Aus dem Institut für Mikrobiologie und Tierseuchen

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Comparative phenotypic and genotypic studies on LA-MRSA CC398 and CC9 of pigs from Germany and China

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Henrike Krüger-Haker, geb. Krüger

Tierärztin

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Dekan:	Univ.-Prof. Dr. Uwe Rösler
Erster Gutachter:	Univ.-Prof. Dr. Stefan Schwarz
Zweiter Gutachter:	Prof. Yang Wang, PhD
Dritte Gutachterin:	Univ.-Prof. Dr. Diana Meemken

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As a research worker, the unforgotten moments of my life are those rare ones which come after years of plodding work, when the veil over nature's secret seems suddenly to lift, and when what was dark and chaotic appears in a clear and beautiful light and pattern.

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Table of contents

List of figures and tables

Figures and tables

Figures and tables – Publication I

Figures and tables – Publication II

Figures and tables – Publication III

Figures and tables – Publication IV

Figures and tables – Publication V

Scientific, Dreieich, Germany), lane + contains the PCR amplicon obtained from *S. aureus* GDC6P096P, and lane – is the negative control, i.e., demineralized water. The sizes of selected fragments of the DNA ladder are indicated in base pairs.

Figures and tables – Publication VI

Figure 1 (a) Structure and organization of plasmid pHKS3860 (GenBank accession number OM362497). The open reading frames (ORFs) are shown as arrows with the arrowhead indicating the direction of transcription. The various colours indicate different functions. A size scale is given outside the circular map and truncated genes are indicated by a Δ symbol. The plasmid had a GC content of 31.4% and comprised 46 predicted ORFs. (b) Phylogeny of known 23S rRNA methylases conferring combined resistance to macrolides, lincosamides and streptogramin B antibiotics. Protein sequences were aligned using MAFFT implemented in Geneious v11.1.4. The maximum likelihood tree was generated with IQ-TREE v1.6.5 using the WAG+F+I+G4 model. The scale bar displays the average number of amino acid substitutions per site. Numbers at nodes indicate IQ-TREE ultrafast bootstrap support values (10000 replicates), which are only shown if greater than 70. The GenBank accession numbers are given for each Erm protein.

Table S1 Comparison of the antimicrobial susceptibility testing data.

List of abbreviations

aa	Amino acid
AMR	Antimicrobial resistance
AST	Antimicrobial susceptibility testing
AUC	Area under the curve
bp	Base pairs
BLAST	Basic local alignment search tool
BST	Biocide susceptibility testing
CA-MRSA	Community-associated methicillin-resistant *Staphylococcus aureus*
CC	Clonal complex
ccr	Cassette chromosome recombinase gene
CGE	Center for Genomic Epidemiology
cgMLST	Core-genome multilocus sequence typing
CLSI	Clinical and Laboratory Standards Institute
CoNS	Coagulase-negative staphylococci
DNA	Deoxyribonucleic acid
DR	Direct repeat
dru	Direct repeat unit
E. faecalis	*Enterococcus faecalis*
HA-MRSA	Healthcare-associated methicillin-resistant *Staphylococcus aureus*
HGT	Horizontal gene transfer
IEC	Immune evasion cluster
IR	Inverted repeat
IS	Insertion sequence
IWG-SCC	International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements
LA-MRSA	Livestock-associated methicillin-resistant *Staphylococcus aureus*

LT	Lag time
MH	Maximum height
MLST	Multilocus sequence typing
PLS_A	Pleuromutilin-lincosamide-streptogramin A
PFGE	Pulsed-field gel electrophoresis
MGE	Mobile genetic element
MIC	Minimal inhibitory concentration
MLS_B	Macrolide-lincosamide-streptogramin B
mRNA	Messenger ribonucleic acid
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSCRAMMs	Microbial surface components recognizing adhesive matrix molecules
MSSA	Methicillin-susceptible *Staphylococcus aureus*
ORF	Open reading frame
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
PM	Phenotype MicroArray™
PT	Plateau time
PVL	Panton-Valentine leucocidin
QAC	Quaternary ammonium compound
RM system	Restriction-modification system
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
S. aureus	*Staphylococcus aureus*
SCC*mec*	Staphylococcal cassette chromosome *mec*
SCVs	Small-colony variants
SMRT	Single-molecule real-time
spa	*Staphylococcus aureus* protein A gene

sPLS-DA	Sparse partial least squares discriminant analysis
ST	Sequence type
Tn	Transposon
TSS	Toxic shock syndrome
TSST-1	Toxic shock syndrome toxin-1
WGS	Whole-genome sequencing

1 Introduction

The Gram-positive bacterium *Staphylococcus aureus* is part of the physiological skin and mucosal flora in humans and animals (Somerville 2016b). As facultative pathogen, it can also cause diverse infectious diseases, depending on the expressed virulence factors and, regarding the host side, on the condition of mechanical (skin and mucosal surfaces) or biological barriers (immune system) (Somerville 2016b). Methicillin-resistant *S. aureus* (MRSA) isolates, which are resistant against β-lactam antibiotics, have become a global health threat in human and veterinary medicine (Somerville 2016b). This resistance trait is mainly conferred by the *mecA* and *mecC* genes located on the staphylococcal cassette chromosome *mec* (SCC*mec*) mobile genetic element (MGE) (Shore and Coleman 2013). Based on adaption to certain niches and the associated clones, MRSA isolates have been assigned to three overlapping epidemiological groups: healthcare-associated (HA-MRSA), community-associated (CA-MRSA) and livestock-associated MRSA (LA-MRSA) (Zarazaga et al. 2018).

Several molecular methods are suitable to trace back the epidemiological origin of certain MRSA clones and to investigate their dissemination among humans, animals and/or the environment (Wendlandt et al. 2013c). Multilocus sequence typing (MLST), *spa* typing and macrorestriction analysis, for example, are applicable to *S. aureus* in general (Wendlandt et al. 2013c). MLST is based on the characterization of deoxyribonucleic acid (DNA) sequence variations in seven stable housekeeping genes that define the sequence type (ST) (Enright et al. 2000). STs are further grouped into a Clonal Complex (CC) by similarity of their allelic profiles (Enright et al. 2000). The *spa* typing targets the variable region X in the *S. aureus* protein A (*spa*) (Wendlandt et al. 2013c; Harmsen et al. 2003). It consists of a differing number of variable repeats that show sequence variations and define the *spa* type with their order (Wendlandt et al. 2013c; Harmsen et al. 2003). In macrorestriction analysis, fragments of the bacterial DNA obtained with rare-cutting endonucleases are separated via pulsed-field gel electrophoresis (PFGE) and the resulting fragment patterns are compared (Wendlandt et al. 2013c). In contrast, *dru* typing and SCC*mec* typing are applicable only to methicillin-resistant staphylococci (Wendlandt et al. 2013c). The *dru* type is defined by number and order of the repeats, that differ slightly in their sequences, located within the direct repeat unit (*dru*) in the SCC*mec* element (Goering et al. 2008), whereas SCC*mec* typing targets multiple regions in the SCC*mec* cassette (see section *1.3.3*) (Wendlandt et al. 2013c). Depending on the objective, a combination of methods might be useful to increase the overall discriminatory power (Wendlandt et al. 2013c).

1.1 LA-MRSA

LA-MRSA was first reported in pigs in the Netherlands in 2005 (Voss et al. 2005). It was initially described as non-typeable MRSA due to the isolates' non-typeability with standard PFGE using the restriction enzyme SmaI (Voss et al. 2005) and limited to ST398 isolates (Butaye et al. 2016). As a consequence of the progressing evolution of LA-MRSA, many more STs assigned to certain CCs are included now (Butaye et al. 2016). The prevalence of the CCs differs geographically. Worldwide, LA-MRSA most commonly belongs to CC398, which dominates in Europe, North and South America as well as Australia (Butaye et al. 2016; Price et al. 2012; Monecke et al. 2011; Kadlec et al. 2009). In contrast, CC9 is the main LA-MRSA lineage in Asia (Butaye et al. 2016; Chuang and Huang 2015). The diversity of subtypes within the CCs is great – in some regions greater than in others – and increasing over time (Butaye et al. 2016). Price et al. have shown that LA-MRSA originated in humans as methicillin-susceptible *S. aureus* (MSSA) (Price et al. 2012). After a host jump of MSSA CC398 to livestock, it subsequently acquired the SCC*mec* element and thus methicillin resistance (Price et al. 2012). Moreover, CC398 also acquired tetracycline resistance after the transfer from humans to livestock (Price et al. 2012). Since LA-MRSA has no particular host specificity and can easily cross species barriers, it has been detected in several livestock species, pet animals as well as humans (Butaye et al. 2016; Graveland et al. 2011). Pigs represent a major reservoir of LA-MRSA (Smith and Pearson 2011). These isolates are mainly involved in colonization of the skin and mucosal surfaces in pigs and only rarely cause diseases, such as skin infections, pneumonia or septicemia (Kadlec et al. 2009). Studies have shown that there exists a substantial risk of LA-MRSA colonization or infection for humans in direct (occupational) contact with LA-MRSA-positive animals (Graveland et al. 2011). The pathogenicity of LA-MRSA for humans has been reported in many studies and although further spread within the healthcare system or the community is less frequent, potential LA-MRSA transmission to other humans and/or animals needs to be considered (Cuny et al. 2015b; Köck et al. 2013; Graveland et al. 2011; Köck et al. 2010; Nienhoff et al. 2009).

1.1.1 LA-MRSA CC398

CC398 is the most common LA-MRSA lineage worldwide (Butaye et al. 2016). It is the main clone found in Europe, North and South America as well as Australia (Price et al. 2012; Monecke et al. 2011; Kadlec et al. 2009). In Africa and Asia, it has also been detected occasionally (Abdulgader et al. 2015; Chairat et al. 2015; Chuang and Huang 2015). LA-MRSA CC398 mainly colonizes pigs and veal calves, but it has also been detected in other livestock species including cattle, sheep, goats, chickens, turkeys, geese, quails, ducks, rabbits, and horses (Silva et al. 2023; Vincze et al. 2014; Feßler et al. 2012). Moreover, CC398 isolates

have been reported from dogs and cats (Vincze et al. 2014; Weiß et al. 2013; Nienhoff et al. 2009), mink (Hansen et al. 2020), rats (Desvars-Larrive et al. 2019) as well as humans (Graveland et al. 2011).

There is a high diversity of SCC*mec* elements, STs, *spa* and *dru* types within CC398, which seems to be increasing over time (Monecke et al. 2018; Butaye et al. 2016; Wendlandt et al. 2013c). The dominant SCC*mec* types among LA-MRSA CC398 are SCC*mec* IV and V, but type III and non-typeable elements have also been reported (Monecke et al. 2018; Butaye et al. 2016). CC398 includes at least 43 STs, but ST398 is described as the main ST colonizing pigs (Butaye et al. 2016). The molecular variability of CC398 seems to differ between countries and the local circulation of specific isolates has been indicated (Butaye et al. 2016; European Food Safety Authority 2009b). A regional high diversity might be consequence of the extensive trading of livestock, suggesting a greater variability of isolates in countries with high trade levels (European Food Safety Authority 2010; European Food Safety Authority 2009a; European Food Safety Authority 2009b).

LA-MRSA CC398 has been found sporadically in pigs suffering from diseases, such as pyoderma or pneumonia (Kadlec et al. 2009). Moreover, such isolates have been involved in bovine mastitis (Silva et al. 2014; Feßler et al. 2010; Vanderhaeghen et al. 2010) and invasive infections (liver, heart, lungs, joints) of poultry (Monecke et al. 2013). Several reports have also described CC398-associated human cases of skin and soft-tissue infections, endocarditis, otomastoiditis, septicemia, ventilator-associated pneumonia, postoperative surgical site infections, and infections after joint replacement (Cuny et al. 2015b; Köck et al. 2013; Köck et al. 2010). Certainly, there is a considerable risk of nosocomial infections for human patients, when LA-MRSA CC398 is introduced into healthcare settings. These isolates are able to cause severe diseases in humans and animals despite the frequent lack of virulence factors typically identified in CA-MRSA and HA-MRSA, which are associated with the human host (Graveland et al. 2011). Several studies demonstrated that LA-MRSA CC398 usually does not harbor the relevant virulence factors, such as the toxic shock syndrome toxin-1 (TSST-1) gene *tst*, the Panton-Valentine leucocidin (PVL) genes *lukF-PV* and *lukS-PV* or exfoliative toxins (Wendlandt et al. 2013c). However, in some cases MRSA CC398 isolates of human origin have been shown to be PVL-positive (Wendlandt et al. 2013c; Stegger et al. 2010; van Belkum et al. 2008). More precisely, CA-MRSA CC398 variants, which display different genetic and epidemiological characteristics, have emerged in humans in the Asia-Pacific region but also in European countries, are highly virulent and transmissible, and carry the human immune evasion cluster (IEC) (Gooskens et al. 2023; Coombs et al. 2022). These isolates cause severe infections more frequently than LA-MRSA CC398 and the affected patients usually did not have contact to livestock (Gooskens et al. 2023; Coombs et al. 2022). In Europe, such patients often have links to the Asia-Pacific region, for example as travel

history (Gooskens et al. 2023; Coombs et al. 2022). In contrast to the detection of the IEC genes in CC398 isolates from human infections, these genes are normally absent in LA-MRSA CC398 isolates adapted to the animal host (Cuny et al. 2015a). IEC genes were also identified in LA-MRSA CC398 from veterinarians treating horses and from nosocomial infections in horses (Cuny et al. 2015a). Moreover, staphylococcal enterotoxin genes have been reported occasionally in LA-MRSA CC398 (Butaye et al. 2016; Wendlandt et al. 2013c). Genes coding for other leucocidins, adhesion factors, hemolysins, proteases and superantigen-like proteins have been found commonly in these isolates (Butaye et al. 2016).

Furthermore, the LA-MRSA CC398 lineage is known for its common multiresistance, which means resistance to at least one agent in at least three antimicrobial classes (Sweeney et al. 2018; Butaye et al. 2016). A broad variety of different antimicrobial resistance (AMR) profiles has been reported from LA-MRSA CC398 of animal origin (Wendlandt et al. 2013c). Besides isolates showing resistance to β-lactams and tetracyclines only, extended AMR patterns have also been described (Wendlandt et al. 2013c). Many AMR genes typically detected in staphylococci from animals have been found in LA-MRSA CC398 isolates, including genes conferring resistance to β-lactams [*blaZ*], tetracyclines [*tet*(K), *tet*(M), *tet*(L)], phenicols [*fexA*], aminoglycosides [*aacA-aphD*, *aadD*, *aphA3*, *aadE*, *str*], macrolides-streptogramin B [*msr*(A)], macrolides-lincosamides-streptogramin B [*erm*(A), *erm*(B), *erm*(C)], lincosamides [*lnu*(A), *lnu*(B)], pleuromutilins-lincosamides-streptogramin A [*vga*(A)], and trimethoprim [*dfrA* (*dfrS1*), *dfrD*, *dfrG*)] (Schwarz et al. 2018; Butaye et al. 2016; Silva et al. 2014). In addition, novel or rare AMR genes have been often identified in LA-MRSA CC398 isolates, such as those mediating resistance against aminocyclitols [*apmA*, *spc*, *spd*, *spw*], macrolides-lincosamides-streptogramin B [*erm*(T)], pleuromutilins-lincosamides-streptogramin A [*vga*(C), *vga*(E), *lsa*(E)], trimethoprim [*dfrK*], and phenicols-lincosamides-oxazolidinones-pleuromutilins-streptogramin A [*cfr*] (Schwarz et al. 2018; Butaye et al. 2016; Wendlandt et al. 2013c). Mutations in the genes *gyrA* and *grlA* as well as in the promoter region of the *norA* gene that are involved in fluoroquinolone resistance were also described for CC398 isolates (Hauschild et al. 2012).

The diversity of the LA-MRSA CC398 clone described above is attributed to its ability to acquire foreign DNA molecules, which has been demonstrated by many studies (Butaye et al. 2016). Due to its low host specificity in contrast to most other *S. aureus* clones, LA-MRSA CC398 may also move easily between different hosts and acquire for example virulence and AMR genes (Butaye et al. 2016). For this reason, it has to be considered that in the course of an ongoing evolution of the CC398 clone novel possibly more virulent and/or resistant variants might emerge (Butaye et al. 2016) that can pose a health risk in human medicine as well as veterinary medicine.

1.1.2 LA-MRSA CC9

CC9 is the main LA-MRSA lineage found in Asia, even though its prevalence may differ among Asian countries (Chuang and Huang 2015). Moreover, LA-MRSA CC9 have been detected occasionally in Europe, Africa, Australia and the Americas (Yu et al. 2021). The lineage has been identified primarily in pigs, but also in cattle, buffalos, chickens and humans (Badua et al. 2020; Chuang and Huang 2015).

