditions (left). Expression ranges from-10 log2FC to +10 log2FC.

system, which concerts virulence factor transcription and synthesis, beyond others (Novick et al., 1993; Gaupp et al., 2016). We noticed a decrease in transcription of the *agr* locus compared to the respective unchallenged control samples for all experiments supplemented with ammonia (up to −4.7 log2FC; Table 2), a result likely related to the reduced growth kinetics associated with these conditions (Supplementary Figure 2). The addition of ammonia reduced the activity of the response regulator of the *agr* locus, AgrA. There was also down-regulation of AgrA-dependent genes/operons such as the phenol soluble modulins (PSMs) encoding operon (*psma* and *psmb*), as well as of the *agr*-inherent effector molecule RNAIII (a larger sRNA which also includes the delta-hemolysin open reading frame (*hld*)) noticed in ammonia-challenged cells (Supplementary Table 2). Reduced levels of RNAIII, are likely to decrease the transcription of toxin encoding genes. This is exemplified by decreased transcript levels of the α-toxin encoding gene *hla* (Supplementary Table 2), presumably *via* reduced degradation of *rot* transcripts, encoding the repressor of toxins (Boisset et al., 2007), which were found to be enhanced by 1.4 to 1.9 log2FC in presence of ammonia, as compared to the controls (Table 2). Reduced RNAIII levels may also explain the decreased transcript levels seen for *mgrA* (encoding the global transcriptional regulator MgrA) in presence of ammonia (Table 2), as RNAIII is known to stabilize the *mgrA* mRNA (Gupta et al., 2015).

When IMT38951 cultures were challenged with ammonia (in presence or absence of serum), clear reductions in *sae* (*S. aureus* exoprotein expression) transcript rates were noticed for both time points monitored (Table 2). Another potential factor contributing to the decrease in *sae* transcripts seen in presence of ammonia is SigB. This stress-induced alternative sigma factor is known to decrease *sae* transcription (Geiger et al., 2008), and is activated among others by a rapid increase in pH (Pané-Farré et al., 2009). Although the *sigB* operon is partially autoregulated, SigB activity is best followed by monitoring alkaline shock protein 23 (*asp*23) transcription (Giachino et al., 2001). Here, the sigma-B regulated *asp*23 gene (Kuroda et al., 1995) was induced after 10 min of exposure to ammonia alone (2.4 log2FC) or the combination of ammonia and porcine serum (1.2 log2FC; Table 1). In addition, transcription of only a part of the SarA regulon was found to be altered by ammonia supplementation in a relevant manner (i.e., *hla*, *hlg*A, *lrg*AB, *nrd*G, *psma*, but not *aur*, *esx*A, *ica*ADBC, *nuc*, *sdr*D, and *sod*M; Supplementary Table 2).

Presence of reactive oxygen species/protein synthesis

Although the equilibrium of the chemical reaction $NH_3 + H_2O \rightleftharpoons NH4^+ + OH$-shifts towards the left site (especially at pH 9), the rather limited number of free protons in bacterial cells (Saito and Kobayashi, 2003) may be captured by the hydroxyl radicals (OH^-).

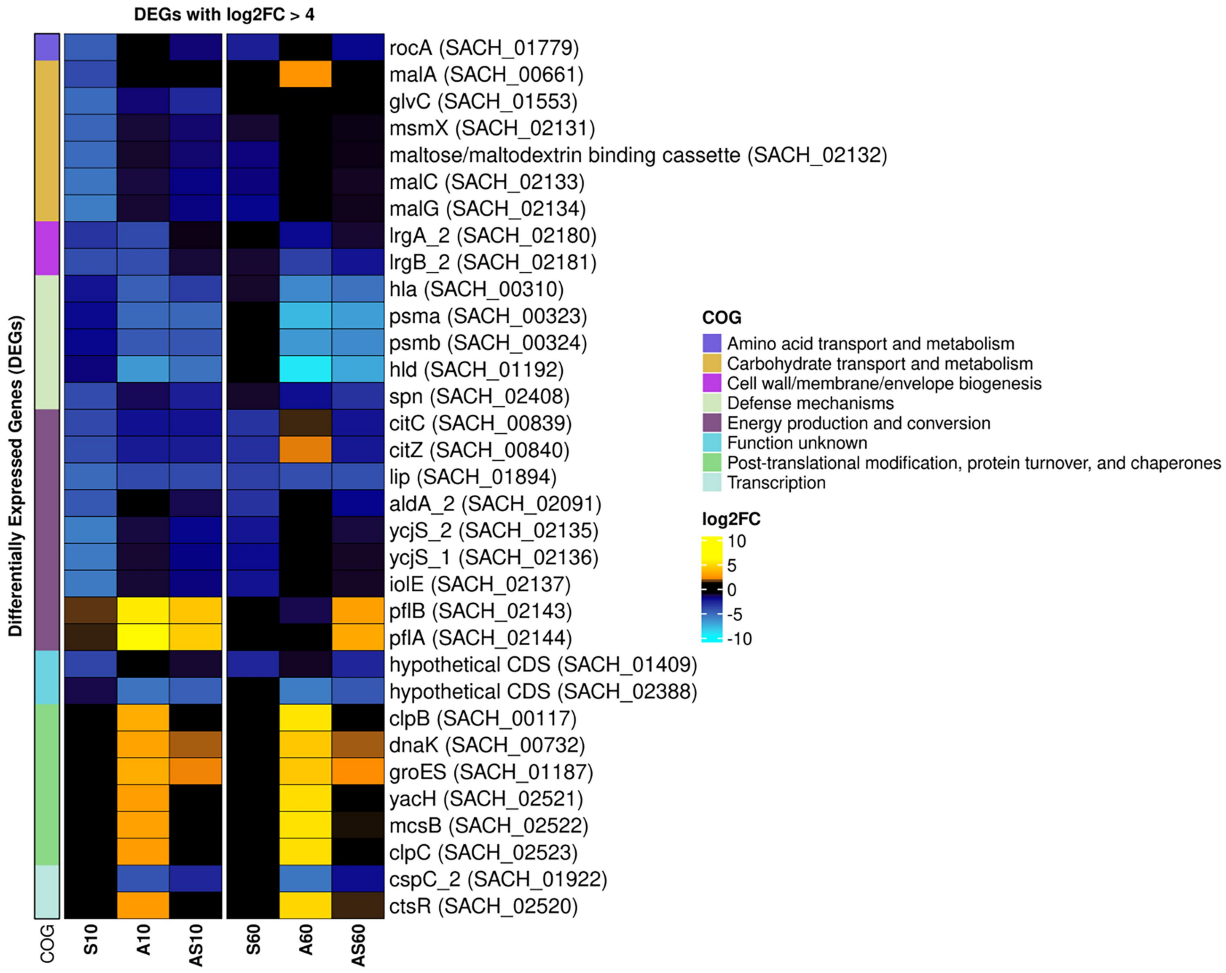

FIGURE 5
Heatmap of the top differentially expressed genes across all sample conditions. Top differentially expressed genes (DEGs) (n=41) are visualized based on their respective log2 fold-change (log2FC) compared to the corresponding control samples. Each column corresponds to a stress condition (S=serum, A=ammonia, AS=ammonia & serum) and time point (10 min and 60 min). Genes are either strongly upregulated with log2FC>5 (yellow) or downregulated with log2FC<−4 (blue). Genes are furthermore annotated with corresponding COG ontologies (left side).

Therefore, the formation of a small fraction of hydroxyl radicals (OH^-) in all samples exposed to ammonia is expected (Saito and Kobayashi, 2003). Here, we noted a transient induction of genes (maximum at 10 min of exposure) known for their response to the presence of reactive oxygen species (ROS) such as hydroxyl radicals (Pandey et al., 2019) including parts of the machinery involved in staphyloxanthin biosynthesis such as the *crtOPQMN* operon (Pelz et al., 2005), a carotenoid thought to protect *S. aureus* from oxidative stress (Clauditz et al., 2006). Similarly, genes encoding catalase (*kat*A) and especially alkyl hydroperoxide reductase (*ahp*C) were most effectively induced after 10 & 60 min of exposure to ammonia alone (Table 1).

Exposure to serum or even the combination of serum and ammonia did not affect transcription of the gene encoding the ribosome-associated translation inhibitor (*rai*A) after either 10 or 60 min, although 60 min of exposure to ammonia alone lead to a 2.0 log2FC, indicating the necessity to decrease overall protein production to cope with the presence of enhanced ammonia concentrations for more than an hour (Supplementary Table 2).

Virulence/defence

Of note, transcription of several virulence-and defence associated genes was transiently induced by exposure of MRSA ST398 to porcine serum only, i.e., genes encoding the von Willebrand-factor binding protein (10/60 min 2.2/1.4 log2FC), the chromosomal staphylococcal complements inhibitor (*scn*; 10/60 min 1.0/0.2 log2FC), an additional pathogenicity island-associated *scn* variant (10/60 min 1.5/1.6 log2FC), staphylocoagulase (*coa*; 10/60 min 2.2/0.6 log2FC) and immunoglobulin G-binding protein (*sbi*; 10/60 min 1.1/0.7 log2FC; Supplementary Table 2).

Transcriptional response of genes associated with SOS responses and horizontal transfer of SCC*mec*

In *S. aureus*, the SOS response following the occurrence of ssDNA is tightly regulated by the interplay of the DNA

TABLE 1 Transcription of genes associated with coping mechanisms in livestock-associated MRSAST398.