Similar to CC398, LA-MRSA CC9 shows a great variety of SCC*mec* elements and *spa* types (Butaye et al. 2016). Distribution of the SCC*mec* cassettes and *spa* types seems to be country-related among CC9 (Butaye et al. 2016). The most predominant SCC*mec* type is XII or XII-like, though SCC*mec* III, IV, V, IX, XI and non-typeable types have also been described (Yu et al. 2021; Chuang and Huang 2015).

In contrast to CC398, LA-MRSA CC9 is a typical pig-associated clone (Butaye et al. 2016). One study reported a CC9 isolate from a pig with signs of infection in Spain (Ruiz-Ripa et al. 2021a). However, LA-MRSA CC9 have also been found infecting other species, such as bovines suffering from mastitis (Wang et al. 2015a) and humans with mild to severe infections (Jin et al. 2020; Chen et al. 2018; Lulitanond et al. 2013; Wan et al. 2013; Liu et al. 2009). It is worth noting that some of the human patients did not have livestock contact prior infection (Jin et al. 2020; Chen et al. 2018) and the LA-MRSA CC9 lineage has emerged in clinical settings in China (Chen et al. 2023).

The virulence gene profiles of CC9 isolates differ from those of other LA-MRSA lineages. According to studies from Asian countries and Germany, more than 90% of LA-MRSA CC9 isolates carried at least one enterotoxin gene (Chuang and Huang 2015). A common feature of the lineage is the presence of the enterotoxin gene cluster *egc* comprising the genes *seg*, *sei*, *sem*, *sen*, *seo*, and *seu*/*y* (Monecke et al. 2011). Moreover, a high detection rate of the *tst* gene was reported in LA-MRSA CC9, but negative expression for the corresponding TSST-1 phenotype (Wan et al. 2013). Similar to CC398, PVL-encoding genes are also usually absent in LA-MRSA CC9 (Chuang and Huang 2015).

A general multiresistance to various classes of antimicrobial agents has also been described for the LA-MRSA CC9 clone (Chuang and Huang 2015). In addition to common AMR genes, CC9 isolates were shown to harbor rare genes, such as those mediating resistance to tetracyclines [*tet*(63), *tet*(T)], aminocyclitols [*spw*], macrolides-lincosamides-streptogramin B [*erm*(33)], lincosamides [*lnu*(B)], pleuromutilins–lincosamides–streptogramin A [*lsa*(E)], and phenicols-lincosamides-oxazolidinones-pleuromutilins-streptogramin A [*cfr*] (Chen et al. 2023; Jiang et al. 2019b; Schwarz et al. 2018; Butaye et al. 2016). Besides the fluoroquinolone-resistance mediating mutations in *gyrA*, *grlA* and the *norA* promoter region, rifampicin-resistance conferring mutations in the *rpoB* gene have also been described for LA-MRSA CC9 (Li et al. 2016; Hauschild et al. 2012).

LA-MRSA CC9 isolates are less widespread compared to the CC398 lineage and, thus, have been studied less frequently (Butaye et al. 2016). However, the CC9 clone also represents a possible public health risk due to its multiresistance and the higher virulence potential. Although adapted to animals, LA-MRSA CC9 colonization and infection has also been demonstrated in the human host. The diversity of CC9 points towards an ability of acquiring foreign DNA molecules and an ongoing clonal expansion similar to that in CC398. Transmission events might also reshape resistance and virulence traits of these isolates in the future, so that the lineage becomes more dangerous and spreads further. This assumption is supported, for example, by a recent study that already reports a multiresistant animal-adapted LA-MRSA ST9 isolate, which has acquired human-specific hypervirulence presumably independently, from a human patient without livestock exposure suffering from a severe infection (Yu et al. 2021).

1.2 AMR properties among LA-MRSA

The discovery of antimicrobial drugs in the 20th century revolutionized the control of bacterial diseases in humans, animals and plants (van Duijkeren et al. 2018). These substances either inhibit bacterial growth (bacteriostatic effect) or kill bacteria (bactericidal effect) (van Duijkeren et al. 2018). The development of AMR is a consequence of natural bacterial evolution allowing bacteria to survive in the presence of less favorable environmental conditions (van Duijkeren et al. 2018). In the last decades, the increased selection pressure resulting from the widespread usage of antimicrobial compounds led to an accelerated rise in emergence and distribution of AMR properties in bacteria (van Duijkeren et al. 2018). AMR can be intrinsic or acquired (van Duijkeren et al. 2018). Intrinsic AMR is associated with the genus or species of a bacterium (van Duijkeren et al. 2018). Common causes are the lack or inaccessibility of target structures, the presence of export systems, the production of inactivating enzymes, or the ability to use alternative metabolic pathways (van Duijkeren et al. 2018). In contrast, acquired AMR is an isolate-specific trait that can be based on the acquisition of AMR genes or mutations of target and regulator genes (van Duijkeren et al. 2018). Furthermore, AMR mechanisms can be divided into three categories: (i) enzymatic inactivation of the antimicrobial agent, (ii) decreased intracellular drug accumulation, and (iii) modification, protection, altered expression or replacement of the antimicrobial's target site (van Duijkeren et al. 2018).

On mucous membranes and the skin, LA-MRSA live in close contact with a great variety of other bacteria. Genetic material can be exchanged across strain, species or even genus boundaries (Schwarz et al. 2018). LA-MRSA have been demonstrated to act as donors or recipients of AMR genes in such exchange events (Kadlec et al. 2012b). Moreover,

transmission of LA-MRSA isolates to other animals and/or humans by direct contact to the bacterial host or its excretions, or by indirect ways e.g. via dust, aerosols, or contaminated environments can contribute to the dissemination of LA-MRSA and their AMR genes (Schwarz et al. 2018). A great number of AMR genes has been detected in staphylococci of human as well as animal origin (Schwarz et al. 2018). In contrast, comparatively small numbers have been identified solely in either human or animal staphylococci (Schwarz et al. 2018) (see also **Figure 2** in section **1.4**). The following sections focus on the AMR mechanisms that have been described for LA-MRSA isolates so far. An overview is given in **Table 1**, also taking into account biocide and heavy metal resistance-mediating genes.

The minimal inhibitory concentration (MIC) value is defined as the lowest concentration of an antimicrobial agent, which prevents visible growth of a bacterium under defined *in vitro* conditions of antimicrobial susceptibility testing (AST) (Clinical and Laboratory Standards Institute 2018). It can be interpreted based on established clinical breakpoints to categorize an organism as "susceptible", "intermediate", "resistant", or "nonsusceptible" (Clinical and Laboratory Standards Institute 2018). The interpretive categories are derived from microbiological characteristics, pharmacokinetic/pharmacodynamic parameters and clinical outcome data, when available (Clinical and Laboratory Standards Institute 2018).

Table 1. Genes and mutations mediating resistance to antimicrobial agents, biocides or heavy metals so far known to occur among LA-MRSA.

Resistance to …	Mechanism	Gene
β-Lactams	Enzymatic inactivation	*blaZ*
	Target site replacement	*mecA*, *mecC*
Tetracyclines (except minocycline and glycylcyclines)	Active efflux	*tet*(K), *tet*(L), *tet*(63)
Tetracyclines (including minocycline, excluding glycylcyclines)	Target site protection	*tet*(M), *tet*(T), *tet*(S)
Phenicols	Active efflux	*fexA*
Aminoglycosides (gentamicin, kanamycin, tobramycin, amikacin)	Enzymatic inactivation	*aacA-aphD*
Aminoglycosides (kanamycin, neomycin, tobramycin)	Enzymatic inactivation	*aadD*
Aminoglycosides (kanamycin, neomycin, amikacin)	Enzymatic inactivation	*aphA3*
Aminoglycosides (streptomycin)	Enzymatic inactivation	*aadE*, *str*
Aminocyclitols (spectinomycin)	Enzymatic inactivation	*spc*, *spd*, *spw*

Aminocyclitols/aminoglycosides (apramycin, decreased gentamicin susceptibility)	Enzymatic inactivation	*apmA*
Macrolides, lincosamides, streptogramin B	Target site modification	*erm*(A), *erm*(B), *erm*(C), *erm*(T), *erm*(33)
Macrolides, streptogramin B	Target site protection	*msr*(A)
Lincosamides	Enzymatic inactivation	*lnu*(A), *lnu*(B)
Pleuromutilins, lincosamides, streptogramin A	Target site protection	*vga*(A), *vga*(A)$_V$, *vga*(A)$_{LC}$, *vga*(C), *vga*(E), *lsa*(E)
Phenicols, lincosamides, oxazolidinones, pleuromutilins, streptogramin A	Target site modification	*cfr*
Oxazolidinones, phenicols	Target site protection	*optrA*, *poxtA*
Trimethoprim	Target site replacement	*dfrA* (*dfrS1*), *dfrD*, *dfrG*, *dfrK*
Fluoroquinolones	Target site modification Active efflux	mutations in *gyrA* and *grlA* mutations in the promoter region of *norA*
Rifampicin	Target site modification	mutations in *rpoB*
Quaternary ammonium compounds	Active efflux	*qacA/B*, *qacC/D*, *qacG*, *qacH*, *qacJ*, *smr*
Heavy metals	Active efflux/Enzymatic modification	*cadD/cadX* (cadmium), *arsRBC* and *arsDARBC* (arsenic), *copB* (copper), *mco/copA* (copper), *czrC* (cadmium/zinc)

1.2.1 Resistance to β-lactams

β-lactams comprise the penicillins, cephalosporins, carbapenems, monobactams and penems, which share the β-lactam ring as part of their chemical core structure (Prescott 2013a). They prevent the final step of bacterial cell wall biosynthesis by inhibiting the activity of penicillin-binding proteins (PBPs), which catalyze the process of synthesizing cross-linked peptidoglycan as the major component of bacterial cell walls (Prescott 2013a). The agents exhibit a bactericidal mode of action, lysing growing cells with an active cell wall biosynthesis (Prescott 2013a).

The methicillin-resistance of LA-MRSA is based on target site replacement by alternative PBPs, which are encoded by the *mecA* and *mecC* genes as part of the SCC*mec* element (Abdullahi et al. 2023; Schwarz et al. 2018). The alternative PBPs have a strongly reduced affinity to almost all β-lactams, except for certain anti-MRSA cephalosporins only approved for human applications (Schwarz et al. 2018). Isolates expressing the *mecA* gene should be regarded as resistant to all β-lactam antibiotics approved for the use in animals (Schwarz et al. 2018). For *mecC*-positive isolates this still needs to be proven (Schwarz et al. 2018). Another β-lactam resistance mechanism in LA-MRSA is the enzymatic inactivation of

β-lactams by a *blaZ*-encoded β-lactamase (Schwarz et al. 2018). This enzyme mediates resistance to penicillins, except for isoxazolyl-penicillins (Schwarz et al. 2018).

1.2.2 Resistance to tetracyclines

The tetracyclines are inhibitors of bacterial protein biosynthesis by binding to the 30S ribosomal subunit and, thus, preventing ribosomal translation (del Castillo 2013). They show bacteriostatic activity, but a time-dependent bactericidal effect has been confirmed at least for doxycycline and tigecycline (del Castillo 2013).

Since tetracyclines are the class of antibiotics with the highest use in veterinary medicine worldwide and they are first-line drugs in food animals (del Castillo 2013), it is not surprising that tetracycline resistance is widespread among LA-MRSA isolates. It is frequently mediated by the genes *tet*(K) and *tet*(L), which code for membrane-associated efflux proteins of the major facilitator superfamily (Schwarz et al. 2018; Roberts 1996). The *tet*(63) gene also encodes such an efflux protein, but has been reported only recently in LA-MRSA (Chen et al. 2023). Moreover, tetracycline resistance is often conferred by *tet*(M), which encodes a ribosome-protective protein (Schwarz et al. 2018; Roberts 1996). The genes *tet*(S) and *tet*(T) also code for ribosome-protective proteins, but have been reported in LA-MRSA by only one publication in each case so far (Jiang et al. 2019b; Moon et al. 2015). Previous studies have shown that among LA-MRSA of various origins *tet*(M), *tet*(K) and *tet*(L) were frequently present in different combinations in the same isolate (Schwarz et al. 2018). The *tet*(T) gene was identified together with *tet*(L) (Jiang et al. 2019b). The *tet*(38) gene can be detected in nearly every *S. aureus* genome, including phenotypically susceptible isolates. Therefore, its presence alone is not necessarily associated with phenotypic tetracycline resistance (Ogundipe et al. 2020; Truong-Bolduc et al. 2005). Although *tet*(38) was shown to be involved in low-level tetracycline resistance when overexpressed (Truong-Bolduc et al. 2005), this has not been demonstrated for LA-MRSA so far.

1.2.3 Resistance to phenicols

Non-fluorinated (e.g., chloramphenicol) or fluorinated (e.g., florfenicol) phenicols inhibit the bacterial protein biosynthesis via binding to the 50S ribosomal subunit, which results in an impaired peptide elongation (Dowling 2013b). The effect is usually bacteriostatic (Dowling 2013b).

In LA-MRSA, phenicol resistance can be based on active efflux via an export pump of the major facilitator superfamily encoded by the *fexA* gene (Schwarz et al. 2018; Kehrenberg and Schwarz 2004). In addition, phenicol resistance may be conferred by the genes *cfr*, *optrA* and *poxtA*, which are described in the section *1.2.7* on resistance to oxazolidinones.

1.2.4 Resistance to aminoglycosides and aminocyclitols

Aminoglycoside antibiotics, including aminoglycosides and aminocyclitols, have bactericidal activity when applied in sufficient concentrations (Dowling 2013a). They bind particularly at the 30S, but also at the 50S ribosomal subunit and interrupt the bacterial protein biosynthesis by inducing a misreading of the genetic code (Dowling 2013a). This leads to the production of abnormal cell membrane channels and, finally, cell death (Dowling 2013a). Different agents of this class interact with varying proteins, therefore extent and type of misreading differ (Dowling 2013a). For example, spectinomycin is often not bactericidal as it cannot induce misreading (Dowling 2013a). In addition, aminoglycoside antibiotics inhibit translation during protein synthesis, affect DNA metabolism, interfere with the cellular electron transport, induce ribonucleic acid (RNA) disintegration and damage the cell membrane (Dowling 2013a).

Several inactivating enzymes that vary in their substrate spectra provide resistance to aminoglycosides and aminocyclitols in LA-MRSA (Schwarz et al. 2018). The product of the gene *aacA-aphD* shows acetyltransferase as well as phosphotransferase activity and mediates resistance to the aminoglycosides gentamicin, kanamycin, tobramycin and, when overexpressed, amikacin (Schwarz et al. 2018; Rouch et al. 1987). The *aadD* gene codes for an adenyltransferase that provides resistance to the aminoglycosides kanamycin, neomycin and tobramycin (Schwarz et al. 2018). A phosphotransferase encoded by the *aphA3* gene confers kanamycin, neomycin and amikacin resistance (Schwarz et al. 2018; Boerlin et al. 2001; Derbise et al. 1996). The gene *aadE* mediates resistance to the aminoglycoside streptomycin via an adenyltransferase (Schwarz et al. 2018; Derbise et al. 1996). It is often located within a multiresistance gene cluster of enterococcal origin (Schwarz et al. 2018; Wendlandt et al. 2014). The gene *str* also codes for an adenyltransferase that confers streptomycin resistance (Overesch et al. 2011; Projan et al. 1988). The genes *spc*, *spw* and *spd* also encode adenyltransferases, but those provide resistance to the aminocyclitol spectinomycin (Schwarz et al. 2018; Jamrozy et al. 2014; Wendlandt et al. 2013b; Murphy 1985). The *spw* gene was identified to be part of the aforementioned multiresistance gene cluster together with *aadE*, as well as the lincosamide resistance gene *lnu*(B) and the pleuromutilin-lincosamide-streptogramin A (PLS_A) resistance gene *lsa*(E) (Schwarz et al. 2018; Wendlandt et al. 2014; Wendlandt et al. 2013b). The gene *apmA* mediates resistance to the aminocyclitol apramycin and reduced susceptibility to gentamicin via an acetyltransferase (Schwarz et al. 2018; Feßler et al. 2011).

1.2.5 Resistance to macrolides, lincosamides, and streptogramins

Macrolides, lincosamides and streptogramins share overlapping binding sites on the 23S ribosomal RNA (rRNA) of the 50S ribosomal subunit (Giguère 2013b). They interfere with bacterial protein biosynthesis by inhibiting the transpeptidation and translocation process, which results in the formation of incomplete polypeptide chains (Giguère 2013b). Macrolides generally have a bacteriostatic effect, but at increased concentrations and against a small number of very susceptible bacteria their activity can be bactericidal (Giguère 2013b). Lincosamides can be bacteriostatic or bactericidal, depending on the bacterial species, the bacterial load and the antibiotic concentration (Giguère 2013a). Streptogramins are divided into group A and B members, which have separate binding sites (Giguère 2013a). Group A streptogramins cause a conformational change that increases the ribosomal affinity for group B components and inhibit peptide bond formation during chain elongation (Giguère 2013a). Group B streptogramins induce the premature detachment of the incomplete polypeptides from the ribosome (Giguère 2013a). The A and B compounds have bacteriostatic activity when applied individually, but they are bactericidal in combination (Giguère 2013a).

Various genes coding for diverse mechanisms of resistance to macrolides, lincosamides and steptogramins including different substrate spectra have been described in LA-MRSA (Schwarz et al. 2018). Combined MLS_B resistance is encoded by *erm* genes, which can be present alone or in varying combinations in a single isolate and code for target site modifying methylases (Roberts 2022; Schwarz et al. 2018). Thereby, the MLS_B agents cannot bind to the A2058 position in the 23S rRNA of the bacterial ribosome (Schwarz et al. 2018). In LA-MRSA, *erm*(A), *erm*(B), *erm*(C), *erm*(T), and *erm*(33), which represents an *in vivo* recombination product between *erm*(A) and *erm*(C), have been described so far (Schwarz et al. 2018; Li et al. 2015; Schwarz et al. 2002). Furthermore, combined resistance to macrolides and streptogramin B is conferred via an ABC-F protein encoded by the *msr*(A) gene (Sharkey et al. 2016). ABC-F proteins seem to protect the bacterial ribosome from the activity of the antimicrobial compound (Sharkey et al. 2016). Moreover, resistance to lincosamides only is mediated by the genes *lnu*(A) and *lnu*(B), which both code for a lincosamide nucleotidyltransferase (Schwarz et al. 2018; Bozdogan et al. 1999; Brisson-Noël and Courvalin 1986). The *lnu*(B) gene was identified within a multiresistance gene cluster together with *lsa*(E), *aadE* and *spw* as described above (Schwarz et al. 2018; Wendlandt et al. 2014). In addition, resistance to lincosamides and streptogramin A may be conferred by the *cfr* gene that is described in the section *1.2.7* on resistance to oxazolidinones.

1.2.6 Resistance to pleuromutilins

Pleuromutilin antibiotics also share overlapping binding sites on the 50S subunit of the bacterial ribosome along with macrolides, lincosamides and streptogramins and, thus, inhibit protein biosynthesis (Giguère 2013a).

Combined PLS_A resistance in LA-MRSA is mediated by the genes *lsa*(E), *vga*(A) – including its variants –, *vga*(C), and *vga*(E), which code for ribosome-protective ABC-F proteins (Schwarz et al. 2018; Sharkey et al. 2016). The *lsa*(E) gene is part of the aforementioned multiresistance gene cluster of enterococcal origin together with *lnu*(B), *aadE* and *spw* (Schwarz et al. 2018; Wendlandt et al. 2014). Pleuromutilin resistance mediated by the *cfr* gene is addressed in the next section on resistance to oxazolidinones.

1.2.7 Resistance to oxazolidinones

Oxazolidinones interfere with bacterial protein biosynthesis by binding to the 23S rRNA of the 50S ribosomal subunit and inhibiting formation of a functional 70S initiation complex so that the translation process cannot begin (Diekema and Jones 2000). In general, these antimicrobial agents are not approved for the use in veterinary medicine. However, via country-specific cascade regulations the extralabel use by veterinarians in non-food-producing animals is possible, when in a certain case there is proof, e.g. via AST, that no other antimicrobial drug approved for the use in animals is effective against the causative bacterium (Committee for Medicinal Products for Veterinary Use 2018). There is great interest in the dissemination of oxazolidinone resistance mechanisms, in particular transferable ones, because oxazolidinones are last-resort antimicrobial drugs in human medicine (Schwarz et al. 2018). This means that they are primarily used to treat infections caused by multiresistant bacteria when there are no other treatment options (Schwarz et al. 2018).

In LA-MRSA, the transferable oxazolidinone resistance genes *cfr*, *optrA* and *poxtA* have been detected so far (Li et al. 2022; Li et al. 2018a; Schwarz et al. 2018). The *cfr*-encoded rRNA methylase modifies the A2503 target site of the oxazolidinones in the 23S rRNA (Kehrenberg et al. 2005; Schwarz et al. 2000). Since this position is located in the overlapping binding region of phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A, the *cfr* gene mediates multiresistance to these five classes of antimicrobial agents (Long et al. 2006). The *optrA* gene encodes a ribosome-protective ABC-F protein that confers resistance to oxazolidinones as well as fluorinated and non-fluorinated phenicols (Li et al. 2018a; Schwarz et al. 2018; Wang et al. 2015b). Previous studies also reported the *cfr* and *optrA* genes together in the same LA-MRSA isolates (Li et al. 2018a) and even on the same MGE (Li et al. 2022). The *poxtA* gene was found in LA-MRSA only recently, in a novel genetic context compared to its detection in other bacterial species (Li et al. 2022). It also codes for a

ribosome-protective ABC-F protein and was initially considered a phenicol-oxazolidinone-tetracycline resistance gene (Antonelli et al. 2018). However, a recent study demonstrated that *poxtA* only confers resistance to phenicols and oxazolidinones, but not to tetracyclines (Crowe-McAuliffe et al. 2022).

1.2.8 Resistance to trimethoprim

Trimethoprim belongs to the antibacterial diaminopyrimidines, which interrupt the production of folic acid by inhibition of the dihydrofolate reductase (Prescott 2013b). Without folic acid the bacteria cannot synthesize purines as central components of the DNA (Prescott 2013b). The greater affinity for the bacterial enzyme, compared to the mammalian one, ensures the selective antibacterial effect (Prescott 2013b). Due to a synergistic bactericidal effect, trimethoprim is in veterinary medicine invariably used in combination with sulfonamides, which inhibit the dihydropteroate synthetase and thus another earlier step of the folic acid biosynthesis (Prescott 2013b). However, in human medicine trimethoprim alone is preferred to combinations in certain cases (Prescott 2013b). Since the activity of diaminopyridines is 20 to 100 times higher than those of sulfonamides, the combined drug preparations contain trimethoprim and a sulfonamide in a fixed 1:5 ratio (Prescott 2013b). Taking pharmacokinetic considerations into account, this is regarded to result in the optimal 1:20 ratio at the site of infection (Prescott 2013b).

Trimethoprim resistance in LA-MRSA is based on trimethoprim-resistant dihydrofolate reductases encoded by the genes *dfrA* (also known as *dfrS1*), *dfrD*, *dfrG*, or *dfrK* (Schwarz et al. 2018). Few studies report the occurrence of *dfrA* in LA-MRSA (Argudín et al. 2011; Lozano et al. 2011). Moreover, there is only one study that reports the occurrence of *dfrD* in one LA-MRSA isolate, together with *dfrA* (Argudín et al. 2011). The genes *dfrG* and *dfrK* have been detected more frequently (Schwarz et al. 2018).

1.2.9 Resistance to fluoroquinolones

Bacterial topoisomerase enzymes, in particular the topoisomerase II – also known as DNA gyrase – and topoisomerase IV are the target structures of fluoroquinolones (Giguère and Dowling 2013). Topoisomerase II comprises two subunits encoded by the *gyrA* and *gyrB* genes and is essential for the dense packaging of the circular, double-stranded DNA double helix into a negative supercoil (Giguère and Dowling 2013). Binding of the antibiotic to a formed DNA gyrase-DNA complex leads to defects in the supercoiling, which results in disruption of the arranged DNA and decreased DNA repair (Giguère and Dowling 2013). Topoisomerase IV consists of *grlA* and *grlB* encoded subunits and mediates the unlinking of novel chromosomes at the end of DNA replication by DNA relaxation (Giguère and Dowling 2013). In summary, by

interfering with the topoisomerases the fluoroquinolones inhibit bacterial DNA replication (Giguère and Dowling 2013). They are normally bactericidal (Giguère and Dowling 2013).

Fluoroquinolone resistance in LA-MRSA is mainly mediated by mutations in the quinolone resistance-determining regions of *gyrA* and *grlA*, for example those resulting in the amino acid (aa) exchanges Ser84Leu, Ser84Ala or Ser84Val and Ser80Phe or Ser80Tyr, respectively (Yu et al. 2021; Hauschild et al. 2012). Moreover, mutations in the promoter region of the *norA* gene, which encodes a multidrug efflux pump, leading to an *norA* overexpression are involved in fluoroquinolone resistance in LA-MRSA (Hauschild et al. 2012).

1.2.10 Resistance to rifampicin

Rifampicin is a semisynthetic derivative of the natural rifamycins that has a bacteriostatic mode of action (Dowling 2013c). It interferes with bacterial RNA synthesis by inhibiting the RNA polymerase and blocking the initiation of transcription (Dowling 2013c).

The β-subunit of the bacterial RNA polymerase, with which rifampicin interferes, is encoded by the *rpoB* gene (Li et al. 2016; Aboshkiwa et al. 1995). Rifampicin-resistance mediating mutations within *rpoB* have been detected in LA-MRSA, for example at the positions Asp471Tyr, Ala473Glu, His481Asn, Ile527Met and Ser529Leu (Yu et al. 2021; Li et al. 2016). However, not all mutations have the same effect on the rifampicin MIC values and the presence of multiple mutations has occasionally been demonstrated to result in higher MICs compared to a single *rpoB* mutation (Li et al. 2016).

1.2.11 Resistance to vancomycin

Vancomycin is a glycopeptide antibiotic, currently only available as last-resort drug for the use in human medicine (Dowling 2013d). It interferes with bacterial cell wall peptidoglycan synthesis by inhibiting formation of glycan chains and subsequent transpeptidation. Thus, vancomycin has bactericidal activity against most Gram-positive species (Dowling 2013d).

In contrast to isolates from humans, vancomycin-resistant staphylococci have not been found in animals so far (Schwarz et al. 2018). However, LA-MRSA displaying reduced vancomycin susceptibility or an intermediate phenotype have been detected in swine, but they were lacking a consistent genetic marker for the low level resistance (Moreno et al. 2016; Kwok et al. 2013). An altered thickness of the bacterial cell wall might be responsible for this phenomenon (Moreno et al. 2016; Kwok et al. 2013).

1.2.12 Other resistances than AMR – Resistance to biocides and heavy metals

Chemical biocides have the ability to decrease the microbial burden on surfaces (Maillard 2018). Therefore, they are used to control microbial contamination and kill pathogens in a wide range of settings and applications (Maillard 2018). In health care settings, for example, biocides are widely used for the disinfection of environmental surfaces or medical devices and antisepsis, with the aim of preventing transmission of microorganisms to patients, staff and objects (Maillard 2018). They are also used in animal husbandry, for better hygiene in farm buildings and of equipment or vehicles (Maillard 2018). Biocides are a chemically diverse group and differ from antimicrobial agents in their often implied lack of target site specificity and selective toxicity (Denyer and Stewart 1998). The total damage to the multiple target sites caused by biocides usually leads to a bactericidal effect, however, self-destructive processes might also be initiated when the overall damage cannot be repaired or worsens (Denyer and Stewart 1998). For example, quaternary ammonium compounds (QACs) interfere with the integrity of the bacterial cytoplasmic membrane and the associated enzymes via electrostatic interaction with phospholipids leading to leakage of essential intracellular components, respiratory inhibition and intracellular material coagulation (Denyer and Stewart 1998).

Decreased susceptibility or resistance to QACs can be mediated for example by efflux pumps through which the biocide concentration is reduced sufficiently, so that it is no longer damaging to the bacterial cell (Maillard 2018). In LA-MRSA, several QAC resistance-mediating genes coding for efflux pumps of the major facilitator superfamily, such as *qacA/B*, *qacC/D*, *qacG*, *qacH*, and *qacJ*, or the small multidrug resistance family, such as *smr*, were identified (Boyce 2023; Schwarz et al. 2018). One study reported the frequent finding of the combination *qacG-qacH-smr* (Slifierz et al. 2015).

The metal compounds copper and zinc are widely used in livestock production (Rensing et al. 2018). On one hand, they are necessary supplements since they are essential trace elements and part of the animals' nutritional requirements (Rensing et al. 2018). On the other, they are also added to the animal feed for therapeutic use or to achieve additional advantages (Rensing et al. 2018). Copper may be added in cases of a deficiency, but it has also antimicrobial properties and has been used for growth promotion (Rensing et al. 2018). Zinc has been considered beneficial for growth promotion as well as the prevention and treatment of diarrhea in young animals (Rensing et al. 2018). Since the copper and zinc usage may also have adverse effects, upper limits have been set up to attenuate their direct impact along the food chain and on the environment (Wang et al. 2021; Rensing et al. 2018).

In some countries of the world, also derivatives from the nonessential metal arsenic have been used as feed supplements in food-producing animals to reduce parasitic infections and to promote growth (Rensing et al. 2018). Although arsenic derivatives are now banned in

the United States and the European Union due to their possible carcinogenicity, this kind of supplementation might be still ongoing in other countries (Rensing et al. 2018). Furthermore, animal feed can be contaminated with undesirable heavy metals from the environment and/or production processes, such as arsenic, mercury, cadmium and lead (Adamse et al. 2017). These contaminants can originate from natural geological sources or result from environmental pollution due to industrial and agricultural developments (Adamse et al. 2017). The contamination is of concern because these compounds have toxic properties and may be transferred to food of animal origin after consumption by the production animal (Adamse et al. 2017). In most parts of the world, there are official limits regulating the maximum presence of such contaminants in animal feed and feed materials (Adamse et al. 2017).

Bacterial cells balance the influx and efflux of metals via metal transport systems to ensure proper cell function but at the same time avoid metal toxicity (Rensing et al. 2018). In recent years, the characterization of bacteria showing reduced susceptibility or increased tolerance to heavy metals has gained increasing attention (Rensing et al. 2018). It is linked to the presence of efflux transporter systems or other enzymes involved in metal homeostasis, such as oxidases (Rensing et al. 2018). In LA-MRSA, various resistance determinants have been found, such as *cadD*/*cadX* (cadmium), *arsRBC* and *arsDARBC* (arsenic), *copB* (copper), *mco*/*copA* (copper), and *czrC* (cadmium/zinc) (Schwarz et al. 2018).

1.3 Spread of AMR among LA-MRSA via MGEs

AMRs continue to spread and diversify globally at an alarming pace (O'Neill 2014). They are considered to become the greatest threat to healthcare by the year 2050 (O'Neill 2014). Knowledge on how (multi)resistant bacteria, such as LA-MRSA, acquire and transmit AMR properties provides an essential contribution to avert the looming global crisis (O'Neill 2014). On one hand, bacteria pass on genetic information within their clonal lineage via vertical gene transfer, which is a rather slow mechanism (Lerminiaux and Cameron 2018). On the other hand, they can acquire genetic material independently from their ancestry and much faster through horizontal gene transfer (HGT) (Lerminiaux and Cameron 2018). Thus, bacteria can share a large pool of genes that may turn out as necessary for survival when facing selection pressures, such as the presence of antimicrobial agents (Lerminiaux and Cameron 2018). HGT is usually also faster than spontaneous mutations and can provide not only AMR genes, but also virulence genes that increase the pathogenicity or tenacity of an organism (Lerminiaux and Cameron 2018). Due to the extended possibilities for the exchange of genetic material and the increased pace of genome diversifications and potential fitness increases, HGT is a major driver of the rapid global spread of AMRs (Lerminiaux and Cameron 2018).

Three mechanisms are involved in HGT (**Figure 1**): Transduction, transformation and conjugation (Lerminiaux and Cameron 2018).

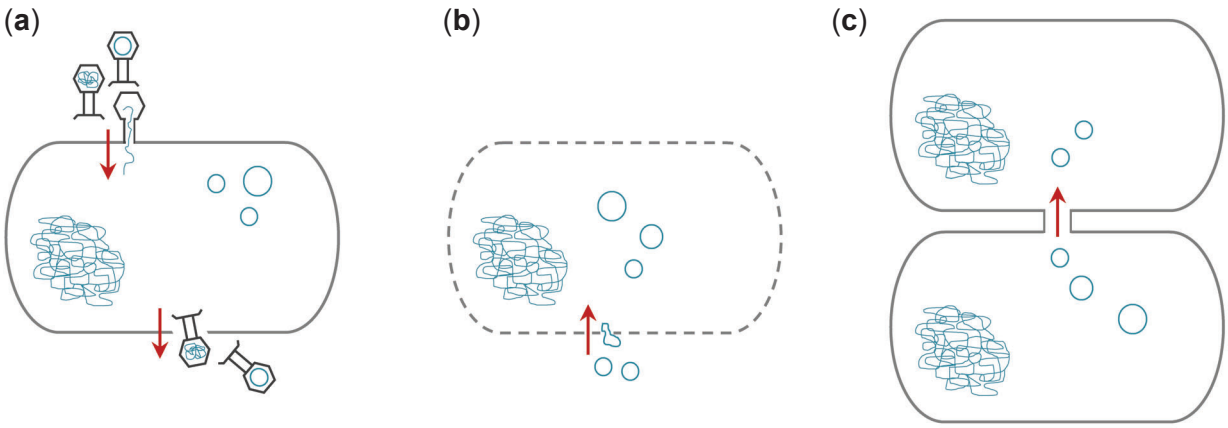

Figure 1. Schematic illustration of the HGT mechanisms among bacteria: (**a**) transduction, (**b**) transformation and (**c**) conjugation/mobilization.