				Gene transcription (log2FC) compared to unchallenged control samples					
				Porcine serum		Ammonia		Porcine serum & ammonia	
ID	Symbol	Product	AA length	10 min	60 min	10 min	60 min	10 min	60 min
SACH_00117	*clp*B	ATPase subunit of an ATP-dependent protease ClpB	870	0.0	−0.1	3.1	5.1	0.9	0.6
SACH_00140	*yjb*H	Disulfid stress effector	269	−0.4	−0.2	0.8	1.0	0.3	0.3
SACH_00499	*cat*A	catalase	506	−1.9	−2.1	2.9	2.7	1.2	0.9
SACH_00731	*dna*J	chaperone protein DnaJ	380	0.9	0.1	2.5	3.2	1.3	1.1
SACH_00732	*dna*K	molecular chaperone DnaK	611	0.3	0.1	2.8	4.1	1.7	1.7
SACH_00733	*grp*E	heat shock molecular chaperone protein	209	0.8	0.7	2.5	3.8	1.6	1.9
SACH_00734	*hrc*A	heat-inducible transcription repressor HrcA	326	0.8	0.3	2.4	3.7	1.6	1.7
SACH_01186	*gro*EL	co-chaperonin GroEL	539	−0.5	0.5	2.8	3.9	1.7	1.8
SACH_01187	*gro*ES	co-chaperonin GroES	95	−0.2	0.9	3.1	4.1	1.9	2.0
SACH_01418	*asp*23	Alkaline shock protein 23	170	−1.9	−1.3	2.4	0.2	1.3	−0.3
SACH_01786	*crt*N	dehydrosqualene desaturase	503	−0.8	−0.9	1.9	1.3	0.6	−0.1
SACH_01787	*crt*B	squalene/phytoene synthase	288	−0.8	−0.7	1.8	0.8	0.8	0.1
SACH_01788	*crt*Q	glycosyl transferase family protein	376	−1.1	−1.1	1.7	0.1	0.5	−0.1
SACH_02361	*ahp*F	alkyl hydroperoxide reductase large subunit	508	−1.9	−1.1	1.5	2.0	0.7	1.3
SACH_02362	*ahp*C	alkyl hydroperoxide reductase subunit C	190	−1.3	−0.9	1.9	2.7	1.0	1.8
SACH_02520	*cts*R	class III stress genes transcriptional repressor	157	−0.7	0.6	2.5	4.6	1.0	1.2
SACH_02521	*mcs*A	putative modulator of heat shock repressor CtsR (UvrB/UvrC motif-containing protein)	189	−0.6	0.2	2.6	4.9	0.4	0.9
SACH_02522	*mcs*B	D-isomer specific 2-hydroxyacid dehydrogenase family protein	337	−0.2	0.5	2.7	5.0	1.0	1.1
SACH_02523	*clp*C	Clp protease ATP binding subunit	819	−0.6	0.4	2.5	4.9	0.7	0.9

Transcriptional changes after 10 min and 60 min of MRSA IMT38951 exposed to Mueller-Hinton (MH) II bouillon supplemented with 10% porcine serum, 0.3 ammonia or both, respectively. ID, sequence identifier; AA length, amino acid length.

10.3389/fmicb.2022.969961

TABLE 2 Transcriptional changes of global regulators in livestock-associated MRSAST398 exposed to ammonia porcine serum or both.

				Gene transcription (log2FC) compared to unchallenged control samples					
				Porcine serum		Ammonia		Porcine serum & ammonia	
ID	Symbol	Product	AA length	10 min	60 min	10 min	60 min	10 min	60 min
SACH_00575	*arl*S	signal transduction histidine kinase	452	−0.4	−0.1	−0.6	−1.8	−0.4	−0.9
SACH_00576	*arl*R	response regulator receiver	220	−0.6	−0.2	−0.7	−1.6	−0.6	−0.9
SACH_00645	*srrB*	integral membrane sensor signal transduction histidine kinase	584	0.1	0.2	−0.4	−0.6	0.6	0.2
SACH_00646	*srr*A	winged helix family two component transcriptional regulator	242	−0.2	0.4	−0.6	−1.1	0.8	0.5
SACH_00713	*rpo*D	RNA polymerase sigma-70 factor RpoD (SigA)	369	0.9	0.1	0.3	−0.1	0.7	0.7
SACH_00905	*rot*	repressor of toxins (Rot)	134	−0.3	−0.1	1.6	1.9	1.5	1.4
SACH_00917	*sig*S	RNA polymerase factor sigma-70	157	1.0	0.5	−0.3	−0.4	−0.2	0.1
SACH_01193	*agr*B	accessory gene regulator B	208	−0.9	−0.4	−2.1	−3.7	−1.8	−4.7
SACH_01194	*agr*D	staphylococcal accessory gene regulator protein D	47	0.3	0.5	−1.6	−3.4	−1.2	−4.5
SACH_01195	*agr*C	accessory gene regulator protein C	431	−0.6	−0.5	−2.1	−4.0	−1.9	−4.6
SACH_01196	*agr*A	autoinducer sensor protein response regulator protein	239	−0.9	−0.5	−2.5	−3.7	−2.1	−4.2
SACH_01284	*sig*B	RNA polymerase sigma factor SigB	257	0.4	−0.3	0.4	−1.3	1.3	0.3
SACH_01524	*sar*R	staphylococcal accessory regulator R SarR	116	0.0	0.5	−1.9	−4.1	−2.8	−2.2
SACH_02036	*sar*S	staphylococcal accessory regulator A SarS	251	0.3	0.4	−1.0	−0.9	0.0	0.6
SACH_02532	*sig*H	RNA polymerase factor sigma-70	190	0.7	0.2	1.2	0.4	0.9	0.9
SACH_02614	*sar*A	staphylococcal accessory regulator family protein	125	0.9	−0.5	1.5	−0.4	1.1	−0.1
SACH_02686	*mgr*A	MarR family transcriptional regulator	148	−0.4	0.1	−1.4	−2.7	−1.4	−0.5
SACH_02705	*sae*S	integral membrane sensor signal transduction histidine kinase	352	−0.3	0.7	−1.6	−3.3	−2.3	−2.6
SACH_02706	*sae*R	winged helix family two component transcriptional regulator	229	−0.1	1.0	−1.5	−3.1	−2.1	−2.4

Transcriptional changes after 10 min and 60 min of MRSA IMT38951 exposed to MH II supplemented with 10% porcine serum. 0.3 ammonia or both, respectively. ID, Sequence identifier; AA length, amino acid length.

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damage-inducible repressor LexA and the recombination protein RecA (Butala et al., 2009; Maslowska et al., 2019; Podlesek and Žgur Bertok, 2020). Activation of RecA by binding to single-stranded DNA promotes transcriptional upregulation of genes belonging to the SOS-regulon by activation of LexA self-cleavage from the respective promoter sites. Although actual activation of the synthesized proteins cannot be followed by transcriptional analysis, some of the genes associated with SOS response in *S. aureus* are transcriptionally induced, i.e., the topoisomerase IV genes *par*E and *par*C at 10 and 60 min (1.3 /0.6 and 1.7 /0.8 log2FC) after exposure to porcine serum (Supplementary Table 2). The LexA-regulated low-fidelity and error-prone DNA polymerase V (Ha and Edwards, 2021) *umu*C (1.7 log2FC) and genes involved in nucleotide excision repair (*uvr*A/B; 1.5/1.2 log2FC) are significantly induced after 60 min of exposure to ammonia. Since a similar SOS-mediated induction of *ccr* genes by cleavage of LexA from its promoter sequence was reported previously (Liu et al., 2017), the transcriptional response of both *ccr* variants was evaluated. Both *ccr* allelic variants in IMT38951 lacked significant induction over the time points and conditions evaluated here, although a minimal induction was noted after 60 min of exposure to ammonia (between 0.1 and 0.2 log2FC; Figure 6). However, this observation seems in overall congruence with the limited but notable induction of genes belonging to the SOS-response after 60 min of exposure to ammonia described above. The transcription of *lex*A, on the other hand, is 0.7 log2FC after 10 and 1.5 log2FC after 60 min of exposure to ammonia, a rate that may indicate that LexA has been consumed by SOS-induction. Of note, *rec*A transcription levels lack significant changes compared to the unchallenged controls at all conditions investigated (Supplementary Table 2).

Data on up-or downregulation of predicted CDSs within the genomic region harboring SCC*mec*, starting with the rRNA large subunit methyltransferase (*rlm*H) and ending with tRNA dihydrouridine synthase (*dus*), is provided in Figure 6 and Supplementary Table 2 (highlighted in green). Considering genes putatively involved in mobilization and recombination of transferrable MGEs present in this particular region, we noticed an increased transcription of transposase *tnp*_2 associated with IS431, located adjacent to *mec*A, after 10 min (0.3 log2FC) and 60 min (1.0 log2FC) of exposure to ammonia (Figure 6). Moreover, the replication initiation gene *rep*N (10/60 min 1.0/0.5 log2FC) and the gene encoding for a plasmid recombination protein (*pre*; 10/60 min 1.7/0.8 log2FC), both belonging to the tetracycline resistance gene (*tetK*)-carrying plasmid pT181 that integrated into the SCC*mec* region downstream of *ccr*C1 (Figure 3A), showed a transcriptional increase after exposure to porcine serum (Figure 6; Supplementary Table 2).

Genes associated with both variants (I and II Figure 3) of the replication machinery CCPol-MP-Cch2 were not significantly induced by the growth conditions investigated here (Supplementary Table 2; Figure 6).

Discussion

Reconstruction of the loss of methicillin resistance in IMT38951_42 revealed deletion of a complete and putatively replicative SCC*mec*Vc element by homologous recombination

Following the loss of methicillin resistance in IMT38951 genomic investigations were hindered by multiple repetitive sequences in the region downstream of *rlm*H. This was overcome by a combination of long read sequences polished by short reads to allow for an accurate genomic inspection of this particular region, including a detailed reconstruction of the process most likely leading to the loss of the complete SCC*mec* element from its chromosomal integration site, outlined in Figure 3.

Previous research indicated that deletion followed by HR, or excision driven by large serine recombinases, allow complete SCC*mec* elements to detach from their chromosomal integration site downstream of *rlm*H (Hanssen and Ericson Sollid, 2006; Chlebowicz et al., 2010; Wang and Archer, 2010; Liu et al., 2017; Gómez et al., 2020). This likely requires the involvement of the serine recombinases (*ccr*A/B or *ccr*C) that facilitate chromosomal integration and excision of SCC*mec*, or other factors belonging to the bacterial recombination machinery. Since SCC*mec* elements are typically flanked by "direct repeats" (DR) of 15 bp length that are recognized by the respective large serine recombinases, *ccr*-driven integration or loss of these elements always leads to changes in the number of DRs at the chromosomal integration site (Ito et al., 1999; Semmler et al., 2016). However, since the number and position of the two direct repeats present in the MRSA predecessor remained unchanged in the descendent, MSSA IMT38951_42, *ccr*-mediated excision of the SCC*mec* element, as described previously (Noto et al., 2008; Wang and Archer, 2010; Zhang et al., 2015, 2016), is unlikely.

A further study identified recombination between the putative primase-encoding (now: helicase Cch2, according to Bebel et al., 2020) genes belonging to the CCPol-MP-Cch2 complex in the ST398 lineage (Gómez et al., 2020). Other authors have linked the loss of SCC*mec* with recombination between *ccr*C allelic variants (Chlebowicz et al., 2010; Vandendriessche et al., 2014), a possibility that cannot be ruled out for the isogenic pair belonging to ST398 in our study (Figure 3, recombination between B and C). However, the actual region prone to recombination described above comprises the end of intergenic spacer region 1 and the initial 48 bp of both *ccr* genes (Figure 3A), a region that harbors four identical short repetitive sequences. Interestingly, both recombination events (A & C and B & D) events would produce an identical SCC*mec* circular element (Figure 3D). Consequently, occasional deletion followed by HR might be of greater biological importance for the spread of methicillin resistance than previously considered, as Bebel et al. reported autonomous replication capabilities for exactly these elements (Bebel et al., 2020) that contribute not only to the maintenance of the circular SCC*mec*

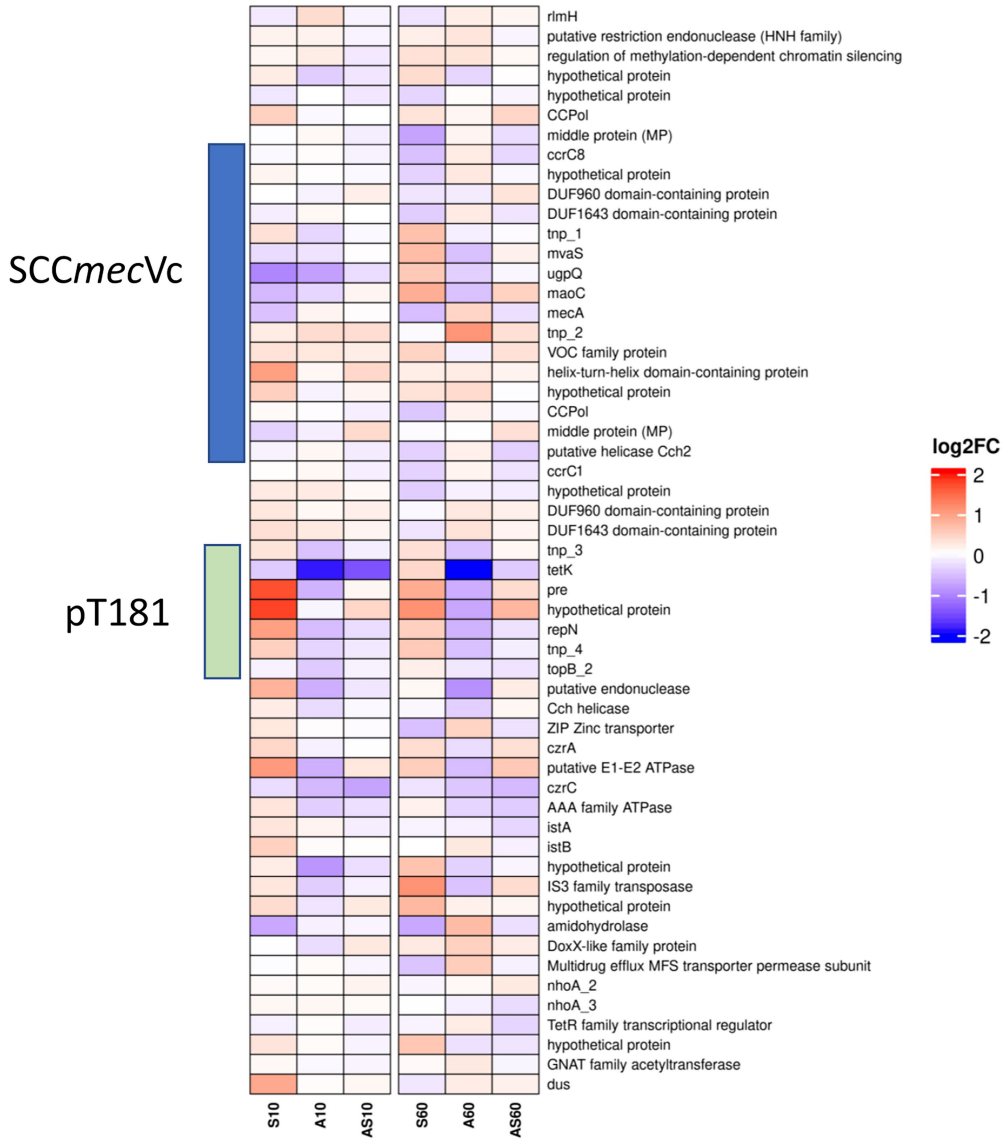

FIGURE 6
Heatmap illustrating transcription of genes at the chromosomal SCC*mec* integration site from *rlm*H ("*orfX*") to *dus* ("*orfY*"). Gene transcripts are visualized based on their log2 fold-change (log2FC) with either red (upregulated), blue (downregulated) or white (no changes) compared to the corresponding control samples. Each column corresponds to a growth condition (S=serum, A=ammonia, AS=ammonia & serum) and time point (10 min and 60 min). Overall expression ranges from-2 log2FC to +2 log2FC.

intermediates, but also to their proliferation within an extra-chromosomal space. Subsequently, novel SCC*mec* elements might be available for horizontal transfer to other suitable recipients - a hypothesis that should be addressed in future research.

In vitro transcriptional response to environmental challenges associated with pig farming highlights the adaptive capabilities of MRSA ST398

Recently, the ability of the MRSA ST398 lineage to survive in pig manure has been demonstrated (Astrup et al., 2021). This particular LA environment is known for high natural ammonia levels - a major selective factor for bacterial community structures (Nordgård et al., 2017). In *S. aureus*, early responses to environmental challenges are often associated with changes in the activity of global regulators (Crosby et al., 2016; Ranganathan et al., 2020; Párraga Solórzano et al., 2021). Activation of components belonging to the heat shock response seems to be among the most important coping mechanisms involved with respect to ammonia-rich environments. The latter is a protective mechanism considered crucial for bacterial survival and adaptation to hostile environmental conditions by degradation of misfolded and denatured proteins and prevention of protein misfolding events (Roncarati and Scarlato, 2017).

The number of free protons present in a bacterial cell depends on the pH: At an alkaline pH above 9, the number of free protons is reduced to less than one per bacterium (calculated for *Escherichia coli*), a fact that hinders the protonation of specific sites in enzymes required for their activity (Saito and Kobayashi, 2003). Consequently, cellular stress including disturbances of pH-sensitive enzymatic reactions, energy metabolism and secondary protein structures were expected to feature in the transcriptomic responses of *S. aureus* cells exposed to ammonia at an alkaline pH (Gutierrez et al., 2022). Moreover, the data suggests that the electron pair of the NH_3 molecule might interfere with hybrid bonds of peptides and/or proteins under these conditions, since addition of more protein (i.e., presence of porcine serum) reduces the effects associated with exposure to ammonia alone, arguing against a pH dependent effect alone. In line with this, the initial pHs of the ammonia-supplemented samples differed only slightly (ammonia: pH 9.0/ammonia & porcine serum: pH 8.9) and did not change significantly over time.

A reduction of the bacterial transcription machinery prevents excessive energy loss from protein synthesis (Roncarati and Scarlato, 2017). As shown in Supplementary Figure 2, viable cell numbers had the smallest increase over time in samples exposed to ammonia only, suggesting that down-regulation of the translation machinery (Supplementary Table 2) contributes to survival and adaptation in ammonia rich environments.

In addition, the *asp*23 expression that is controlled by the alternate sigma factor of RNA polymerase (SigB) was upregulated after 10 min of exposure to 0.3% ammonia and the combination of ammonia and porcine serum, an increase that has been reported for sodium hydroxide-induced pH increases previously (Anderson et al., 2010). However, after 60 min, the *asp23* transcription rates were comparable to the control, suggesting that ammonia supplementation induces a short-lived activation of SigB. This is in line with earlier observations reporting that SigB activity is only transiently induced by stresses such as heat shock (Gertz et al., 1999; Giachino et al., 2001). Further support for a stimulating effect of ammonia on SigB activity is given by our findings of increased transcript rates for several SigB-regulated genes known to be directly controlled by this factor such as the genes *crtMN,* whose products are involved in staphyloxanthin biosynthesis, a carotenoid thought to protect *S. aureus* from oxidative stress (Clauditz et al., 2006), *spoVG,* a transcription factor acting downstream of SigB (Meier et al., 2007), and *sar*A (Bischoff et al., 2001). Notably, SarA is also reported to act as regulatory protein responsive to redox and pH (Fujimoto et al., 2009), and to repress the expression of *rot* (Manna and Ray, 2007).

The observed reduction in *sae* transcription in all samples except those exposed to porcine serum for at least 60 min might be, at least in part, due to increases in SigB and Rot-activities, which were shown to decrease *sae* transcription from the *sae* P1 and P3 promoters, respectively (Geiger et al., 2008; Li and Cheung, 2008), and by a reduction in RNAIII transcripts, which are known to enhance *sae* transcription (Novick and Jiang, 2003; Geiger et al., 2008).

Although not directly regulated by *agr*, gene transcription of a few virulence factors located on mobile genetic elements that can be considered as a specific response to host (porcine) proteins such as *scn* and the von Willebrand Factor binding protein were decreased in all samples exposed to ammonia with or without porcine serum, indicating that the pH of 8.9–9.0 and/or the NH_3 molecule interferes with (host-)protein recognition, requires energy saving or both.

Transcription of genes involved in capsular polysaccharide biosynthesis in ammonia supplemented media was not recorded, although their upregulation due to increased alkaline conditions has been demonstrated before (Anderson et al., 2010).

Taken together, LA-MRSA ST398 seems fully equipped to endure pig-farm associated environmental factors such as ammonia and porcine serum, helping to support their long-term survival within that environment.

Transcription of genes involved in SCC*mec* mobility

In *S. aureus*, the genomic region between origin-of-replication (*ori*) and the SCC generally seems prone to HR, with the integration site for SCC representing a particular "hotspot" in this regard (Everitt et al., 2014; Semmler et al., 2016). A recent study investigated the stability of four different SCC*mec* elements (I, II, III, IV) during 3 months of serial subculturing at room temperature, revealing that SCC*mec* stability was influenced both by internal mobile elements (IS431) as well as the cell environment (Scharn et al., 2022). Exposure to pig-farming associated environmental challenges did not significantly alter the expression ratios of genes likely to be involved in the mobilization of complete or partial SCC*mec* elements, including transposases associated with IS431 and the *ccr* recombinases (Wang and Archer, 2010; Wang et al., 2017; Scharn et al., 2022) in this study when compared to unchallenged controls. These results suggest that the aforementioned conditions do not strongly contribute to SCC*mec* mobility to a large extent - at least not within the timeframe investigated here.