Transduction (**Figure 1** (**a**)) describes the transfer of genetic material via bacteria-specific viruses, the bacteriophages (Lerminiaux and Cameron 2018). After infection of a bacterial cell, bacterial DNA may be additionally packaged into a newly formed bacteriophage during assembly (Lerminiaux and Cameron 2018). This bacteriophage can inject its DNA into another recipient cell and the transferred bacterial genes can be recombined into the recipient's genome (Lerminiaux and Cameron 2018). Transduction is limited by the size of the phage's head into which the genetic material is packaged and the requirement for specific receptors on the recipient cell (Schwarz et al. 2017). For this reason, only a limited amount of DNA, which is approximately 45,000 base pairs (bp) for staphylococci, can be transduced and this mainly happens between isolates of the same or closely related species (Schwarz et al. 2017). However, also extrachromosomal MGEs, such as plasmids, which are present in the cytoplasma, may be accidentally packaged into the bacteriophage heads during phage assembly (Schwarz and Noble 1999). The formed "pseudophages" can inject their plasmid DNA into new recipient cells as the regular bacteriophages but this does not result in the emergence of new bacteriophages because the "pseudophages" do not contain phage DNA (Schwarz and Noble 1999).

Transformation (**Figure 1** (**b**)) describes the uptake of extracellular DNA by competent bacteria (Lerminiaux and Cameron 2018). The genetic material is then recombined into the bacterial genome (Lerminiaux and Cameron 2018). Although transduction and transformation

are estimated to play a smaller role than conjugation in AMR dissemination, they are both important since this way chromosomally as well as MGE-encoded AMR genes can be transmitted (Lerminiaux and Cameron 2018). The presence of antimicrobials is suggested to increase the transfer rates of both mechanisms (Lerminiaux and Cameron 2018).

Conjugation (**Figure 1** (**c**)) describes the transfer of MGEs that carry genes for a conjugative system from a donor to a recipient cell mediated by cell-to-cell junctions and a pore (Thomas and Nielsen 2005). The *tra* gene complex encodes the transfer apparatus (Schwarz et al. 2017). It may also be used by non-conjugative MGEs that co-reside in the same donor cell to move to the recipient cell. This process is referred to as mobilization (Schwarz et al. 2017). Conjugation can be stimulated by the presence of antimicrobial agents, which can act as chemical signals modulating transcription of DNA transfer, DNA repair, and virulence genes, or indirectly modify cell wall composition or stimulate the expression of conjugative phenotypes (Lerminiaux and Cameron 2018). However, conjugation experiments have also shown that antimicrobials might reduce the conjugation potential by decreasing the number of sensitive recipients (Lerminiaux and Cameron 2018).

The term MGE refers to genetic elements that enable intracellular as well as intercellular DNA mobility (Partridge et al. 2018). They can be self-transmissible or dependent on the transfer via another MGE in whose genome they integrate (Partridge et al. 2018). Different MGE types can be distinguished, for example transposons, plasmids and genomic islands.

1.3.1 Transposons

A transposon (Tn) is a DNA sequence that can “jump” to different positions of the genome (Babakhani and Oloomi 2018). In bacteria, DNA transposons are divided into the four subgroups insertion sequence (IS), composite transposons, non-composite transposons, and transposing bacteriophages such as phage Mu (Babakhani and Oloomi 2018; Bennett 2008). They play a major role in the transmission of AMR in bacteria: Phages, composite and non-composite transposons commonly carry additional AMR genes, while insertion sequences can also be involved in composite transposons or influence AMR via their integration at specific target sites (Babakhani and Oloomi 2018).

Insertion sequences are the simplest and smallest type of transposons (Partridge et al. 2018). They usually carry only one, sometimes two, *tnp* gene(s) encoding transposase(s) for catalyzation of the insertion sequence movement (Partridge et al. 2018). Their transposition can occur conservatively by a “cut-and-paste” mechanism, where the insertion sequence is simply excised and inserted into its target site, or be replicative via a “copy-and-paste” or “copy-out-paste” mechanism (Partridge et al. 2018). The most common insertion sequence types are

flanked by inverted repeat (IR) sequences (Partridge et al. 2018). The transposase binds to the IRs and mediates the insertion of the insertion sequence into its target site (Partridge et al. 2018). Since cuts in the DNA occur during this process that need to be repaired, many insertion sequences generate flanking direct repeat (DR) sequences on insertion, also known as target site duplications (Partridge et al. 2018).

A composite transposon is, traditionally, a gene region flanked by two identical or related insertion sequences that can move as a whole (Partridge et al. 2018). Non-composite transposons also contain internal genes, including *tnp*, but are bounded by IRs instead of insertion sequences (Partridge et al. 2018). Transposing bacteriophages use transposition to replicate (Bennett 2008).

Several AMR genes found in LA-MRSA have been found to be associated with transposons. The *blaZ* gene was detected as part of the *blaZ-blaI-blaR1* operon on the non-composite Tn*552* (Rowland and Dyke 1989) in the chromosomal DNA and on plasmids (Schwarz et al. 2018; Lyon and Skurray 1987). Furthermore, the *tet*(M) gene is commonly found in the chromosomal DNA as part of conjugative transposons of enterococcal origin, such as the non-composite Tn*916* (Schwarz et al. 2018; Roberts 1996). The gene *fexA* is located on non-composite Tn*558* that has been detected in the chromosomal DNA or on (multi)resistance plasmids (Schwarz et al. 2018; Kehrenberg and Schwarz 2005). The *aacA-aphD* gene is part of composite Tn*4001*, whose AMR gene region is flanked by two IS*256* elements in opposite orientations (Schwarz et al. 2018; Byrne et al. 1989). The *spc* gene can be associated with non-composite Tn*554* together with *erm*(A), or non-composite Tn*6133* together with *erm*(A) and *vga*(E) (Schwarz et al. 2018; Schwendener and Perreten 2011). The *dfrK* gene can be part of non-composite Tn*559* (Schwarz et al. 2018; Kadlec and Schwarz 2010). The *cfr* gene that is regularly carried by plasmids, is frequently flanked by insertion sequences of different types, such as IS*21-558* or IS*Enfa4*, which can mediate the mobilization processes accounting for the spread of *cfr* (Brenciani et al. 2022).

1.3.2 Plasmids

Plasmids are small, extrachromosomal DNA molecules that can replicate independently of the chromosome (Bennett 2008). Instead of essential core genes they rather harbor genes that may be beneficial for niche adaptation (Bennett 2008). Thus, plasmids are important carriers of acquired AMR genes, and other MGEs, such as transposons or smaller plasmids, are also known to be integrated into larger plasmids (Partridge et al. 2018). Conjugative plasmids have the genetic equipment to mediate their own transfer and the transmission of other plasmids from one bacterial cell to the other via conjugation (Bennett 2008). They can also be integrated into the bacterial chromosome (Partridge et al. 2018).

Staphylococci have been reported to frequently carry one or more plasmids that mediate not only (multi)resistance to antimicrobial agents, but also resistance to heavy metals and/or biocides (Partridge et al. 2018).

Among LA-MRSA, several AMR genes are frequently located on plasmids, for example as part of transposons as described in the previous section, or independent from other MGEs. The genes *str*, *spd*, *erm*(C), and *lnu*(A) are commonly associated with small plasmids lacking additional resistance genes (Schwarz et al. 2018; Jamrozy et al. 2014; Lozano et al. 2012). The *tet*(K) gene has also often been identified on such small plasmids in staphylococci, thus very likely also in LA-MRSA, which can even be integrated into larger plasmids via IS*257* (Schwarz et al. 2018). Other AMR genes have been found, in part regularly, together with further resistance genes on large (multi)resistance plasmids in LA-MRSA, such as *tet*(L), *tet*(T), *tet*(63), *aadD*, *apmA*, *erm*(T), and *erm*(33) (Chen et al. 2023; Jiang et al. 2019b; Schwarz et al. 2018; Li et al. 2015; Wendlandt et al. 2013a; Feßler et al. 2011). The *apmA* gene was at least once also detected on a small plasmid without additional resistance genes (Kadlec et al. 2012a). Furthermore, *cfr* is most frequently carried by plasmids, which either harbor at least one AMR gene in addition to *cfr*, often *fexA* or an *erm* gene, or even several additional AMR determinants (Li et al. 2022; Li et al. 2018a; Li et al. 2015; Shen et al. 2013). The *optrA* gene has also been detected on a multiresistance plasmid together with *cfr* and other AMR genes (Li et al. 2022). Prior to the characterization of Tn*559*, *dfrK* was initially identified linked to *tet*(L) on multiresistance plasmids (Schwarz et al. 2018). Polymerase chain reaction (PCR) assays can be applied to distinguish between the presence of *dfrK* within Tn*559* and its linkage with *tet*(L) (Schwarz et al. 2018). Moreover, the aforementioned multiresistance gene cluster of enterococcal origin including the AMR genes *aadD*, *spw*, *lnu*(B,) and *lsa*(E), has not only been identified in the chromosomal DNA, but also on at least one plasmid (Schwarz et al. 2018). Considering resistance to biocides, QAC resistance mediating genes were found in large part on plasmids among staphylococci (Bjorland et al. 2005). There are no explicit reports available to date, but the respective genes found might therefore also in many cases be part of plasmids in LA-MRSA. Furthermore, the heavy metal resistance genes *cadD*/*cadX* and/or *mco*/*copA* were detected on multiresistance plasmids together with AMR genes and, in one study, also with a novel biofilm gene cluster (Feßler et al. 2017; Gómez-Sanz et al. 2013).

1.3.3 Genomic Islands – SCCmec elements

Genomic islands are segments of the bacterial chromosome that have been acquired by HGT (Partridge et al. 2018). They are often bounded by DRs and can be categorized based on their mediated phenotype, for example as resistance islands or pathogenicity islands

(Partridge et al. 2018). The SCC*mec* element can be considered a genomic island that is excised from the chromosome and may move horizontally via phage-mediated transfer (Partridge et al. 2018). Next to the methicillin-resistance determinant, which is part of a *mec* gene complex, SCC*mec* elements contain cassette chromosome recombinase (*ccr*) genes within a *ccr* gene complex (Partridge et al. 2018). The site-specific *ccr* genes mediate excision and integration of SCC*mec* at the 3' end of the *rlmH* gene, also known as *orfx*, in the core genome (Partridge et al. 2018). Three joining regions, between *ccr* and the chromosomal bounding region of the element (J1), between *mec* and *ccr* (J2), and between *rlmH* and *mec* (J3), commonly harbor AMR and heavy metal resistance genes that are frequently linked to co-integrated MGEs or remnants thereof (Partridge et al. 2018). So far, 15 highly diverse SCC*mec* types have been classified based on *mec* and *ccr* gene complex diversity (Wang et al. 2022; International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC) 2009). SCC*mec* subtypes are defined by the structure of the joining regions (International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC) 2009).

The SCC*mec* types commonly found among LA-MRSA CC398 and CC9 are given in sections *1.1.1* and *1.1.2*, respectively. Considering resistance genes among LA-MRSA, *cfr* has been found integrated into the SCC*mec* element (Li et al. 2015) and small *tet*(K)-carrying plasmids can be integrated into the cassettes via IS*431* (Schwarz et al. 2018). Several heavy metal resistance genes were also frequently identified as part of SCC*mec* elements, such as *cadD*/*cadX*, *arsRBC* and *arsDARBC*, *copA*, *copB*, and *czrC* (Wang et al. 2022; Schwarz et al. 2018).

1.4 LA-MRSA in the One Health context

Worldwide it has been recognized that the health of people, wild and domestic animals, plants, and ecosystems are closely linked. For this reason, a common "One Health" approach taking into account this connection is necessary to face future public health challenges (One Health High Level Expert Panel (OHHLEP) 2021). This certainly also applies to dealing with LA-MRSA as a frequently multiresistant opportunistic pathogen that can colonize and/or infect a wide variety of different host species worldwide, including humans.

The previous sections have shown that LA-MRSA harbor numerous different resistance genes and resistance-mediating mutations. Since they do not live in isolation, they share resistance genes with other bacterial species, also of human origin, which is illustrated in **Figure 2** (Schwarz et al. 2018). As has been demonstrated, these resistance properties are frequently linked to MGEs, and as such might be easily transferred via HGT across strain, species or even genus boundaries. Acquisition at different times and MGEs carrying several

resistance genes might explain the concurrent presence of multiple resistance genes that mediate the same resistance trait in the same isolate, such as the different *tet* or *erm* genes in various combinations (Schwarz et al. 2018).

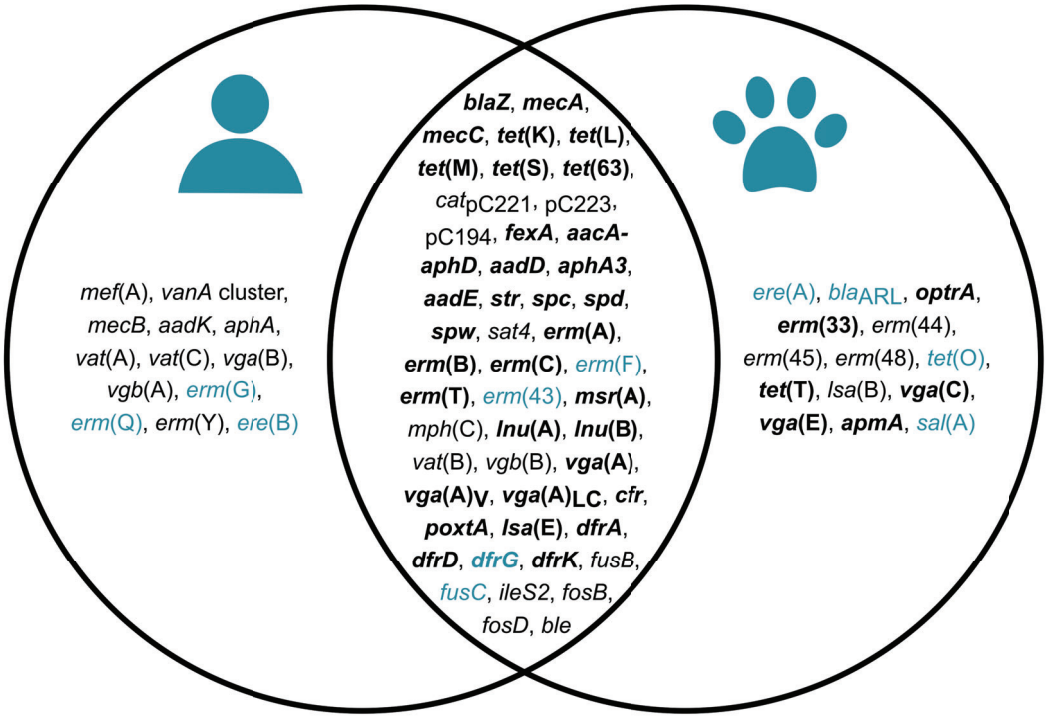

Figure 2. AMR genes present in staphylococci of humans (left), animals (right), and both origins (overlapping area in the middle) adapted from Schwarz et al. 2018. The genes displayed in black are associated with MGEs. The genes depicted in bold letters have been identified in LA-MRSA.

The use of antimicrobial agents contributes to the presence of AMR properties in LA-MRSA. The selection pressure affects not only the causative pathogens of a disease, but also the physiological microbiota. Thus, genes mediating AMR to agents applied to treat other than staphylococcal infections in livestock, such as apramycin or florfenicol, have also been detected in LA-MRSA (Schwarz et al. 2018). However, in addition to a direct selection pressure resulting in the establishment of specific resistance properties, also co-selection processes need to be considered (Schwarz et al. 2018). As a consequence, resistance genes can persist within a bacterial population even in the absence of a direct selection pressure (Schwarz et al. 2018). The reason for this can be a physical linkage of resistance genes due to a location on the same (multi)resistance MGE or the existence of resistance genes as part of multiresistance gene clusters that are commonly co-transferred (Schwarz et al. 2018). It is important to note that not only AMR genes, but also heavy metal or biocide resistance genes on the same MGE, such as a SCC*mec* cassette or multiresistance plasmid, might add to these co-selection events

(Schwarz et al. 2018). As a consequence, the ban or limitation of the application of specific antimicrobial agents, disinfectants or heavy metals might not lead to the intended loss of certain resistance properties (Schwarz et al. 2018). Furthermore, previous analyses of the resistance genes among LA-MRSA, including their genetic environment as well as the possible MGE carrying them, already pointed towards gene exchange events among Gram-positive bacterial species (Schwarz et al. 2018).

Finally, LA-MRSA lineages continue to diversify and the ongoing evolution might result in the emergence of novel even more resistant and/or virulent variants, possibly posing a threat to veterinary as well as human medicine (Butaye et al. 2016). Therefore, a comprehensive investigation and surveillance of the known epidemic LA-MRSA lineages is important, including the detailed analysis of detected (multi)resistance mediating MGEs in order to understand how resistance properties persist within bacterial populations.