Moreover, exposure to ammonia, porcine serum or both failed to significantly induce *rec*A transcription that would lead to de-repression of LexA-controlled genes including allelic variants of *ccr*C, as reported before (Liu et al., 2017). Although some genes tightly regulated by LexA such as *uvr*AB and *umu*C showed a clear log2FC increase after 60 min of exposure, the corresponding transcription rate noted for both *ccr* genes (0.1/0.2 log2FC) lacked significant difference compared to the controls. Since autocatalysis of LexA is required for derepression of some genes within the SOS regulon (Ha and Edwards, 2021) including *lex*A, others are only derepressed when the N-terminal domain is further digested by Clp proteases including ClpCP. Here we noted an induction between 2.5 log2FC after 10 min and 4.9 log2FC after 60 min for *clp*C in cells exposed to ammonia only (Table 1), once more suggesting a fine-tuned activation of the SOS machinery in MRSA ST398 strain IMT38951. Therefore, a massive cleavage of LexA from the *ccr*C promoter region may depend on more invasive DNA

damaging conditions, i.e., the presence of distinct antibiotics, as reported before (Cirz et al., 2007; Liu et al., 2017). It seems worth considering that DNA-damaging effects attributed to ammonia rich environments and further challenging circumstances, for instance, exposure to antibiotics, may accumulate *in vivo*. In light of the recent results of Scharn et al. (2022), we speculate that prolonged exposure to livestock-associated conditions might induce a more prominent transcription of the *ccr* genes.

Conclusion

Here we followed the deletion of a complete and fully functional SCC*mec* Vc element from its chromosomal integration site downstream of *rlm*H in an MRSA ST398 isolate (IMT38951) and highlighted the most important transcriptional responses of the bacteria required to endure pig-farm associated environmental challenges such as increased ammonia concentrations and porcine serum. A detailed reconstruction of SCC*mec* loss from its genomic integration site raises concerns with respect to the autonomous replication capabilities of the predicted circular SCC*mec* element and its potential to spread to other susceptible bacterial hosts. It seems likely that this particular method of SCC*mec* conservation and transmission might have been underestimated, especially considering that, at least so far, only severe DNA-damaging events seem to trigger an increased activity of the *ccr*C variants at the population level. However, further studies are required to evaluate which factors and co-factors may trigger and support the autonomous replication machinery present in the excised elements.

Data availability statement

The datasets presented in this study can be found in online repositories. Raw sequencing reads, including RNA-Seq and long-read genomic data, were uploaded to NCBI and deposited within BioProjects PRJNA891722 and PRJNA449454.

Author contributions

CH, AL-B, LW, LE, SW, and BW contributed to conception and design of the study. CH, JB, JA, and BW performed laboratory experiments and analysis. Genomics and transcriptomics were carried out by LE, SW, AT, and TS. CH, MB, MH, AB, WZ, and BW analysed the results. CH and BW wrote the first draft of the manuscript. LE, SW, WZ, and MB wrote sections of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.969961/full#supplementary-material

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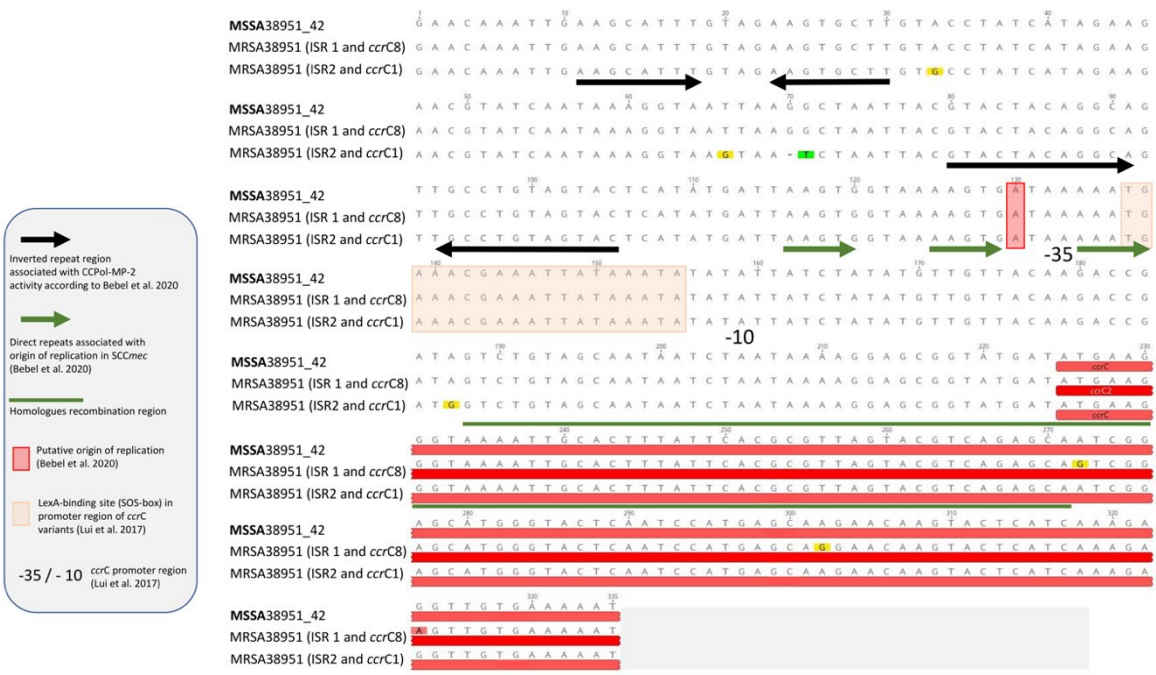
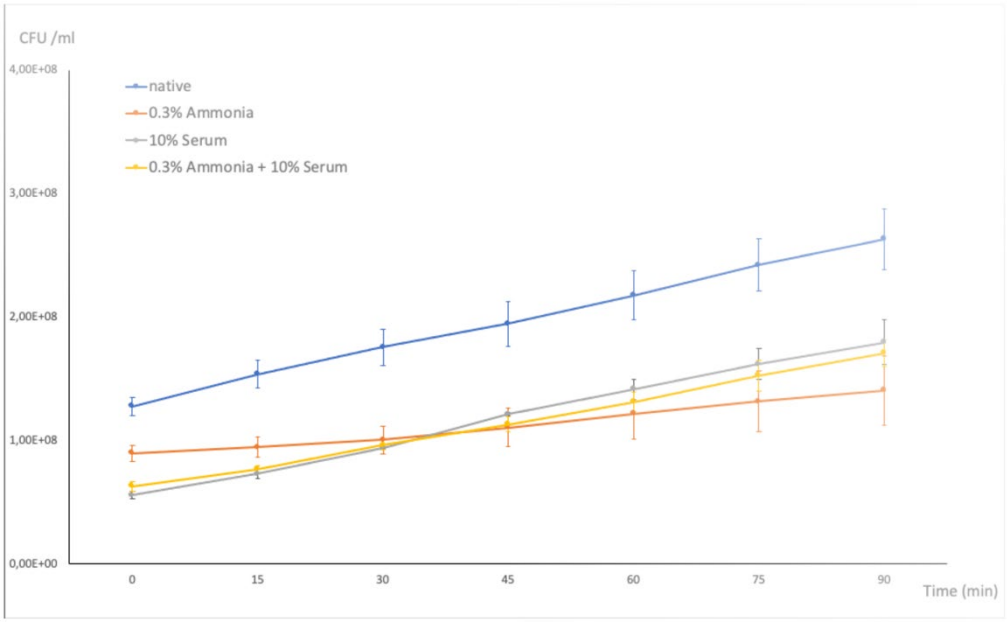
Supplementary Information

Supplementary Figure 1| Comparison of intergenic spacer regions (ISR1 and ISR2), *ccr*C8 and *ccr*C1 (first 48 bp each)

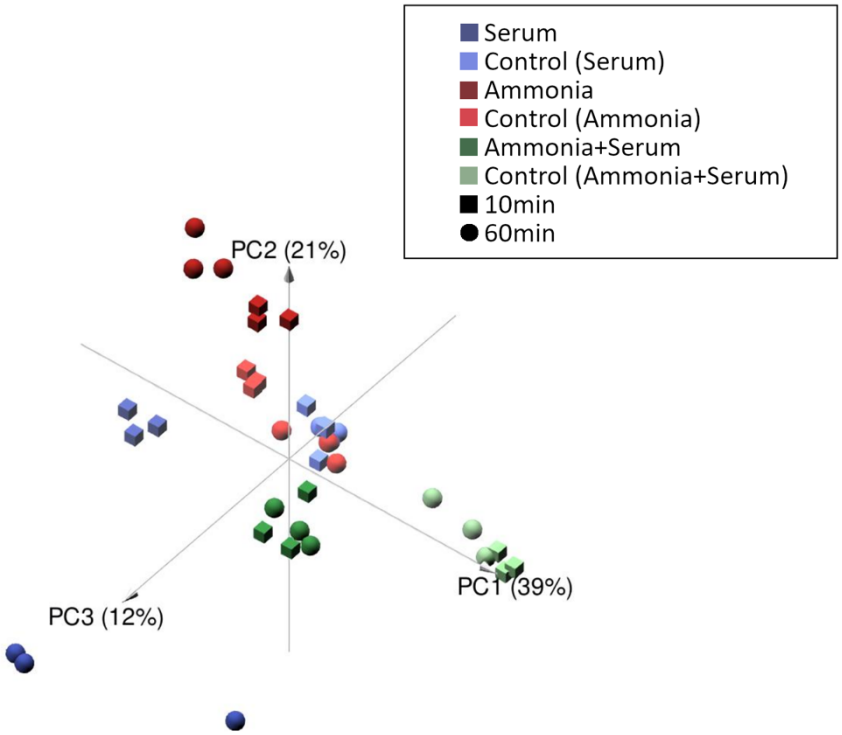


Supplementary Figure 2| Viability assay

Recovered MRSA IMT38951 are shown as colony forming units (CFU) per ml on a log scale (start: 0.4 OD_{600}). Bacteria were challenged by different supplements mimicking pig-farming environmental conditions such as enhanced ammonia, porcine serum and a combination of both for 90 min. Three independent experiments in technical triplicates are shown.