1.5 Aims of the present study

The reasons for the success of two different porcine LA-MRSA lineages in Germany (CC398) and China (CC9) were unknown. Therefore, the main objective of a German-Chinese joint project was to understand factors that could have led to the development of different epidemic LA-MRSA lineages in pigs in the two countries. In particular, the aims of the present study were to

I. investigate whether the respective dominant LA-MRSA lineages could have had an advantage in host colonization due to certain metabolic properties compared to the non-successful clones within the same CC (**Publication I**),
II. understand population dynamics of the current epidemic LA-MRSA lineage in pigs in Germany (CC398) over time (**Publication II**), and
III. analyze novel MGEs and/or AMR genes detected within the German and Chinese porcine LA-MRSA isolate collection (**Publications III – VI**).

Porcine LA-MRSA CC398 and CC9 isolates from China and Germany were subjected to comparative pheno- and genotypic studies within the framework of the joint project. Lead of the work packages was divided between the project partners from Germany and China, respectively, and the tasks were accomplished in close cooperation. The following work was carried out in order to achieve the goals of the present study. First, a representative selection of 20 whole-genome-sequenced isolates was investigated for metabolic variations applying Biolog Phenotype MicroArray™ (PM) technology, a microtiter plate system that allows the parallel testing for bacterial growth responses to various nutrients (**Publication I**). In addition,

178 whole-genome sequenced CC398 isolates from Germany were characterized comprehensively (**Publication II**). They were subjected to molecular typing and phylogenetic analysis, and investigated for MGEs, virulence and resistance properties. Furthermore, the nucleotide sequence of a SCC*mec* cassette in a ST398 isolate from China that differed from all those known so far was analyzed in detail (**Publication III**). Moreover, the nucleotide sequences of two new members of the Tn*554* family were characterized and their functional activity was investigated searching for their circular forms. One new transposon originated from a Chinese ST9 isolate (**Publication IV**). The other novel transposon was found in a Chinese ST398 isolate and also harbored a novel variant of the *spc* gene, which was studied further (**Publication V**). Lastly, a novel *erm* gene located on a plasmid in a ST398 isolate from Germany was characterized, including its genetic environment (**Publication VI**). The gene's transferability and functionality were also investigated and a specific PCR-based detection method was developed.

2 Publications

The contribution of Henrike Krüger-Haker to the following articles was evaluated according to the following scheme:

A: Has contributed (0 – 33%)
B: Has contributed significantly (34 – 66%)
C: Has worked essentially independent (67 – 100%)

2.1 Publication I

Krüger-Haker H, Ji X, Bartel A, Feßler AT, Hanke D, Jiang N, Tedin K, Maurischat S, Wang Y, Wu C, Schwarz S

Metabolic Characteristics of Porcine LA-MRSA CC398 and CC9 Isolates from Germany and China via Biolog Phenotype MicroArray™

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Authors' contributions:

1. **Conceptualization**, K-HH, WY, WC, FAT, SS — B
2. **Methodology**, K-HH, BA, FAT, TK, SS — B
3. **Validation**, K-HH, BA, FAT, SS — B
4. **Formal Analysis**, K-HH, BA, FAT, HD — B
5. **Investigation**, K-HH, FAT — C
6. **Resources**, JX, JN, TK, MS, WY, WC, SS
7. **Data Curation**, K-HH, JX, BA, FAT, HD — C
8. **Writing – Original Draft Preparation**, K-HH, FAT, SS — C
9. **Writing – Review & Editing**, K-HH, JX, BA, FAT, HD, JN, TK, MS, WY, WC, SS — B
10. **Visualization**, K-HH, BA, FAT — C
11. **Supervision**, FAT, WY, SS
12. **Project Administration**, WY, WC, SS
13. **Funding Acquisition**, WY, WC, SS

All authors have read and agreed to the published version of the manuscript.

microorganisms

MDPI

Article

Metabolic Characteristics of Porcine LA-MRSA CC398 and CC9 Isolates from Germany and China via Biolog Phenotype MicroArray™

Henrike Krüger-Haker [1,2], Xing Ji [3], Alexander Bartel [4], Andrea T. Feßler [1,2,*], Dennis Hanke [1,2], Nansong Jiang [5], Karsten Tedin [1,2], Sven Maurischat [6], Yang Wang [5], Congming Wu [5] and Stefan Schwarz [1,2,5]

1 Institute of Microbiology and Epizootics, Department of Veterinary Medicine, Freie Universität Berlin, 14163 Berlin, Germany
2 Veterinary Centre for Resistance Research (TZR), Freie Universität Berlin, 14163 Berlin, Germany
3 Jiangsu Key Laboratory for Food Quality and Safety, State Key Laboratory, Cultivation Base of Ministry of Science and Technology, Institute of Food Safety and Nutrition, Jiangsu Academy of Agricultural Sciences, Nanjing 210000, China
4 Institute for Veterinary Epidemiology and Biostatistics, Department of Veterinary Medicine, Freie Universität Berlin, 14163 Berlin, Germany
5 Key Laboratory of Animal Antimicrobial Resistance Surveillance, MARA, College of Veterinary Medicine, China Agricultural University, Beijing 100193, China
6 Department Biological Safety, German Federal Institute for Risk Assessment (BfR), 10589 Berlin, Germany
* Correspondence: andrea.fessler@fu-berlin.de; Tel.: +49-30-838-63074

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Abstract: Livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) is an important zoonotic pathogen, often multi-resistant to antimicrobial agents. Among swine, LA-MRSA of clonal complex (CC) 398 dominates in Europe, Australia and the Americas, while LA-MRSA-CC9 is the main epidemic lineage in Asia. Here, we comparatively investigated the metabolic properties of rare and widespread porcine LA-MRSA isolates from Germany and China using Biolog Phenotype MicroArray technology to evaluate if metabolic variations could have played a role in the development of two different epidemic LA-MRSA clones in swine. Overall, we were able to characterize the isolates' metabolic profiles and show their tolerance to varying environmental conditions. Sparse partial least squares discriminant analysis (sPLS-DA) supported the detection of the most informative substrates and/or conditions that revealed metabolic differences between the LA-MRSA lineages. The Chinese LA-MRSA-CC9 isolates displayed unique characteristics, such as a consistently delayed onset of cellular respiration, and increased, reduced or absent usage of several nutrients. These possibly unfavorable metabolic properties might promote the ongoing gradual replacement of the current epidemic LA-MRSA-CC9 clone in China with the emerging LA-MRSA-CC398 lineage through livestock trade and occupational exposure. Due to the enhanced pathogenicity of the LA-MRSA-CC398 clone, the public health risk posed by LA-MRSA from swine might increase further.

Keywords: *Staphylococcus aureus*; metabolic properties; area under the curve (AUC); sparse partial least squares discriminant analysis (sPLS-DA); whole-genome sequencing (WGS)

1. Introduction

Livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) has shown an increase in food-producing animals since the mid-2000s [1]. This MRSA variant was first recognized in pigs [2,3] and later in other livestock, such as cattle or poultry [1]. LA-MRSA are considered a matter of public health concern since such isolates are often multi-resistant to antimicrobial agents and can easily cross species barriers [1,4]. Several reports on LA-MRSA transmission from animals to humans point out the increased risk of also becoming MRSA carriers for persons in close contact with LA-MRSA-positive animals [1,4–7]. Healthy, colonized pigs represent one of the main reservoirs for LA-MRSA [5]. Such isolates only

rarely cause diseases in swine ranging from skin infections to pneumonia or septicemia [8]. However, LA-MRSA can also cause a broad spectrum of mild to severe infections in humans although outbreaks have been reported only sporadically [4–6].

Detailed molecular analyses revealed that LA-MRSA colonizing pigs in Europe, Australia as well as North and South America belong predominantly to the clonal complex (CC) 398 [8–10]. In contrast, CC9 was identified as the dominant pig-associated clonal lineage in most Asian countries, particularly in China [11–13]. In line with this, LA-MRSA CC9 isolates in European countries and LA-MRSA CC398 isolates in China were only rarely isolated from swine [9,14]. However, reports of LA-MRSA CC398 isolates from China have recently increased [15–17]. Previous studies on LA-MRSA CC398 and CC9 mainly investigated host adaptation and pathogenicity of the isolates or focused on genetic analyses and epidemiological relationships [8–10,16–22]. Although prior studies have helped to clarify the impact of genomic characteristics, fitness and virulence of LA-MRSA CC398 and CC9 isolated from China and Germany on the development of two pig-associated epidemiologically successful LA-MRSA clones in different regions in a comparative study [23], some questions remain unanswered.

Biolog Phenotype MicroArray^{TM} (PM) technology for microbial cells offers a high-throughput method for the extensive analysis of cellular phenotypes [24,25]. Responses of a microbial community or an individual isolate to numerous classes of chemical compounds can be measured using a colorimetric redox reaction that mirrors the extent of cellular respiration. By investigation of susceptibility to antimicrobial agents and metabolic pathways along with ionic, osmotic as well as pH effects, the metabolic and chemical sensitivity properties of microbial cells can be determined. The Biolog PM system has been widely used in a variety of fields to gain an overview of a microbial community's metabolic profile or to study specific gene functions by detecting phenotypic changes associated with gene knockouts [25,26]. The *S. aureus* small-colony variants (SCVs), for example, are increasingly found in antibiotic-refractory as well as recurrent infections and are known to exhibit several phenotypic changes [27]. Von Eiff et al. conducted Biolog PMs of SCV mutants to define metabolic variations between SCVs and wild-type *S. aureus* more precisely, revealing defects in ATP generation via electron transport due to defects in carbon metabolism and proving growth of SCV mutants utilizing carbon sources that provide ATP independently from electron transport [27]. Another study focused on phenotypic characterization of *S. aureus sarR* mutant isolates [28]. The staphylococcal specific *sar* family genes control the expression of factors associated with virulence, antimicrobial resistance and survival under adverse conditions [28]. Biolog PM analyses allowed identification of altered utilization of multiple substrates, which suggested that *sarR* might play a role in controlling cell wall or membrane related functions [28]. Furthermore, Marchi et al. performed Biolog PM studies to investigate antimicrobial resistance and susceptibility phenotypes associated with isolates harboring mutations up-regulating different efflux pumps [29]. They identified compounds to which the mutant isolates showed enhanced susceptibility, suggesting potential opportunities to overcome the antimicrobial resistance problem [29]. Most recently, Vaillant et al. investigated the use of the Biolog PM system for antimicrobial susceptibility testing in comparison to broth microdilution using clinical *S. aureus* isolates [30]. Despite these prior applications regarding the connections between virulence, antimicrobial resistance and metabolic traits in *S. aureus*, to the best of the authors' knowledge, Biolog PM analyses have not been used to characterize LA-MRSA isolates.

In this study, we examined a representative collection of whole-genome sequenced LA-MRSA CC398 and CC9 isolates of swine from Germany and China for possible differences in their metabolic properties applying Biolog PM technology to evaluate whether metabolic variations could have played a role in the development of two different epidemic LA-MRSA clones in swine.

2. Materials and Methods

2.1. Selection of Representative Bacterial Isolates

In total, 20 whole-genome sequenced porcine LA-MRSA isolates were chosen as representative test collection, including five dominant MRSA-CC398 from Germany (GER-MRSA-CC398) and five dominant MRSA-CC9 from China (CHN-MRSA-CC9) as well as five rare MRSA-CC9 from Germany (GER-MRSA-CC9) and five rare MRSA-CC398 from China (CHN-MRSA-CC398). Their characteristics are displayed in Table 1. All isolates were obtained from swine during the period between 2004 and 2017 in various regions of Germany and China [23]. As only the five CHN-MRSA-CC398 isolates investigated were available to us during the study period, the other three groups were set up to be comparable in isolate number. Here, independent isolates were selected based on preferably diverse pheno- and genotypic antimicrobial resistance profiles. The antimicrobial susceptibility testing data were obtained in a previous study [23]. The 15 antimicrobial agents tested included cefoxitin, tetracycline, minocycline, tigecycline, erythromycin, clindamycin, virginiamycin M1, gentamicin, ciprofloxacin, florfenicol, tiamulin, trimethoprim, linezolid, vancomycin and fusidic acid. Furthermore, preliminary Biolog PM experiments confirmed, in general, a uniform metabolic behavior of the isolates within each of the four groups. Therefore, the selected isolates were considered as representative for the respective LA-MRSA category.

2.2. Whole-Genome Sequencing, Assembly and Annotation

Genomic DNA was extracted using a HiPure Bacterial DNA Kit (Magen, Guangzhou, China). The libraries were prepared using the KAPA Hyper Prep Kit (Kapa Biosystems, Boston, MA, USA) and sequencing was performed on the Illumina HiSeq X-Ten System (Annoroad Genomics Co., Beijing, China). For each isolate, 300-bp paired-end reads with a minimum of 250-fold coverage were obtained. SPAdes (version 3.12.0) [35] was used for assembly of the DNA sequence reads, which were then run through an automatic annotation pipeline via RAST [36]. The whole-genome sequences of the 20 MRSA isolates included in this study have been deposited at GenBank under the accession numbers JAMYYK000000000, JAMYYJ000000000, JAMYYI000000000, JAMYYH000000000, JAMYYG000000000, JAMYYF000000000, JAMYYE000000000, JAMYYD000000000, JAMYYC000000000, JAMYYB000000000, JAMYYA000000000, JAMYXZ000000000, JAMYXY000000000, JAMYXX000000000, JAMYXW000000000, JAMYXV000000000, JAMYXU000000000, JAMYXT000000000, JAMYXS000000000, and JAMYXR000000000 (see also the Data Availability section).

2.3. Molecular and Phylogenetic Analyses

Known genes conferring antimicrobial resistance were located by directly mapping the DNA sequence reads against the ResFinder database [37] applying a procedure implemented in the SRST2 tool [38]. The contigs generated with SPAdes were also checked for the respective genes using AMRFinderPlus [39]. Results obtained from both databases were compared and verified using Geneious v11.1.4 (Biomatters Ltd., Auckland, New Zealand). Moreover, chromosomal point mutations associated with antimicrobial resistance were located applying PointFinder [40], which is implemented in the ResFinder tool [37] of the Center for Genomic Epidemiology (CGE). Multilocus sequence typing (MLST), *spa* typing and SCC*mec* typing were carried out using the MLST 2.0 [41], *spa*Typer 1.0 [42] and SCC*mec*Finder 1.2 [43] CGE online analysis tools. The *dru* types were determined from the dru-typing.org database [44] by using the blast function in Geneious v11.1.4. Ridom SeqSphere+ (version 7.5.5) [45] was used for phylogenetic analysis via the *S. aureus* core genome MLST (cgMLST) approach [46]. In order to illustrate the clonal relationship between the representative isolates, a minimum spanning tree was built based on a distance matrix of the core genome allelic profiles including 1749 of 1861 possible target genes by removing 112 columns with missing values from the comparison table.

Table 1. Characteristics of the representative bacterial isolates of swine origin selected for Biolog PM assays.

ID	Year	MLST	*spa*	*dru*	SCC*mec*	Resistance Phenotype [1]	Resistance Genotype [2]
CHN-MRSA-CC9							
DL44	2009	ST9	t899	dt12w	XII(9C2)	bla, tet, ery, cli, gen, cip, ffn, tia, tmp	*mecA*, *blaZ* (2x), *tet*(L), *erm*(C), *lnu*(B), *lsa*(E), *aacA-aphD*, *aadD*, *aadE*, *spw*, *cat(pC221)*, *fexA*, *dfrG* [3,4]
QDT9	2014	ST9	t899	dt12w	XII(9C2)	bla, tet, ery, cli, gen, cip, ffn, tia, tmp	*mecA*, *blaZ* (2x), *tet*(L), *erm*(C), *lnu*(B), *lsa*(E), *aacA-aphD*, *aadD*, *aadE*, *spw*, *fexA*, *dfrG* [3,4]
PNB35	2009	ST9	t899	dt12w	XII(9C2)	bla, tet, ery, cli, gen, cip, ffn, tia, tmp	*mecA*, *blaZ*, *tet*(L), *lnu*(B), *lsa*(E), *aadD*, *aadE*, *spw*, *fexA*, *dfrG* [3,4]
SF30	2013	ST9	t899	dt8av	XII(9C2)	bla, tet, ery, cli, gen, cip, ffn, tia, tmp	*mecA*, *blaZ* (2x), *tet*(L), *lnu*(B), *lsa*(E), *aadD*, *aadE*, *spw*, *fexA*, *dfrG* [3,4]
DY82	2014	ST9	t1939	dt10du	XII(9C2)	bla, tet, ery, cli, gen, cip, ffn, tia, tmp	*mecA*, *blaZ*, *tet*(K), *tet*(L), *erm*(C), *lnu*(B), *lsa*(E), *aacA-aphD*, *aadD*, *aadE*, *spw*, *str*, *fexA*, *dfrG* [4]
CHN-MRSA-CC398							
GDC1 [5]	2016	ST398	t034	dt11ax	Vc(5C2&5)	bla, tet, ery, cli, ffn, tia, tmp	*mecA*, *blaZ*, *tet*(K), *tet*(M), *erm*(C), *lnu*(B), *lsa*(E), *spc*, *fexA*, *dfrG*, *cfr*
SHP1 [5]	2016	ST398	t571	dt9bw	V(5C2)	bla, tet, ery, cli, gen, cip, tia, tmp	*mecA*, *blaZ* (2x), *tet*(L), *tet*(M), *erm*(T), *lnu*(B), *lsa*(E), *aacA-aphD*, *aadD*, *aadE*, *spw*, *fexA*, *dfrG* [3]
YN471	2017	ST398	t034	dt6j	Vc(5C2&5)	bla, tet, ery, cli, tia, tmp	*mecA*, *blaZ*, *tet*(K), *tet*(M), *erm*(A), *vga*(E), *spc*, *dfrG*
YN523	2017	ST398	t034	dt6j	Vc(5C2&5)	bla, tet, ery, cli, tia, tmp	*mecA*, *blaZ*, *tet*(K), *tet*(M), *erm*(A), *vga*(E), *spc*, *dfrG*
YN502	2017	ST398	t034	dt6j	Vc(5C2&5)	bla, tet, ery, cli, tia, tmp	*mecA*, *blaZ*, *tet*(K), *tet*(M), *erm*(A), *erm*(C), *vga*(E), *spc*, *dfrG*
GER-MRSA-CC9							
DG34	2015	ST9	t15199	dt10a	IV(2B)	bla, cip	*mecA*, *blaZ*, *str* [3,4]
DG36	2015	ST9	t1430	dt10a	IV(2B)	bla, cip	*mecA*, *blaZ*, *str* [3,4]
DG37	2015	ST9	t1430	dt10a	IV(2B)	bla, cli, cip	*mecA*, *blaZ*, *str* [3,4]
DG39	2015	ST9	t1430	dt10a	IV(2B)	bla, tet, ery, cli, cip, tmp	*mecA*, *blaZ*, *tet*(L), *erm*(B), *aadD*, *dfrK* [3,4]
DG41	2017	ST9	t1430	dt10a	IV(2B)	bla, cli, cip	*mecA*, *blaZ*,*str* [3,4]
GER-MRSA-CC398							
DG4	2008	ST398	t011	dt11a	Vc(5C2&5)	bla, tet	*mecA*, *blaZ*, *tet*(K), *tet*(M), *str*
DG9	2008	ST398	t011	dt11a	Vc(5C2&5)	bla, tet, tmp	*mecA*, *blaZ*, *tet*(K), *tet*(L), *tet*(M), *str*, *dfrK*
DG12	2008	ST398	t011	dt11a	Vc(5C2&5)	bla, tet, ery, cli, gen, tmp	*mecA*, *blaZ*, *tet*(K), *tet*(M), *erm*(C), *str*, *dfrG*
DG17	2008	ST398	t011	dt11a	Vc(5C2&5)	bla, tet, ery, cli, cip	*mecA*, *blaZ*, *tet*(K), *tet*(M), *erm*(C), *str* [3]
DG29	2004	ST398	t011	dt11a	Vc(5C2&5)	bla, tet, cli, gen	*mecA*, *blaZ*, *tet*(K), *tet*(M), *aacA-aphD*, *str*

[1] bla, β-lactam antibiotics; tet, tetracycline; ery, erythromycin; cli, clindamycin; gen, gentamicin; cip, ciprofloxacin; ffn, florfenicol; tia, tiamulin; tmp, trimethoprim. Despite the lack of clinical breakpoints approved by the Clinical and Laboratory Standards Institute (CLSI), isolates that showed high minimal inhibitory concentration values for ffn (≥32 mg/L) and tia (≥16 mg/L) were considered as resistant. [2] All isolates carried the gene *tet*(38), which may confer resistance to tetracycline when overexpressed [31]. Its presence alone is not associated with tetracycline resistance, because it can be found in nearly every *S. aureus* genome including phenotypically susceptible isolates. [3] These isolates harbored fluoroquinolone resistance-mediating point mutations in *gyrA* and *grlA* (S84A/S80F—DL44, QDT9, PNB35, SF30; S84L/S80Y—SHP1; S84L/S80F—DG34, DG36, DG37, DG39, DG41, DG17) [32,33]. [4] These isolates harbored the quinolone resistance-mediating type A mutation in the *norA* promoter region [34]. [5] GDC1 corresponds to GDC6P096P and SHP1 corresponds to SHP6P021P in a previous study [23].

2.4. Biolog PM Assay Performance and Data Evaluation

Materials, chemicals, instrumentation and consumables for the Biolog PM assays were obtained from Biolog Inc., Hayward, CA, USA. The Biolog PM assays were conducted using the 96-well PM1 (carbon utilization), PM2A (carbon utilization), PM9 (osmotic and ionic effects), and PM10 (pH effects) microplates, which represent different metabolic capabilities and/or growth conditions [24]. The experiments were carried out at least in independent duplicates according to the manufacturer's PM procedures for Gram-positive bacteria with slight modifications of the incubation conditions. Bacteria from the frozen stock cultures

were grown overnight at 37 °C on BUG + B (Biolog Universal Growth Medium + 5% sheep blood) agar plates. After subculturing for a second time, bacterial cells were removed from the BUG + B plate and transferred into sterile IF-0a GN/GP Base fluid. Uniform cell suspensions with a final cell density of 81% T (transmittance) were combined with the respective inoculating fluid depending on the microplate to be used, according to the manufacturer's recommendations. The recipes varied slightly in the composition of IF-0a GN/GP Base and IF-10b GN/GP Base fluid, the redox dye tetrazolium violet (Dye H), and different additive solutions. The Biolog PM microplates were inoculated with the final cell suspension (100 µL/well) and incubated at 37 °C in the OmniLog incubator. The OmniLog PM system (OmniLog Data Collection software version 2.3.01) recorded the colorimetric data every 15 min for 48 h. In order to detect abiotic "false-positive" reactions, an additional set of control Biolog PM microplates (PM1, PM2A, PM9, PM10) was incubated using the same assay protocol described above but without the bacterial cells.

The final kinetic Biolog PM data were processed and evaluated using OmniLog Data File Converter, OmniLog FM (version 1.20.02), OmniLog PM (version 1.20.02), and Biolog Data Analysis (version 1.7.1.51) software. Kinetic graphs obtained from the colorimetric reaction in each well were designated as "respiration curves" as they display the bacterial cells' respiration over time. Respiration curves obtained after 24 and 48 h of incubation were compared visually between the four investigated groups. Moreover, metabolic substrate utilization was quantified via the parameters: area under the curve (AUC), lag time (LT), maximum height (MH), plateau time (PT) and slope of the respiration curves. Two statistical approaches were applied to obtain possible selections of the most informative substrates and/or conditions tested. Firstly, sparse partial least squares discriminant analysis (sPLS-DA) [47] was employed considering two independent AUC measurements obtained after 24 h of incubation for each isolate and all wells/conditions investigated. sPLS-DA provides variable selection (Lasso) for datasets with a large number of highly correlated variables [47]. The optimal number of selected variables was determined using 10-fold cross validation. This approach revealed that four components containing 30 of the 379 substrates and/or conditions provided optimal discriminatory power to differentiate the four MRSA groups. Components combine similarly behaving variables into groups (instrumental variables). This aids in selecting variables according to recurring patterns and reduces the risk of selecting variables based on randomly occurring differences in 'omics datasets. Based on the selected variables, a heatmap was generated consisting of one row per isolate and one column per substrate. The colored tiles represent the average z-score for each isolate and substrate combination. A hierarchically clustered tree was generated using the Euclidian distances between isolate (rows) and substrate (columns) z-scores. Secondly, sPLS-DA [47] analysis was carried out for each of the parameters LT, MH, PT and slope considering independent duplicate measurements obtained after 24 h of incubation for each isolate and all wells investigated. Parameters were ranked according to variable importance in each of the analysis for every parameter. Here, 40 of the 379 substrates and/or conditions tested were selected based on the lowest combined rank between all four analyses. This combined sPLS-DA variable selection was performed since AUC values can differ due to varying reasons, such as clearly different curves or just minor changes in the maximum height. This analysis favors variables with differences in respiration curves in more than one curve parameter. All of the statistical analyses were performed using the R package mixOmics (version 6.18.1) [48] and R version 4.1.3 (R Foundation for Statistical Computing, Vienna, Austria).

3. Results and Discussion

3.1. Molecular and Phylogenetic Analyses

Molecular typing results and antimicrobial resistance data are displayed in Table 1. All porcine LA-MRSA from Germany and China displayed *spa* and SCC*mec* types that are typically associated with CC9 and CC398, respectively [8,21,49]. An exception is *spa* type t571, which was found in isolate SHP1 and has so far rarely been reported in MRSA

sequence type (ST) 398 isolates [50,51]. However, a recent study has shown that swine-associated MRSA ST398 circulating in a slaughterhouse in China were predominantly associated with *spa* type t571 [17]. The methicillin-susceptible *S. aureus* (MSSA) t571 clonal lineage also predominated in swine, human, and environment-associated isolates [17]. Moreover, MSSA of ST398 and *spa* type t571 have been reported from human infections in Belgium [52], China [53], Colombia [54], France [55,56], Germany [51], the Netherlands [50], and the United States [57]. It is worth noting that several of the affected patients had no or only indirect contact with livestock prior to infection. Interestingly, *spa* type t571 harbors only one repeat (r24) less than type t034, which is commonly detected in MRSA-CC398.

The antimicrobial resistance data were used for selection of representative isolates for Biolog PM assays as described above. In general, antimicrobial resistance patterns of LA-MRSA from China were more complex than those of LA-MRSA from Germany (Table 1). All tested isolates harbored several antimicrobial resistance genes, which differed in number and distribution among the isolates. The Chinese isolates displayed higher numbers of antimicrobial resistance genes, especially the CHN-MRSA-CC9. As MRSA, all isolates were resistant to beta-lactams and carried the *mecA* gene as well as at least one copy of the *blaZ* gene. In addition, all CHN-MRSA-CC9 and -CC398 were resistant to tetracycline, erythromycin, clindamycin and tiamulin. All GER-MRSA-CC398 furthermore revealed tetracycline resistance. However, all isolates were susceptible to linezolid and vancomycin and the German isolates additionally to florfenicol and tiamulin. The isolates' resistance profiles mirror the selection pressure imposed by antimicrobial agents commonly used in the pig industry. Usage of tetracyclines, tiamulin, florfenicol, as well as macrolides, lincosamides and streptogramins (MLS) is associated with selection of multi-resistant isolates in China [58]. In Germany, MRSA isolates have adapted to selection pressure imposed by tetracyclines by acquiring the respective antimicrobial resistance genes [59].

The cgMLST analysis revealed two different clusters, in accordance with the MLST and CC assignments (Table 1), and 20 varying allelic profiles for the 20 MRSA isolates (Figure 1, Table S1). Cluster 1 comprised the CC398 isolates and included five rather closely related allelic profiles each from Germany and China. The GER-MRSA-CC398 differed in 14 to 42 and the CHN-MRSA-CC398 in 8 to 241 target genes (Table S1). The two most closely related German (DG29) and Chinese (YN523) CC398 isolates varied in 103 target genes. Cluster 2 represented the CC9 sequences, also assigned to five more closely related allelic profiles each from Germany and China. The GER-MRSA-CC9 varied in 25 to 41 and the CHN-MRSA-CC9 in 43 to 108 target genes (Table S1). The two most closely related German (DG39) and Chinese (DL44) MRSA-CC9 differed in 191 target genes. According to a previous study on the phylogenetic analysis of three MRSA outbreaks applying the SeqSphere+ cgMLST approach, *S. aureus* isolates displaying 0 to 8 allelic differences should be considered as related; isolates with 9 to 29 allelic differences as possibly related; and those with $\geq$30 allelic variations as unrelated [60]. Thus, two CHN-MRSA-CC398 seemed to be related, while four GER-MRSA-CC398, four GER-MRSA-CC9 and three CHN-MRSA-CC398 showed varying possible relationships among each other (Table S1). Moreover, the differences in 1559 of 1749 alleles included in the comparison reflected the considerably distant clonal relationship between Cluster 1 and 2. As the isolates SHP1 within CHN-MRSA-CC9, and relatively higher numbers of allelic differences between the groups, the division of isolates into four groups for the Biolog PM assay based on their CC and origin was in accordance with the phylogenetic analysis (Figure 1, Table S1).

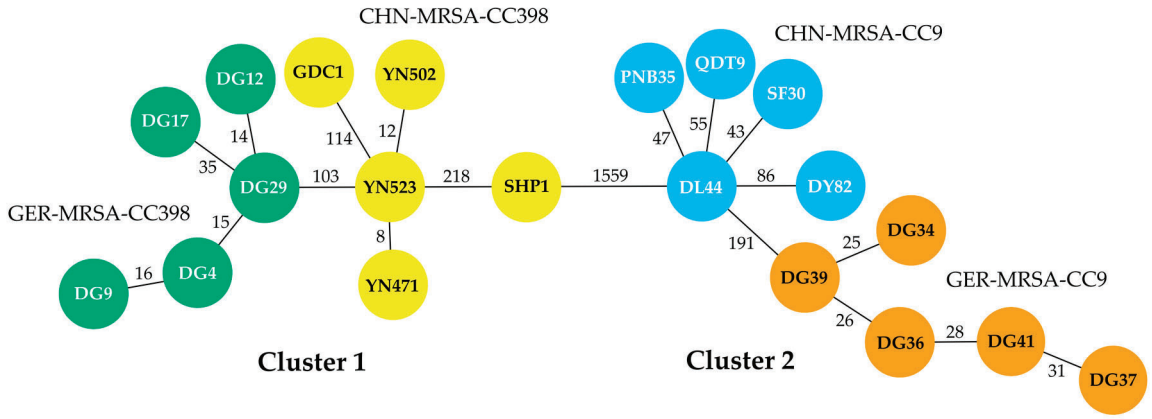

Figure 1. Minimum spanning tree displaying the phylogenetic relationship of 20 porcine MRSA isolates from Germany and China based on cgMLST analysis including 1749 alleles using the SeqSphere+ software. The circles represent distinct allelic profiles and the count of varying target genes between the different profiles is shown next to the connecting lines. The isolate IDs are given within the circles; origin and CC are indicated by color: GER-MRSA-CC398 in green, CHN-MRSA-CC398 in yellow, CHN-MRSA-CC9 in blue and GER-MRSA-CC9 in orange.

3.2. Biolog PM Assay Analysis

If not further specified, all findings described in this section relate to an incubation period of 24 h. Some of the chemical compounds used in the Biolog PM microplates can directly reduce Dye H even when no bacterial cells are added resulting in the formation of purple color [24]. These wells will give abiotic "false-positive" reactions without bacteria present because the dye is chemically reduced. There is no final list of abiotic wells available because the dye reduction depends on the definitive test parameters. Therefore, a set of Biolog PM microplates was run without bacteria. Wells that showed abiotic reactions under the study's experimental conditions are given in Table S2. Comparison of the obtained curves with the reduction kinetics resulting from the Biolog PM assays involving bacterial cells enabled assessment of the "false-positive" reactions. Biological reduction was gradual over time, whereas abiotic reduction occurred rapidly, more like a step function. In addition, biological exceeded chemical reduction in the abiotic wells. In the end, abiotic reactions did not affect further evaluation of the Biolog PM assay data as they were easy to distinguish from biological reduction due to bacterial respiration.

3.2.1. General Metabolic Properties of Porcine LA-MRSA from Germany and China

Overall, the porcine MRSA isolates were able to metabolize a broad variety of macromolecules, including a wide spectrum of different carbon sources. These nutrients comprised sugars (D-galactose, D-trehalose, D-mannose, etc.), sugar alcohols (sorbitol, adonitol, maltitol, etc.) or (di-, tri-, hydroxy-) carboxylic acids (formic acid, succinic acid, citric acid, glycolic acid, etc.). Furthermore, the bacteria utilized the emulsifier tween, a wide range of amino acids, a number of other acids (a-keto-valeric acid, caproic acid, quinic acid, etc.) and nucleosides such as adenosine, thymidine or uridine. *S. aureus* requires endogenous and/or exogenous sources of biosynthetic precursors, e.g., carbohydrates, amino acids, nucleic acids, for assembly reactions generating DNA, RNA and proteins [61]. Moreover, in *S. aureus* the de novo synthesis of precursors requires 13 biosynthetic intermediates, which are provided by central metabolic pathways [61]. Central metabolism is both catabolic, i.e., energy-generating, and anabolic, i.e., energy-requiring, and involves glycolysis, gluconeogenesis, the pentose phosphate pathway and the tricarboxylic acid cycle in *S. aureus* [61]. In line with our findings, it is known that *S. aureus* is able to catabolize many different macromolecules, e.g., amino acids or nucleic acids, to produce the 13 intermediates, but typically prefers to use carbohydrates when available [61]. The process of carbohydrate translocation into the cytoplasm involves a group of different enzymes/transporters, where specificity

for a certain sugar is provided by particular components. However, *S. aureus* has at least 15 enzyme II components encoded within its genome, which also highlights the pathogen's versatility in carbohydrate utilization, consistent with our results [61]. In some cases, substrate metabolism was slightly delayed (α-keto-butyric acid, N-acetyl-neuraminic acid, 2-hydroxy benzoic acid, etc.), or was reduced compared to other substances (i-erythritol, citraconic acid, D-tartaric acid). Only a few carbon sources could not be metabolized at all by the MRSA isolates, including glyoxylic acid, capric acid and itaconic acid, which emphasizes the remarkable versatility in nutrient utilization.