Supplementary Figure 3| Principal Component Analysis (PCA) of transcription levels between samples. Inter-sample distances were calculated based on the transcripts per million (TPM) expression values of their respective gene content. PC1 (39%), PC2 (21%) and PC3 (12%) were used to visualize the similarity of the samples in a three-dimensional space. Samples were color-coded according to the distinct exposure conditions (serum = blue, ammonia = red, ammonia & serum = green) and different symbols illustrate the corresponding time points (ball = 10min, cube = 60min).



Supplemental Table 1: IMT38951 OD600 values obtained from growth in Mueller-Hinton II supplemented with either 0.3% ammonia, 10% serum or a combination of both. OD600 values of samples selected for RNA sequencing are bold.

	0.3% Ammonia							nativ						
time [min]	1	2	3	4	5	6	7	1	2	3	4	5	6	7
0	0.403	0.423	0.447	0.418	0.426	0.406	0.423	0.457	0.437	0.469	0.463	0.484	0.459	0.471
10	0.464	**0.474**	0.492	0.468	**0.477**	0.459	**0.474**	0.547	0.531	**0.557**	**0.55**	0.567	0.547	**0.555**
60	0.577	**0.584**	0.606	0.577	**0.585**	0.57	**0.583**	0.808	0.779	0.808	**0.799**	0.819	**0.799**	**0.798**
	10% Serum							nativ						
time [min]	1	2	3	4	5	6	7	1	2	3	4	5	6	7
0	0.405	0.425	0.391	0.422	0.408	0.415	0.41	0.465	0.435	0.481	0.473	0.45	0.467	0.488
10	**0.526**	0.545	0.506	0.544	**0.529**	0.534	**0.521**	**0.542**	0.517	0.555	**0.549**	0.531	**0.543**	0.558
60	**1.261**	**1.278**	1.214	**1.268**	1.249	1.236	1.252	**0.776**	0.744	0.786	**0.773**	0.746	0.761	**0.77**
	0.3% Ammonia + 10% Serum							nativ						
time [min]	1	2	3	4	5	6	7	1	2	3	4	5	6	7
0	0.453	0.446	0.4	0.39	0.397	0.411	0.394	0.398	0.387	0.408	0.411	0.422	0.394	0.416
10	0.526	0.52	**0.459**	**0.451**	**0.459**	0.481	0.46	**0.429**	**0.421**	0.443	0.443	0.45	**0.427**	0.444
60	**0.873**	**0.87**	0.788	0.767	0.799	**0.898**	0.808	**0.577**	**0.567**	0.587	0.595	0.597	**0.57**	0.592

Supplemental Table 2: Due to the size of the Table, Supplemental Table 2 is not shown here. The data can be found online at https://www.frontiersin.org/articles/10.3389/fmicb.2022.969961/full#supplementary-material or in the printed version on the enclosed data medium.

3 Discussion

Both publications on *S. aureus* presented in this cumulative thesis provide insights into different bacterial adaptation strategies, i.e., core genome alterations and uptake or loss of MGEs. Despite their different nature, both strategies support the survival and subsequent adaptation of the bacteria to changing or challenging hosts and environmental conditions.

The data presented in **Publication I** demonstrated core genome changes that cause modulation or even silencing of the accessory gene regulator (*agr*) quorum sensing (QS) system in *S. aureus*. Furthermore, the publication has disclosed that these changes occur independently in strains belonging to completely different genomic backgrounds, clearly indicating the importance of the *agr* system alterations with respect to adaptive changes and not at least bacterial evolution. These adaptive modulatory changes enable the bacteria, beyond others, to survive among competing staphylococci of the host microbiota [151], simultaneously reducing their perceptibility by the host's immune system [152] while enhancing their biofilm formation capabilities [153, 154]. Consequently, *S. aureus*, lacking a functional *agr* system, can survive in challenging environments since it clearly favours inter-species spread including, and not at least, humans (**Publication I**).

A mechanism which leads to the loss of complete SCC*mec* elements from its genomic integration site has been reconstructed in detail for an MRSA ST398 strain in **Publication II**, revealing, beyond others, the presence of an operon conferring autonomous replication capabilities associated with the excised SCC*mec*Vc element. Although the putative impact of LA environmental conditions on the loss of SCC*mec* elements in MRSA ST398 had been a focus of scientific discussions, cell exposure for 10 min and 60 min to typical LA conditions, i.e. an increased concentration of ammonia, the presence of porcine serum, or a combination of both, did not significantly induce the transcription of these particular genes (large serine recombinases, see Chapter 1.4.2.3) known for their association with the elements' mobility compared to the unchallenged controls (**Publication II**). Moreover, a strong stimulus in terms of severe DNA damage seems necessary to induce the *ccr* genes on a population scale [155, 156]. Thus, the occasional loss of an element that harbours the machinery to reproduce itself autonomously seems of epidemiological importance concerning the origin, maintenance and spread of methicillin resistance (**Publication II**).

3.1 Core genome alterations promote the survival and adaptation of *S. aureus* to changing environmental conditions

Changes in genes belonging to the core genome (see Chapters 1.2 and 1.3) are one of the most important drivers of bacterial adaptation with respect to changing growth conditions.

Beyond others, the core genome comprises global transcriptional regulators, such as the *agr* system, Rot (repressor of toxins) and SigB (alternative sigma factor). These regulators are known for their important role regarding the recognition of changing environmental conditions [157]. Consequently, genomic changes in these regions often result in variations of several essential pathways, allowing a broad range of phenotypes to emerge.

Alterations of the *agr* system enable *S. aureus* to rapidly undergo a diverse spectrum of situation-specific regulatory modifications. The QS system of *S. aureus* relays on the capability of bacteria to produce and release chemical signal molecules, i.e., autoinducing peptides (AIP, Figure 4) in *S. aureus* [158, 159]. Briefly, staphylococci usually produce basal levels of AIPs, that act as autoinducers in the QS circle. AIPs are encoded by *agr*D, a part of the *agr*BDCA operon that is transcribed by the *agr* operon promoter P2. The *agr* locus consists of a second transcription unit which is responsible for the transcription of RNAIII via promoter P3. Subsequently, the AIPs are exported by the transmembrane endopeptidase (AgrB) and trimmed by a type I extracellular signal peptidase (SspB). If the extracellular concentration of AIP is sufficient, the peptide binds to its sensor histidine kinases (AgrC), resulting in phosphorylation of the response regulator AgrA (AgrA-P). AgrA-P, in turn, binds to the promoters P2 and P3, resulting in the transcription of AgrBDCA and RNAIII, which subsequently regulates several toxins [159-161]. At present, four distinct *agr* locus variants (*agr* types I – IV) are known, with differences in *agr*B, *agr*C and *agr*D. The latter leads to the production of individual AIPs, responsible for autoinduction or the cross-inhibition of other *agr* types (reviewed in [159]).

S. aureus recognises the increasing population density through elevated concentration of AIPs via the *agr* system and subsequently regulates important pathways according to the current (community) situation [158, 159]. Cell wall-associated proteins involved in local or tissue attachment, for example, are mainly expressed at low cell density. Increased activity of the *agr* system indicated by higher cell densities initiates a “switch” from the initial phase of self-establishment of the invading bacteria to the expression of numerous toxins (reviewed in [159, 161]).

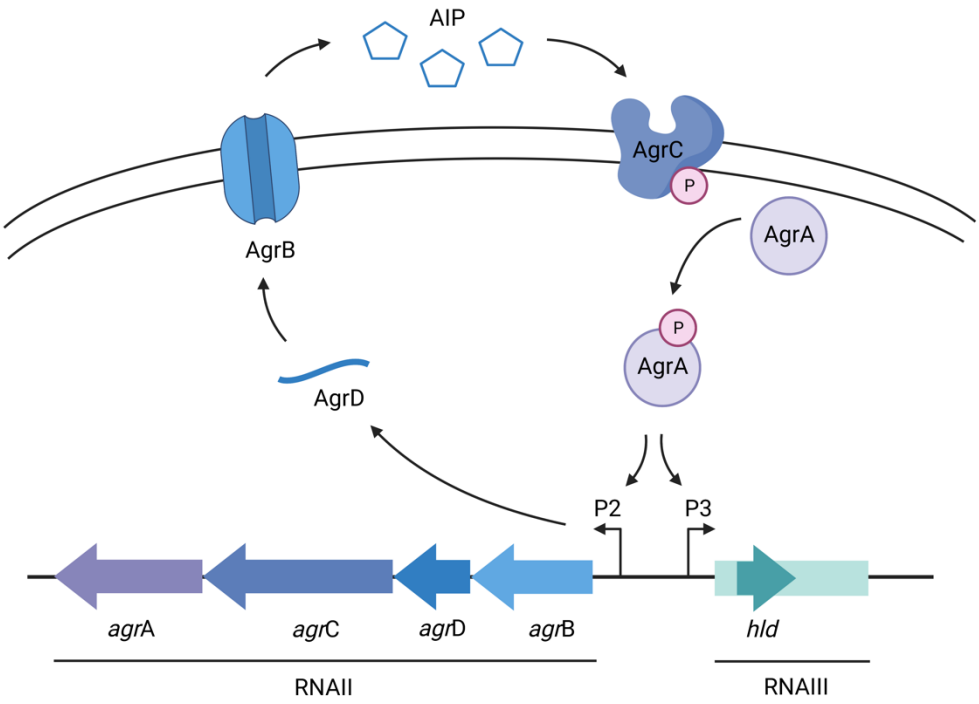


Figure 4: Overview of the accessory gene regulator (*agr*) quorum sensing system

Small autoinducing peptides (AIPs) are encoded by *agr*D of the *agr*BDCA operon and exported by the transmembrane endopeptidase (AgrB). Extracellularly, AIPs accumulate and eventually bind to the sensor histidine kinases (AgrC), resulting in phosphorylation of the response regulator AgrA (AgrA-P), which in turn bind to the promoters P2 and P3, resulting in the transcription of AgrBDCA and RNAIII [159-161]. Created with BioRender.com (October 2022).

Different *agr* activity levels were described for either acute or chronic states of infection. Acute cases of infection such as endocarditis, skin and soft tissue infections, pneumonia, septic arthritis, and osteomyelitis are usually caused by MRSA with a functional *agr* system. Thereby, *S. aureus* switches from an asymptomatic colonising bacterium to an invasive pathogen (reviewed in [159]), which seems to be particularly important for the development of acute infections. However, chronic diseases seem to be often associated with *agr* defective strains, which can result, for example, in an increased biofilm formation (reviewed in [159]).

Single nucleotide polymorphisms (SNPs) in the *agr*A and *agr*C genes of strains belonging to different MRSA lineages associated with all four *agr* types were investigated in **Publication I**. The various alterations resulted in different *agr* activity levels, including but not limited to non-functional (silenced) *agr* systems (**Publication I**). These results are in line with already published *agr* mutations affecting the QS system, as highlighted with some examples in Table 3.

Table 3: Examples for alterations in genes of the accessory gene regulator (*agr*) system leading to different *agr* activity levels

aa: amino acid; nt: nucleotide

Strain	Origin	Genomic alteration	Impact	*agr* activity	Reference
RN4220	derivative of *agr*+ NCTC8325	frameshift caused by an extra A residue in a run of seven A's at the 3' end of *agr*A → results in the addition of three amino acyl residues to the C-terminal end of the protein	delayed *agr* activation by 2 – 3 h, results in failure to translate α- and δ-haemolysins → non-haemolytic phenotype	delayed	[162]
RN9771	cellulitis	intergenic G218A *agr*C L245S (aa)	RNAIII was produced at wild-type levels, lack of haemolytic activity	delayed	[163]
RN9764	prosthesis	*agr*B V119L (aa), *agr*C E220D (aa)	produced only a trace of RNAIII, transcribed *hla* extremely weakly	none	[163]
RN9900	foot ulcer	*agr*C aa151 stop	little or no detectable RNAIII, lack of haemolytic activity	none	[163]
RN9901	pneumonia	*agr*B V119L (aa); *agr*C aa127 (nt378) frameshift; aa145 stop	little or no detectable RNAIII, lack of haemolytic activity	none	[163]
RN9903	osteomyelitis	RNAIII T228C, *agr*C IS*256* insertion	no RNAIII, lack of haemolytic activity	none	[163]
RN9904	pneumonia	*agr*A, aa63 (nt487) frameshift; aa175 stop	no RNAIII, lack of haemolytic activity	none	[163]

In addition, the *agr* system also adapts to further environmental stimuli, especially changes of the pH value. A maximum of *agr* activity occurs in the range of a neutral pH (reviewed in [164]). In contrast, the *agr* system is inhibited by extremes, either a strong alkaline (i.e. pH 8.0, [165]) as well as acidic pH (i.e. pH 5.5, [166]). Due to the high-protein diets fed to pigs, their manure is very rich in ammonia. In industrial pig farming, the toxic gas (NH_3) is released from manure due to bacterial degradation activities, a fact that leads to an accumulation of the gas in the barn air and, due to its solubility in fluids, harms the airways of exposed pigs as well as pig farm workers [167, 168]. Furthermore, invasive *S. aureus* are often confronted with blood serum, a challenge that seems to downregulate the *agr* system (reviewed in [164]).

Consequently, these growth conditions were investigated in **Publication II** by challenging the MRSA CC398 isolate IMT38951 through its exposure to an increased ammonia concentration (pH 9.0), porcine serum (pH 7.3) and a combination of these (pH 8.9). A decrease in transcription of the *agr* locus compared to the respective unchallenged control was associated with samples supplemented with ammonia (up to -4.7 log2FC, **Publication II**). Furthermore, the downregulated response regulator AgrA decreased AgrA-dependent genes / operons such as the phenol soluble modulins (PSMs) encoding operon (*psm*a and *psm*b), as well as the effector molecule RNAIII. For samples exposed to porcine serum, a rather discrete change was noted regarding the transcription of genes belonging to the *agr* locus (up to -0.9 log2FC, **Publication II**).

As mentioned earlier, the *agr* system generally shows substantial differences in activity depending on the genomic background and *agr* type, respectively. The CC398 isolates [169], including IMT38951 ([169], **Publication II**), exhibited an increased secretion of α- and β-hemolysins in a previous study [169], indicating, among others, an enhanced *agr* activity in CC398 isolates compared to the investigated CC8 and CC22 isolates [169]. Furthermore, biofilm formation was significantly reduced in these particular CC398 isolates compared to CC8 and CC22 isolates [169].

A further example for core genome alterations that contribute to the bacteria's capability to cope with changing environments are adaptive mutations in genes belonging to the mevalonate (MVA) pathway. Since the MVA pathway is involved in cell wall biosynthesis, respiratory energy generation, and oxidative stress protection, it harbours multiple "adjusting screws" with respect to changing growth conditions / environmental challenges [170]. Reduced bacterial cell growth, for example, is usually observed after expression downregulation of each of the MVA pathway genes [170]. Yu et al. [171] generated an *S. aureus* Hydroxy-3-methylglutaryl-Coenzyme A (HMG-CoA) synthase (*mva*S) deletion mutant that was initially auxotrophic for MVA and, therefore, unable to grow in the absence of

MVA. This mutant was generated as a representative for *S. aureus* isolates that exhibit growth delays due to, for example, starvation or acid stress [171, 172]. However, *S. aureus* Δ*mva*S adapted to nutrient deficiency and reverted to prototrophy. Whole genome analysis revealed that two sequential point mutations were necessary for this adaptation [173]. The first point mutation occurred at nucleotide position 32 of the coding region of the global transcriptional regulator Spx. This resulted in a non-synonymous amino acid exchange (see Chapter 1.3.1) from threonine to isoleucine at position 11 in *spx* [173]. A second mutation appeared in the lactonase-encoding gene *drp*35. The two sequenced genomes had either a base exchange from cytosine to thymine 41 nucleotides upstream of the predicted transcription start site (TSS) or from adenine to guanine 52 nucleotides from the TSS [173]. Both mutations are the adaptational results of a partially missing MVA pathway and are therefore necessary for recurring cell growth and proliferation of *S. aureus* Δ*mva*S [173]. Thus, core genome alterations can enhance the chances of bacteria to survive and overcome unfavourable circumstances in a specific habitat and then further adapt to novel conditions.

Another example of adaptation is the acquired resistance against vancomycin. This particular antibiotic is regarded as a last-resort drug in human medicine to treat life-threatening MRSA infections [145, 174, 175]. However, isolates with reduced susceptibility to vancomycin have also been reported for animal samples (reviewed in [175]). Depending on their resistance levels, *S. aureus* can be classified as vancomycin intermediate *S. aureus* (VISA), which has a moderate minimum inhibitory concentration (MIC) of 4 – 8 µg/ml or vancomycin resistant *S. aureus* (VRSA) with a MIC ≥ 16 µg/ml (reviewed in [175, 176]). The resistance is either mediated by the acquisition of the *van*A gene by MGEs or by the accumulation of several SNPs (reviewed in [175, 176]). Individual mutations of genes are already known to contribute to the emergence of VISA, for example, in the *rpo*B gene, which encodes a subunit of the RNA polymerase or in genes encoding two-component regulatory systems, such as *gra*SR, *wal*KR and *vra*SR (reviewed in [175, 176]). Furthermore, a recent study [175] identified genetic variations responsible for VRSA. A vancomycin susceptible strain was exposed to vancomycin to become VRSA. The whole genome sequencing revealed eight SNPs in the VRSA compared to its predecessor, namely *rim*M (G16D), *ssa*A2 (G128A), *rps*K (P60R), *rpo*B (R917C), *wal*K (T492R), D-alanyl-D-alanine carboxypeptidase (L307I), *vra*T (A152V), and chromosome segregation ATPase (T440I), leading to vancomycin resistance. These involved genes are related to cell wall metabolism and growth defects, which goes in line with the development of a thicker cell wall in the VRSA strain (34.96 ± 7.73 nm) compared to the vancomycin susceptible strain (17.71 ± 1.97 nm) [175].

3.2 The role of SCC*mec* elements in the adaptation process of *S. aureus* to novel habitats and environmental challenges

MGEs have an important impact on the adaptive genome evolution [20] by including or excising the MGEs into or from the bacterial genome (see Chapter 1.3.2). The HGT-mediated frequent exchange of MGEs (see Chapter 1.3.2) harbouring genes encoding resistance, virulence, and / or host-specific factors results in the diversification of *S. aureus* populations and in an advantage during changing environmental conditions [177-179]. An *in-vivo* study by McCarthy et al. [177] examined the co-colonization of *S. aureus* populations belonging to CC398 of porcine and human origin in gnotobiotic piglets. Overall, the transfer of MGEs from the porcine to the human isolate was detected within four hours of co-colonization. In particular, two bacteriophages and three plasmids spread extensively and repeatedly [177]. This resulted in a large number of mobilomes rather than just a single successful variant. However, bacteria were rarely transferred between individual piglets since each animal carried unique populations of bacterial variants [177]. The results of this study highlight that MGEs can be spread at high frequency by HGT and that adaptation to changing environmental conditions can occur more rapidly due to the possible co-existence of multiple MGEs.

The SCC*mec* element belongs to the group of MGEs which are of significant clinical relevance due to its ability to confer resistance to β-lactam antibiotics and other anti-infective agents (see Chapters 1.4.2 and 1.5.2). Furthermore, the obvious spread of SCC*mec* elements led to a global increase of MRSA in the past (see Chapter 1.5). Consequently, it is not surprising that scientists began investigating the processes involved in the mobilisation, proliferation and spread of SCC*mec* elements [33, 36, 77, 90, 180-184].

Previously, mobilisation of the SCC*mec* element was primarily attributed to the activation of the *ccr* genes [77, 90, 181]. Excision of SCC*mec*, for example, can be triggered by cell exposure to antibiotics including, beyond others, sulfamethoxazole, ciprofloxacin, trimethoprim or the β-lactam antibiotic oxacillin [155, 156]. The antibiotics inflict DNA damages that provoke an SOS response in the bacteria [156]. Initially, the SOS box (5'-TGAAACGAAATTATAAATA-3') in the promoter region of *ccr*AB and *ccr*C is bound by LexA (repressor), which constantly blocks the RNA polymerase activity at that particular location and thus hinders the transcription of the recombinase genes [156]. During the SOS response, RecA (bacterial recombinase A) is activated (RecA*), which subsequently leads to the autocleavage of LexA from the promotor region that is followed by an increased expression of *ccr*AB and *ccr*C, respectively. Afterwards, the recombinases are able to bind the chromosomal binding sites (see Chapter 1.4.2.3), causing the excision of the SCC*mec*

element via *ccr* activation from the genome [156]. Liu et al. [156] demonstrated that the frequency of the SCC*mec* excision by *ccr* depends on the type of antibiotic. The excision rate of SCC*mec* during unchallenged growth in trypticase soy broth was < 10^{-11}. However, during exposure to antibiotics compounds that are targeting DNA replication and repair, it increased to 3.4×10^{-5}, 2.3×10^{-6} and 1.1×10^{-5}, respectively [156]. Whereas the β-lactam antibiotic oxacillin, targeting the cell wall synthesis of the bacterium, achieved only an approximately 100-fold higher excision rate of SCC*mec* compared to the control [156].