S. aureus has also evolved a number of cellular stress responses allowing it to persist in different host species and to survive in varying environmental settings [62]. The bacterium's high osmotolerance, for example, supports growth in high-osmolarity body sites, e.g., skin and mucosal surfaces, or in food with high salt concentrations [62]. Consistent with this, the porcine LA-MRSA from Germany and China showed pronounced metabolic activity in the presence of many osmolytes in various and even high concentrations. In several studies, the role of cytoplasmic accumulation of K^+ and solutes as glutamine or proline in the adaptation to settings with increased osmolarity is discussed [62]. Here, all isolates were resilient to increasing concentrations of potassium chloride, sodium sulfate and ethylene glycol up to 6%, 5% and 20%, respectively. Their metabolism was also invariable to sodium phosphate and ammonium sulfate in all concentrations tested. Except for a minor delay, increasing concentrations of sodium chloride up to 10% had no substantial effect on bacterial metabolic activity. Similar to sodium chloride, rising concentrations of urea up to 7% resulted only in slightly delayed metabolic activity. However, individual substances in certain concentrations could significantly delay, reduce or even inhibit cellular respiration. The combination of sodium chloride 6% and dimethylsulphonylpropionate appeared to significantly decelerate the onset of metabolism more for some isolates and less for others. Moreover, increasing concentrations of sodium formate up to 6% reduced metabolic capacity and notably delayed bacterial metabolism. The presence of sodium lactate at concentrations of 3% to 12% also led to increasing inhibition of metabolic activity. Already at 4%, the metabolic activity of a few isolates was only recognizable during a 48 h incubation period. The isolates also showed reduced metabolism in the presence of sodium benzoate at a pH value of 5.2. The reduced metabolism was apparent at 20 mM, only a few isolates were able to maintain metabolism at 50 mM, and no activity was detected at 100 mM or 200 mM except for single isolates during a 48 h incubation period. In addition, rising concentrations of sodium nitrate had only a minor impact on the extent of metabolic activity, while those of sodium nitrite significantly reduced metabolism.

The extent of bacterial metabolism was also dependent on ambient pH conditions. Values between 5 and 10 did not pose significant difficulties, except for a slight decrease in metabolic activity at acidic values of 5 to 6. In contrast, no metabolic activity was detected at acidic pH values of 3.5 to 4.5. Provision of different nutrients such as amino acids and others at a pH of 4.5 did not affect this observation with the exception of urea. Here, some isolates were able to fully develop their activity, after a period of adaptation, which was mainly visible only over 48 h of incubation. Many studies investigated the effect of pH on the growth of *S. aureus*, for example to develop strategies to control *S. aureus* growth and enterotoxin production in food-manufacturing processes. Lanciotti et al. showed that among various bacterial species examined, *S. aureus* was characterized by the highest sensitivity to pH changes [63]. Considering an interactive effect of pH, water activity, temperature and ethanol concentrations on the pathogen's growth, predicted critical pH values ranged between 5 and 7 [63], which matches the pH values allowing bacterial metabolism observed in our study. A pH sensitivity within the range of 5 to 7 was also shown for *S. aureus* by Iyer et al. emphasizing the advantages of keeping the skin pH within an acidic range for maintenance of a balanced skin microbiome, including a lower level of *S. aureus* compared with other commensals [64].

In contrast to a previous study [23], no differences were recognized between isolates belonging to CC398 and CC9 regarding the survival under acidic and hypertonic conditions.

3.2.2. Statistical Evaluation of the Biolog PM Assay Data

3.2.2.1. AUC sPLS-DA

The 30 sPLS-DA selected substances are displayed in Figure 2 and mainly included sugars, sodium salts and amino acids combined with varying pH conditions. The two independent AUC values considered in each case were reproducible. The branching diagram shows that differentiation of the isolates depending on their CC and origin into the four groups CHN-MRSA-CC9, CHN-MRSA-CC398, GER-MRSA-CC9 and GER-MRSA-CC398 was possible on the basis of the most remarkable differences in metabolic substrate utilization. Moreover, the selected substrates and/or conditions could be assigned to eight differing groups. Regarding the 30 most informative substrates, the heat map suggested that the groups GER-MRSA-CC398, GER-MRSA-CC9 and CHN-MRSA-CC398 harbored more similar metabolic profiles compared to the CHN-MRSA-CC9 (Figure 2). The Euclidian distance based on z-scores is for every Chinese CC9 isolate larger than that between the isolates of the other three groups. However, evaluation of respiration curves and mean AUC values revealed the following substrates as particularly interesting for the study's research question: pH 4.5 + urea, D-arabitol, pH 9.5 + agmatine, sodium benzoate pH 5.2 50 mM, sodium formate 3%, sodium formate 4%, sodium formate 6%, sedoheptulosan, pH 9.5 + L-glutamine, pH 9.5 + hydroxy-L-proline, pH 9.5 + L-leucine, pH 9.5 + L-isoleucine, and pH 9.5 + L-threonine. The four groups also displayed crucial differences in the metabolism of several nutrients beyond the sPLS-DA selection, which are described in the following sections. Overall, the selection of substrates and/or conditions using this method allowed grouping of the isolates, but a more detailed insight into growth kinetics considering additional parameters to the AUC was required to evaluate metabolic differences between the four groups.

3.2.2.2. Combined LT, MH, PT, and Slope sPLS-DA Variable Selection

The combined sPLS-DA variable selection also allowed a preselection of the most informative substrates and/or conditions tested. Similar to the AUC sPLS-DA selection, the 40 nutrients mainly included sugars, (amino) acids and sodium salts. In each case, the parameters LT, MH, PT and slope were reproducible and could be assessed in detail. Interestingly, the four isolate groups showed recurring patterns regarding the usage of many substances, which allowed classification of these metabolic differences as described in the following sections. However, for several substrates and/or conditions, the patterns were different across all parameters making classification impossible. Moreover, similar to the AUC sPLS-DA, relevant deviations between the four isolate groups were detected regarding various nutrients beyond the selection, which are also described below.

3.2.3. Relevant Metabolic Varieties between the Four MRSA Groups

3.2.3.1. Metabolic Differences between the Dominant and Rare Lineage within a CC

Different colonization and infection sites within a pathogen's host require unique metabolic pathways due to the varying availability of nutrients. For example, *S. aureus* would likely have access to lactate, urea and amino acids, but not carbohydrates at skin surfaces, and to serum glucose at deep tissue sites [65]. Moreover, several studies have shown that the metabolic state of *S. aureus* has an impact on the activity of major virulence factor regulators, proving the linkage of *S. aureus*' metabolism to its overall pathogenesis [65]. Therefore, it seemed likely that the dominant LA-MRSA CC9 from China and CC398 from Germany might have an advantage in host colonization due to certain metabolic properties in relation to the non-successful pig colonizing isolates.

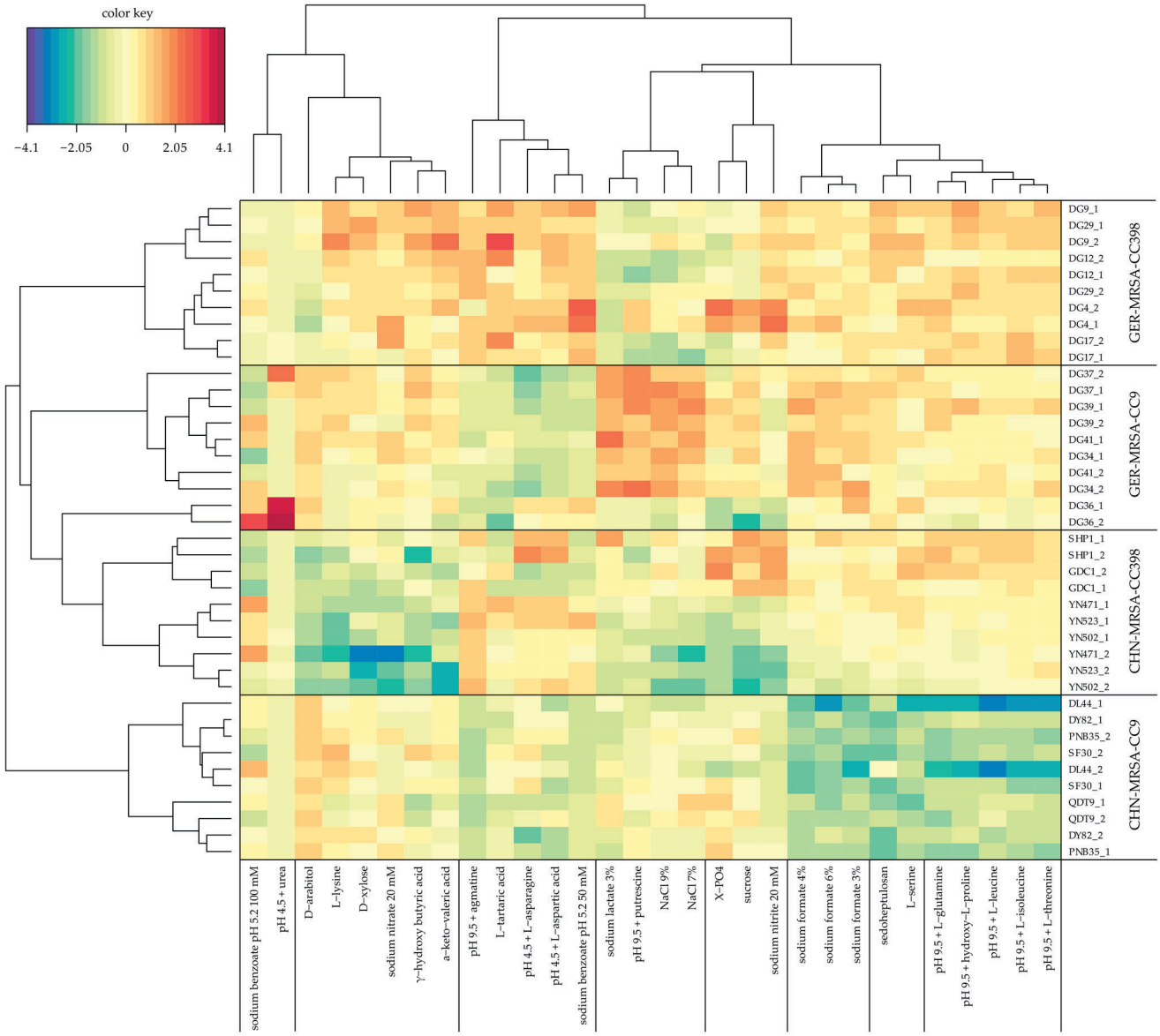

Figure 2. Heat map constructed via sPLS-DA using four components to differentiate porcine MRSA isolates from Germany and China based on CC and origin. Two independent area under the curve (AUC) values obtained after 24 h of incubation were considered for each of the isolates displayed on the right side. Based on the most remarkable differences selected nutrients are shown at the bottom. The color key mirrors the extent of metabolic substrate utilization (z-scaled) by the isolates, with red color displaying more successful and blue color less successful usage of a nutrient. The branching diagram provides classification of isolates and substrates into different groups.

Lower AUC values indicated a less successful substrate metabolization by the rare lineages compared with the respective dominant clone within the same CC for the carbon sources phenylethylamine, D,L-octopamine and 3-hydroxy 2-butanone (Figure 3a). More precisely, the combined sPLS-DA revealed that the associated respiration curves of the dominant clones displayed greater MH values (Figure 3b). In addition, beyond the sPLS-DA selections, lower AUC values indicated a less successful substrate metabolization by the rare lineages compared with the respective dominant clone within the same CC regarding the carbon sources acetamide, L-phenylalanine and 2,3-butanediol (Figure 3a).

In contrast to initial expectations, only a few relevant metabolic differences were found between the dominant and rare lineages within a CC. Thus, the dominant lineages might

have only single advantages in environments with limited nutrient availability. However, due to *S. aureus*' versatility in nutrient utilization and since several substrates can be used alternatively in the same essential metabolic pathways, we speculate that these variations did not play a role in the emergence of two different epidemic LA-MRSA clones in swine. Advantages such as an enhanced biofilm forming ability and an increased tolerance to desiccation, which we revealed in a previous study [23], more likely allow the dominant clones to outcompete the non-successful lineages in extreme environments.

3.2.3.2. Distinctive Metabolic Features of CHN-MRSA-CC9

The CHN-MRSA-CC9 showed varying metabolic capacities regarding several substrates compared with the other three groups. Major differences were detected for the carbon sources sedoheptulosan, xylitol and oxalomalic acid. Here, lower AUC values pointed towards a reduced usage in relation to the other groups. According to the combined sPLS-DA, higher PT and lower slope values were recognized for all three substrates. Moreover, an extended LT was observed in the metabolization of sedoheptulosan and xylitol and lower MH values were identified for sedoheptulosan as well as oxalomalic acid (Figure 4a). Sedoheptulosan was also included in the AUC sPLS-DA selection. Regarding the usage of oxalomalic acid, evaluation of the respiration curves provided additional information. Although Figure 4a indicated that the utilization of this nutrient by the CHN-MRSA-CC9 was the lowest, one isolate of the group could use it better than the others (see Section 3.2.3.6). The GER-MRSA-CC9 also seemed to have more difficulties using the substance compared to the MRSA-CC398. Therefore, the differences between the four groups considering oxalomalic acid could also have been discussed in Section 3.2.3.3. Interesting changes in the further progression of the respiration curves were only evaluable considering those resulting from an incubation period of 48 h (see Section 3.2.3.6).

Furthermore, relevant differences were detected beyond the sPLS-DA selections for the carbon sources m-tartaric acid and D-malic acid. Again, lower AUC values indicated a reduced usage in relation to the other groups (Figure 4b). In contrast, respiration curves and AUC values suggested a comparatively increased metabolization of the substrate 2-deoxy-d-ribose (Figure 4b). The AUC values also pointed towards a distinctly increased metabolization of the substrate 3-0-β-D-galactopyranosyl-D-arabinose by the CHN-MRSA-CC9. Evaluation of the respective respiration curves revealed that only these isolates were able to initiate cellular respiration within an incubation period of 24 h (see Section 3.2.3.6). However, the observation of single non-CHN-MRSA-CC9 isolates also being able to metabolize the substance was only detectable over a period of 48 h (see Section 3.2.3.6).

The reduced metabolic capacity and the delay of metabolism resulting from rising concentrations of sodium formate as mentioned in Section 3.2.1 was recognized for all four groups, but was most distinct for the CHN-MRSA-CC9. Here, the respiration curves displayed lower AUC values, but also an extended LT, lower MH as well as PT values and a greater slope as shown by the combined sPLS-DA (Figure 4b,c). The AUC sPLS-DA selection also suggested sodium formate at concentrations of 3%, 4% and 6% as relevant. As mentioned above, rising concentrations of urea also led to a delay of bacterial respiration, which was notably more distinct for the CHN-MRSA-CC9 compared to the other groups (Figure 5a). Sodium benzoate 50 mM at a pH of 5.2 was also included in the AUC sPLS-DA selection. As discussed in Section 3.2.1, not all isolates could maintain metabolism under these conditions. While lower respiration curves and the delayed onset of metabolic activity displayed in Figure 5a indicated that isolates belonging to all groups struggled using this substance, no metabolic activity was detected for the CHN-MRSA-CC9. Thus, the CHN-MRSA-CC9 showed an increased sensitivity to rising concentrations of sodium benzoate in relation to the other groups (see Section 3.2.1).