Since the loss of SCC*mec* elements has also been described under pig-farming conditions [130, 181], LA-MRSA IMT38951 was exposed to porcine serum and enhanced ammonia concentration or a combination of both to mimic these settings (**Publication II**). However, exposure to these factors achieved a rather discrete change in the *ccr* genes compared to unstressed controls (**Publication II**). Thus, the applied growth conditions did not induce the *ccr* genes at the examined time points 10 min and 60 min after exposure. More research on the subject is necessary to investigate whether longer exposure times might induce the SOS response and therefore lead to *ccr* activation.

Besides a precise SCC*mec* excision, partial losses of SCC*mec* elements or even extensive adjacent deletions up to 201 kb were described after three months of serial subculturing [35]. The loss of these SCC*mec* elements was suggested to be influenced by IS*431* elements as well as the cell environment [35]. However, in **Publication II**, exposure to pig-farming-associated environmental challenges lacked significant changes in the transposase tnp_2 associated with IS*431*, located adjacent to *mec*A, compared to the unchallenged controls within the investigated timeframe.

Furthermore, SCC*mec* excision has been described as a result of a recombination of the recombinase genes [181] or the primases [130] present in that particular region. The investigated loss of the complete SCC*mec*Vc element in **Publication II** is likely the result of homologous recombination within a repetitive sequence between the end of intergenic spacer region 1 and the first 48 bp of the *ccr* genes (Figure 3 in **Publication II**). Moreover, **Publication II** demonstrated that the excised SCC*mec* element also encodes a recently described conserved domain (replication unit) of pattern 2, in addition to the already well-known *ccr* gene complex and *mec* gene complex (see Chapter 1.4.2.2). The replication unit assigned as pattern 2 comprises a putative A-family DNA polymerase (CCPol), a small “middle” protein with no conserved domains (MP) and a putative helicase (Cch2) followed by *ccr*C [185, 186] (Figure 5). In contrast, the main characteristics of pattern 1 are a short ssDNA-binding protein (LP1413) and a helicase (Cch) [185, 186] (Figure 5). Additionally, both patterns harbour three small origins of replication (ORFs) containing domains of

unknown function (DUFs) 950 (renamed SaUGI, staphylococcal uracil-DNA glycosylase inhibitor), 960, and 1643 (not illustrated in Figure 5) [186].

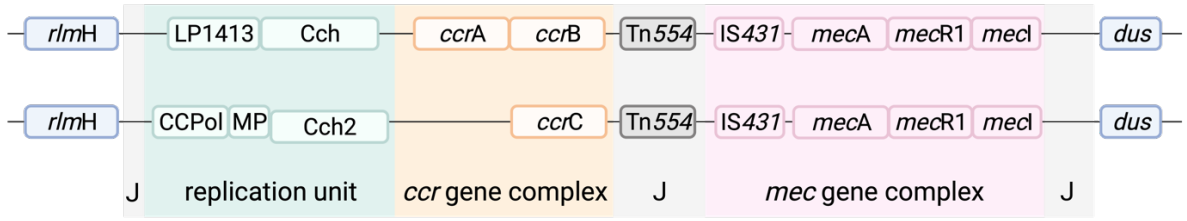


Figure 5: Illustration of the genomic organisation of SCC*mec* elements

The region of interest, i.e., the SCC*mec* integration site usually starts with *rlm*H (previously *orf*X) and ends with *dus* (previously *orf*Y). The SCC*mec* element comprises at least three distinct parts, the replication unit (green) comprising pattern 1 (first line) or pattern 2 (second line), which are associated with the respective *ccr* gene complex (yellow), either *ccr*A and *ccr*B (first line) or only *ccr*C (second line) and a *mec* gene complex (light red) mediating methicillin resistance. Further areas of the SCC*mec* element, like the flanking regions, are labelled with "J" (light grey) and may contain various insertion sequences (IS), transposons, direct repeats, antibiotic- as well as heavy metal-resistance- and virulence factor-encoding genes. Created with BioRender.com (October 2022).

The replication unit of pattern 2, i.e. a helicase, a primase and a recognised ORF, are also commonly associated with the replication of other replicative MGEs, like plasmids (reviewed in [186]). Cch2 is a 3'-to-5' helicase that probably binds the ORF, opens the bubble, and unwinds the DNA. The resulting ssDNA serves as a template for priming by the CCPol-MP complex [186]. Bebel et al. [186] further suggested that a host polymerase would presumably complete the replication of the element using the CCPol-MP-generated primers. Furthermore, all SCC elements described so far encode one of the two distinct replication units [186]. For example, all isolates investigated in **Publication I** harbour a replication unit resembling pattern 1 (Figure 6). This conserved domain putatively enables excised SCC*mec* elements to replicate independently in the extra-chromosomal space. Thus, SCC*mec* elements are available for genomic re-integration after or during excision [185, 186]. This self-replication potential of the SCC element might explain the widespread dissemination of SCC*mec* within staphylococci. Rolo et al. [187] investigated 106 isolates belonging to the *S. sciuri* group, namely *S. sciuri*, *S. vitulinus*, and *S. fleurettii*, collected in different time periods in various geographic regions and obtained from humans as well as animals. Analysing the *rlm*H (previously *orf*X) region of these isolates provided evidence that all three staphylococcal species were involved in the development of the SCC*mec* element. *S. vitulinus* and *S. fleurettii* contributed to the assembly of the *mec* complex, and *S. sciuri* most likely provided the mobile element in which *mec*A was later incorporated [187].

Furthermore, the SCC*mec*III-like element in *S. sciuri*, probably protecting the bacteria from natural antibiotics produced by other microorganisms, was subsequently transferred to *S. aureus* over time [187].

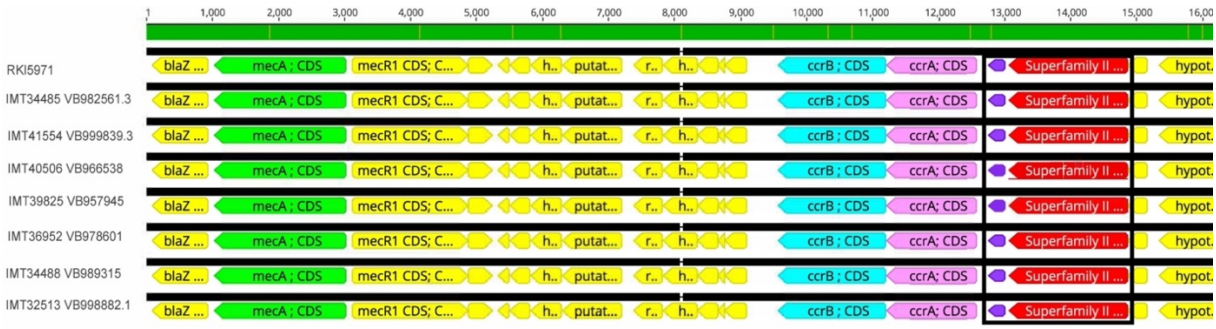


Figure 6: Selection of SCC*mec* elements from isolates investigated in Publication I

Illustration of the replication unit (black frame) belonging to pattern 1 according to [185, 186] of selected *mec*C-MRSA isolates investigated in Publication I. All isolates share a pattern 1 replication unit in their SCC*mec* element. Created with Geneious 11.1.5 [188].

Besides SCC*mec* elements and the resistance against β-lactam antibiotics, also plasmids harbour genes encoding antibiotic resistance, heavy metal resistance or virulence factors [81]. One prominent example is the resistance against tetracyclines, an antibiotic that is frequently used in pig farming [81, 135, 189]. Consequently, it is not surprising that MRSA CC398, belonging to predominant isolates in livestock animals in Europe, has adapted to this drug exposure. Price et al. [135] assumed that during a host switch from humans to pigs and the associated high selection pressure associated with the use of antibiotics caused CC398 isolates to acquire a tetracycline resistance gene. In **Publication II**, this gene is carried by the plasmid pT181 and is in tight proximity to the SCC*mec* element of the investigated LA-MRSA IMT38951 isolate (Figure 1 in **Publication II**). Furthermore, heavy metals can be impurities of animal feed additives, commonly added to meet the mineral requirements and improve the growth performance of the animals (reviewed in [190]). The animals only partially absorb these supplements, and non-absorbed heavy metal residues are excreted with the faeces (reviewed in [190]). Exposure of *S. aureus* to these heavy metals eventually leads to the adaptation of the bacteria to this specific environmental condition. In porcine *S. aureus* CC398 isolates, several plasmids contain resistance genes, for example, against cadmium [191, 192] and / or copper [192]. However, heavy metal resistance, especially towards zinc, can also be mediated by SCC elements (Figure 6 in **Publication II**).

Phages are another important group of MGEs, which seem essential with respect to host adaptation processes. Several virulence factors can be incorporated into the phage genome

and thus further distributed via HGT [193]. For instance, incorporating phages in the genome of *S. aureus* often allows the bacteria to cope with factors produced by the host's immune system [193]. A well-known example in this context is the bacteriophage ΦSa2, which consists of functionally colinear major modules encoding genes for lysogeny, DNA processing, head morphogenesis and packaging, tail morphogenesis, and lysis (reviewed in [194]). In addition, this phage can transport the *lukS-PV* and *lukF-PV* genes coding for PVL (reviewed in [194]). PVL is a pore-forming two-component toxin that lyses human macrophages, polymorphonuclear leukocytes, and monocytes (reviewed in [194]). Initially, the presence of PVL distinguished CA-MRSA from HA-MRSA, although this distinction has become increasingly obsolete over time (see Chapter 1.5.2). PVL-positive *S. aureus* isolates are frequently isolated from necrotising skin lesions and pneumonia, among others (reviewed in [195]).

3.3 The interplay between the core genome and MGEs

During an *S. aureus* infection, the bacteria produce several virulence factors to defend and evade the host's immune system and thereby adapt to the new environment. Among these are secreted toxins and exo-proteases, for example, sphingomyelinase, which is referred to as β-hemolysin [196]. This toxin hydrolyses the sphingomyelin of plasma membranes to ceramides and phosphorylcholines [197, 198], a process that explains why β-hemolysin is also called phospholipase [199].

In *S. aureus*, the *hlb* gene coding for β-hemolysin is part of the core genome [199]. However, this gene can be interrupted by the integration of a β-hemolysin-converting phage, for example, ΦSa3 or Φ13 (reviewed in [199]). Furthermore, ΦSa3 carries an immune evasion cluster (IEC), which is thus incorporated into the bacterial genome [30, 31]. The IEC harbours the genes for staphylokinase (*sak*), the chemotaxis inhibitory protein (*chip*) and the staphylococcal complement inhibitory protein (*scn*), as well as genes for enterotoxins like enterotoxin A or enterotoxin P [31]. The incorporated IEC thereby enable *S. aureus* to evade the immune system of humans and, to varying degrees, some animals as well [30, 107, 200-202]. Since *S. aureus* "sacrifices" the β-hemolysin for a phage containing an IEC, the phage integration appears to be more beneficial regarding host adaptation and spread.

The uptake or release of different MGEs in combination with changes in the core genome contributes to the adaptation of *S. aureus* to diverse and challenging hosts and environments. As mentioned before (see Chapter 1.5.2), MRSA belonging to CC8 was a common CA-MRSA phylogenetic lineage [122]. In the meantime, this lineage was also reported to be associated with bovine mastitis [203, 204]. During the jump from humans to

bovine hosts, the strains lost the β-hemolysin converting prophage, which is typical for human strains and acquired a new bovine-specific SCC element, which lacked the *mec*A gene conferring methicillin resistance, but carried a gene coding for a new surface-associated protein [203].

Another example is the host jump of MRSA CC5 from humans to poultry. The adaptation to the new host occurred through at least 44 recombinations affecting 33 genes [140, 141]. During the process, the poultry isolates not only integrated genes but also lost human-specific genes [140]. In addition to humans and poultry, MRSA belonging to CC5 are common in other animals, including pigs [205] and companion animals such as cats and dogs [206].

3.4 Conclusion and Outlook

S. aureus, as well as its methicillin resistant variants, have various strategies to cope with new, changing, or challenging hosts and environmental conditions. Changes in the core genome and the uptake or release of different MGEs promote the adaptive capabilities of *S. aureus* and thus contribute to their clinical importance.

In this thesis, core genome alterations of the *agr* system were examined revealing similar modulatory variations affecting *agr*A and *agr*C in completely distinct genomic lineages, leading to different *agr* activity levels including completely non-functional variants. This might contribute to the survival strategy of the bacteria to establish emerging clonal lineages in new hosts and habitats.

Genomic changes with regard to MGEs were investigated in terms of SCC*mec* elements. *S. aureus* ST398 lost a complete SCC*mec*Vc element including a unit conferring autonomous replication capabilities from its chromosomal integration site downstream of *rlm*H. Environmental conditions associated with pig farming such as increased ammonia concentrations and porcine serum did not induce a significant transcription of *ccr* genes that is required for *ccr*-mediated SCC*mec* excision in this experimental setting, although this has been a subject of speculation [143, 207]. Considering the results reported in this thesis, the SCC*mec* element may occasionally be lost during homologues recombination at particular molecular “breaking points” (Figures 2 and 3 in **Publication II**). Moreover, due to its auto-replicative potential and ability to be incorporated into susceptible genomes, further spread of resistance to β-lactam antibiotics via SCC*mec* are likely.

The two distinct adaptive strategies investigated here highlight the fact that the evolution of *S. aureus* will continue unabated, a fact that is highlighted by the WHO’s recommendation to enhance efforts towards the development of novel antibiotics against *S. aureus* [150].

However, considering the adaptative potential of *S. aureus*, neither the development of further antibiotics, drugs that interfere with several metabolic processes of *S. aureus*, nor possible vaccinations (alone) will probably hold or combat the pathogen completely, at least not in the long term. The development of completely new treatment options, which consider the current knowledge on the adaptation strategies of *S. aureus* are therefore of considerable clinical importance.

In addition, a more holistic approach to counteract *S. aureus* including MRSA is necessary. Since the bacteria can ubiquitously colonise humans, animals and abiotic materials, scientists from the sectors of human, veterinary as well as environmental health are required to cooperate in a one-health approach [208], to prevent further transmission and reduce the burden of diseases associated with infections caused by *S. aureus*.

4 Literature

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5 Further Publications and Conference Contributions

5.1 Collaboration Publications

2022

Genome-wide association reveals host-specific genomic traits in *Escherichia coli*

Sumeet K. Tiwari*, Boas C.L. van der Putten*, Thilo M. Fuchs, Trung N. Vinh, Martin Bootsma, Rik Oldenkamp, Roberto La Ragione, Sebastien Matamoros, Ngo T. Hoa, Christian Berens, Joy Leng, Julio Álvarez, Marta Ferrandis-Vila, Jenny M. Ritchie, Angelika Fruth, Stefan Schwarz, Lucas Domínguez, María Ugarte-Ruiz, Astrid Bethe, **Charlotte Huber**, Vanessa Johanns, Ivonne Stamm, Lothar H. Wieler, Christa Ewers, Amanda Fivian-Hughes, Herbert Schmidt, Christian Menge, Torsten Semmler, Constance Schultsz

preprint at BioRxiv. 2022. doi: 10.1101/2022.02.08.479532

2021

Genome-wide insights into population structure and host specificity of *Campylobacter jejuni*

Lennard Epping, Birgit Walther, Rosario M Piro, Marie-Theres Knüver, **Charlotte Huber**, Andrea Thürmer, Antje Flieger, Angelika Fruth, Nicol Janecko, Lothar H Wieler, Kerstin Stingl, Torsten Semmler

Sci Rep. 2021 May 14;11(1):10358. doi: 10.1038/s41598-021-89683-6

2019

Comparison of different technologies for the decipherment of the whole genome sequence of *Campylobacter jejuni* BfR-CA-14430.

Lennard Epping, Julia C Golz, Marie-Theres Knüver, **Charlotte Huber**, Andrea Thürmer, Lothar H Wieler, Kerstin Stingl, Torsten Semmler

Gut Pathog. 2019 Dec 16;11:59. doi: 10.1186/s13099-019-0340-7

2018

Equine Methicillin-Resistant Sequence Type 398 *Staphylococcus aureus* (MRSA) Harbor Mobile Genetic Elements Promoting Host Adaptation

Birgit Walther, Katja-Sophia Klein, Ann-Kristin Barton, Torsten Semmler, **Charlotte Huber**, Roswitha Merle, Karsten Tedin, Franziska Mitrach, Antina Lübke-Becker, Heidrun Gehlen

Front Microbiol. 2018 Oct 24;9:2516. doi: 10.3389/fmicb.2018.02516

Occurrence and molecular composition of methicillin-resistant *Staphylococcus aureus* isolated from ocular surfaces of horses presented with ophthalmologic disease

Tanawan Soimala, Antina Lübke-Becker, Stefan Schwarz, Andrea T Feßler, **Charlotte Huber**, Torsten Semmler, Roswitha Merle, Heidrun Gehlen, Johanna C Eule, Birgit Walther

Vet Microbiol. 2018 Aug;222:1-6. doi: 10.1016/j.vetmic.2018.06.009

Extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* and *Acinetobacter baumannii* among horses entering a veterinary teaching hospital: The contemporary "Trojan Horse"

Birgit Walther, Katja-Sophia Klein, Ann-Kristin Barton, Torsten Semmler, **Charlotte Huber**, Silver Anthony Wolf, Karsten Tedin, Roswitha Merle, Franziska Mitrach, Sebastian Guenther, Antina Lübke-Becker, Heidrun Gehlen

PLoS One. 2018 Jan 30;13(1):e0191873. doi: 10.1371/journal.pone.0191873

5.2 Participation in Scientific Conferences

2019

A kaleidoscope of phenotypes: *mec*C-positive Methicillin-resistant *Staphylococcus aureus* (MRSA)

Charlotte Huber, Lennard Epping, Ivonne Stamm, Wilma Ziebuhr, Gabriella Marincola, Markus Bischoff, Birgit Strommenger, Christiane Cuny, Wolfgang Witte, Jörg Döllinger, Christoph Schaudinn, Torsten Semmler, Antina Lübke-Becker and Birgit Walther

National Symposium on Zoonoses Research 2019, Berlin, 16.10.-18.10.2019

2018

Loss of Staphylococcal Cassette Chromosome *mec* V in Methicillin-resistant *Staphylococcus aureus* progeny

Charlotte Huber, Torsten Semmler, Lennard Epping, Antina Lübke-Becker, Lothar H. Wieler, Birgit Walther

Diplomanden- und Doktoranden-Meeting, Robert Koch-Institut, Berlin, 21.11.2018

Loss of Staphylococcal Cassette Chromosome *mec* V in Methicillin-resistant *Staphylococcus aureus* progeny

C. Huber, T. Semmler, L. Epping, A. Lübke-Becker, L.H. Wieler, B. Walther

National Symposium on Zoonoses Research 2018, Berlin, 17.10.-19.10.2018

Epidemiology of *mec*C-positive methicillin-resistant *Staphylococcus aureus*
Charlotte Huber, Ivonne Stamm, Torsten Semmler, Antina Lübke-Becker, Birgit Walther
Junior Scientist Zoonoses Meeting, Hamburg, 07.06.-09.06.2018

Molecular characteristics of *mec*C-positive Methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from various animal species
Charlotte Huber, Ivonne Stamm, Torsten Semmler, Antina Lübke-Becker, Birgit Walther
Tagung der Deutsche Veterinärmedizinische Gesellschaft (DVG) Fachgruppe „Bakteriologie und Mykologie“, Hannover, 30.05.-01.06.2018

2017

Development of a high-throughput method for identification of Methicillin-susceptible *Staphylococcus aureus* (MSSA) progeny from epidemic MRSA
C. Huber, A. Lübke-Becker, T. Semmler, L.H. Wieler, B. Walther
National Symposium on Zoonoses Research 2017, Berlin, 12.10.-13.10.2017

Verification of resistance loss in methicillin-resistant *Staphylococcus aureus* (MRSA) progeny using a high-throughput screening procedure
Charlotte Huber, Torsten Semmler, Antina Lübke-Becker and Birgit Walther
10. Doktorandensympsosium & DRS Präsentationsseminar „Biomedical Sciences“, Berlin, 22.09.2017