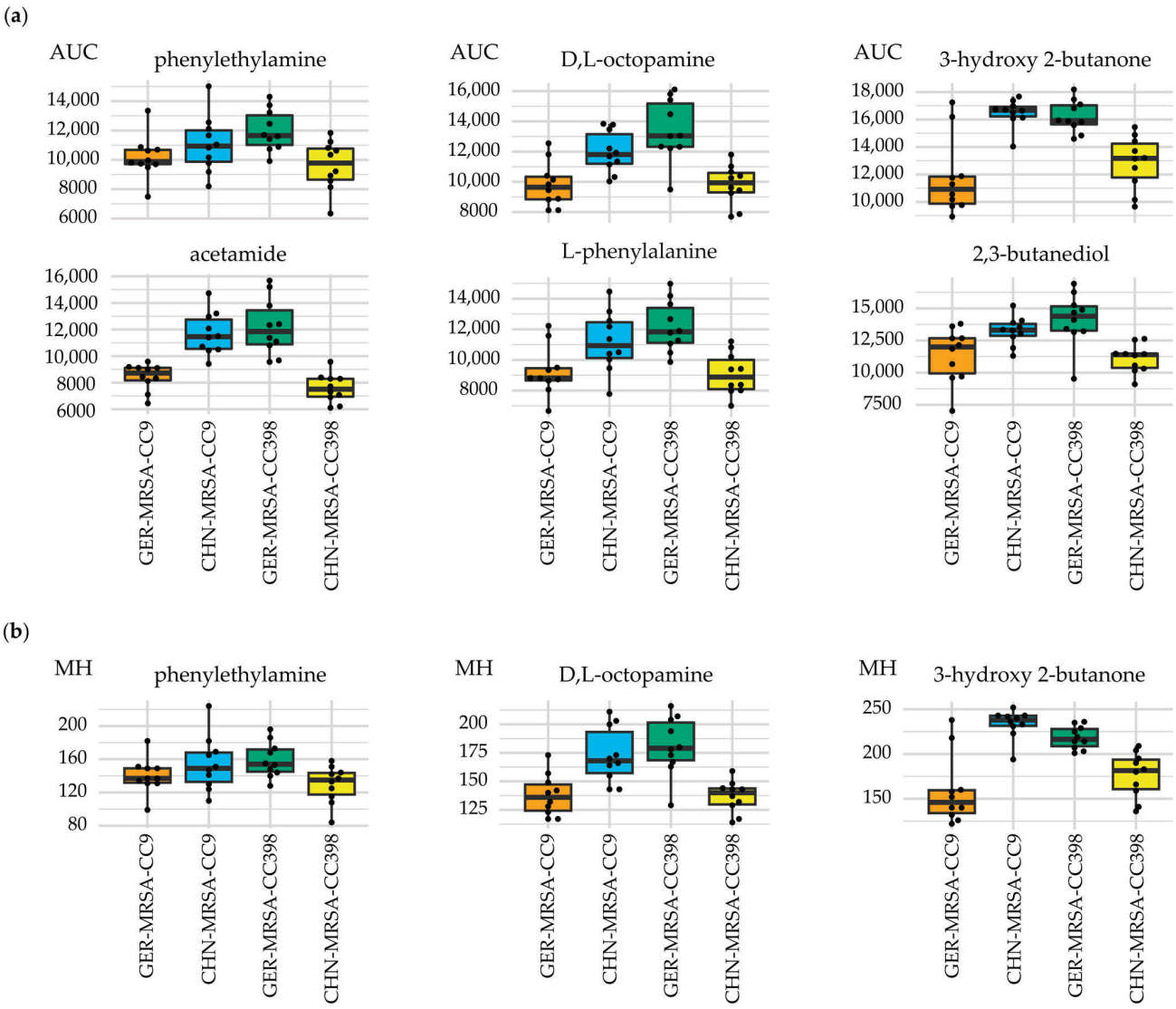

Figure 3. Relevant metabolic differences between the dominant and rare lineage within a CC regarding the usage of the substances given above the individual diagrams. The box plots display varying (**a**) area under the curve (AUC) values and (**b**) maximum height (MH) values of the four isolate groups.

In addition, the respiration curves of the CHN-MRSA-CC9 suggested the requirement for a longer adaptation time to the surrounding conditions before onset of nutrient utilization. Not all findings can be shown here, but the observation was particularly evident considering the usage of 2,3-butanedione (Figure 5a) as well as of amino acids and derivatives thereof at basic pH values. The examples given in Figure 5b are representative for all amino acids and derivatives at pH 9.5 and several of them were also emphasized by the AUC sPLS-DA (see Section 3.2.2.1). The availability of amino acids, which can be catabolized to produce the essential biosynthetic intermediates, is of importance for *S. aureus*, as the bacterium is frequently described as an amino acid auxotroph [61]. However, *S. aureus* often has the ability to revert to a prototrophic state, which is important for the adaptation to host/environmental niches where nutrients are limited [61]. Moreover, Halsey et al. have shown that certain amino acids serve as central carbon sources for *S. aureus* critical for growth in medium lacking glucose [66]. This supports a model describing the ability to metabolize secondary carbon sources, such as amino acids, as essential for survival in niches where preferred nutrients, such as glucose, are limited, as

for example within a staphylococcal abscess [66]. Thus, the CHN-MRSA-CC9 might be inferior in niche adaptation due to their delayed amino acid catabolism.

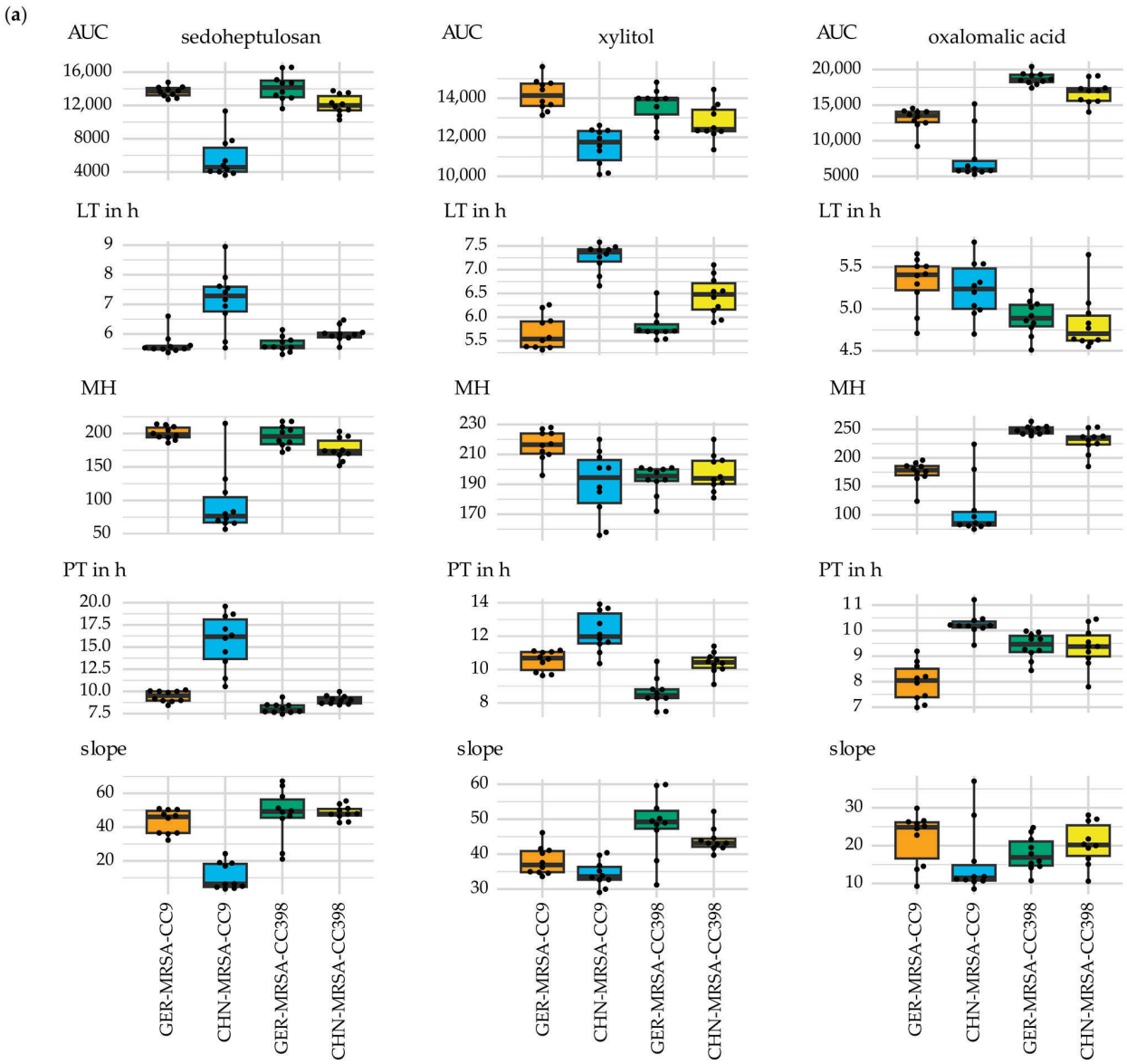

Figure 4. *Cont.*

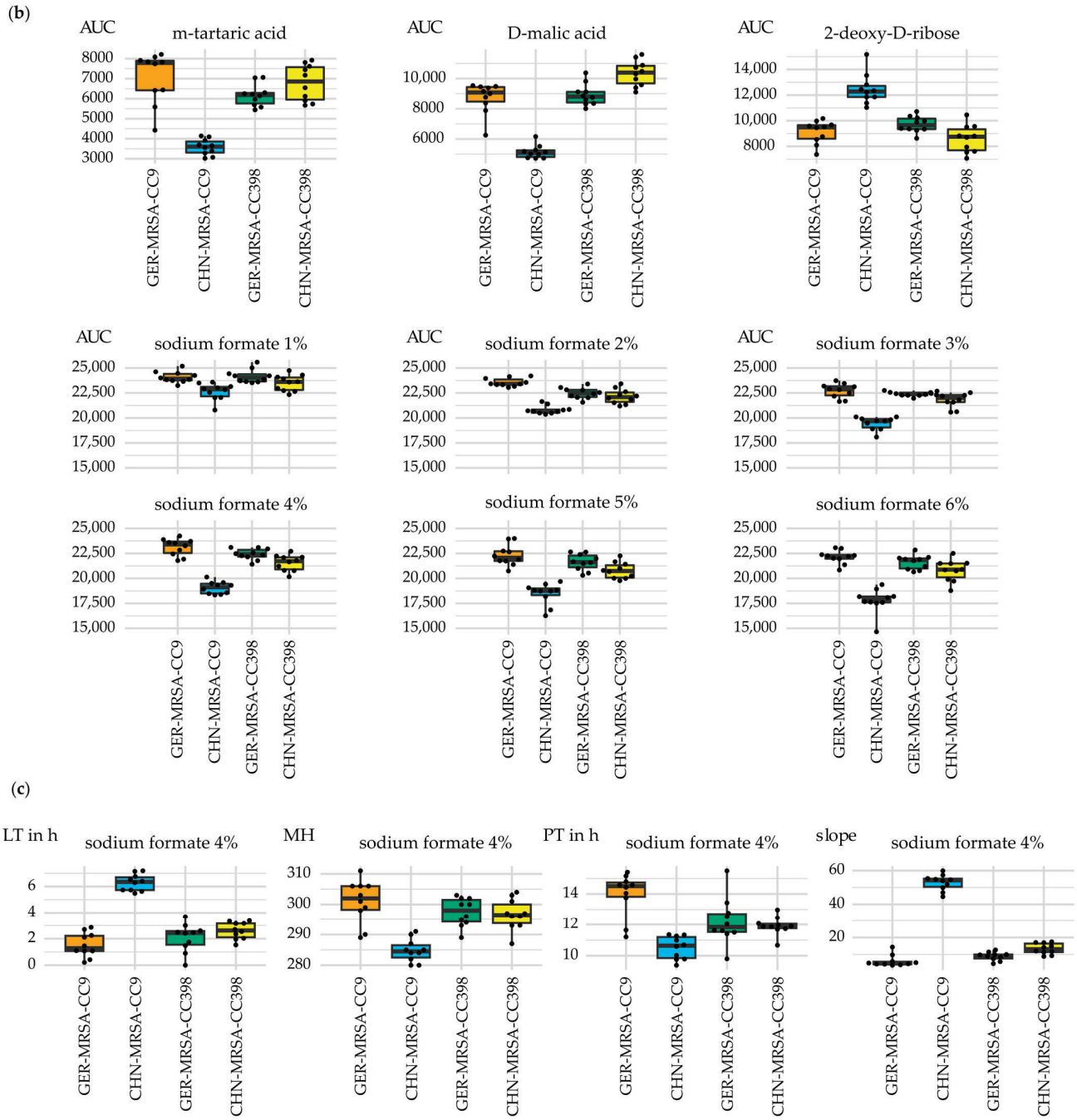

Figure 4. Major differences in the metabolic properties of the CHN-MRSA-CC9 compared with the other three groups regarding the substances or conditions given above the individual charts. The box plots display the varying (**a**) area under the curve (AUC), lag time (LT), maximum height (MH), plateau time (PT) and slope values, (**b**) AUC values and (**c**) LT, MH, PT and slope values of the four isolate groups. LT and PT are given in hours and sodium formate 4% in (**c**) is representative for sodium formate 1% to 6%.

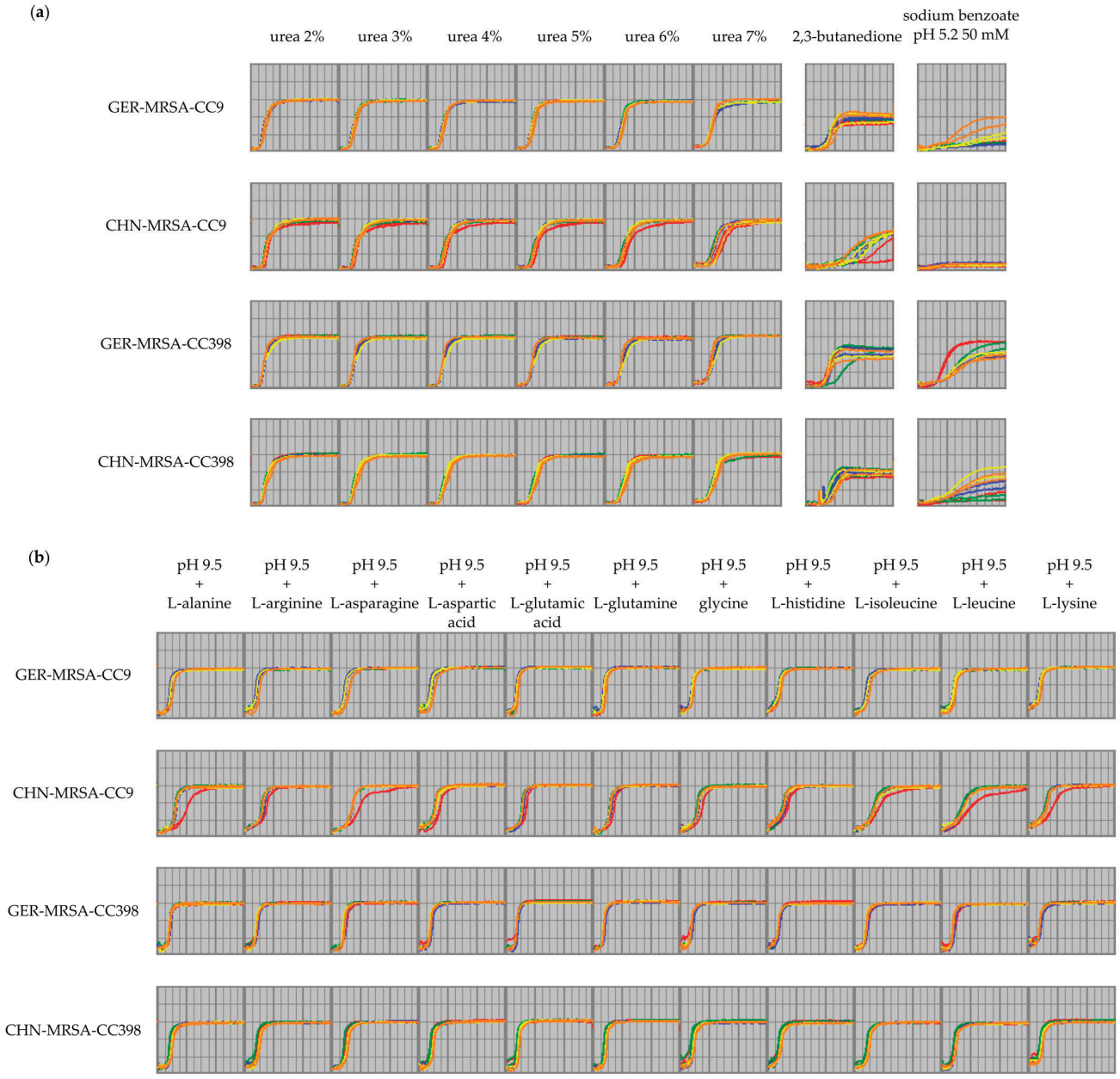

Figure 5. Curves displaying cellular respiration of the four isolate groups over an incubation period of 24 h including two test runs. The isolates within each group are indicated in different colors. (**a**) The CHN-MRSA-CC9 show a delayed onset of metabolization in the presence of urea 2% to 7% as well as 2,3-butanedione and no metabolic activity in the presence of sodium benzoate 50 mM at pH 5.2. (**b**) The CHN-MRSA-CC9 show a delayed onset of cellular respiration considering the usage of different amino acids at a pH value of 9.5.

As expected, the cgMLST revealed a closer relationship between the isolates within the two CCs regardless of their geographical origin. Nevertheless, the CHN-MRSA-CC9 showed major metabolic differences to the other three groups. The greater similarities of the CHN-MRSA-CC398, GER-MRSA-CC398 and GER-MRSA-CC9 are also displayed in Figure 2. In a previous study, we observed that the MRSA-CC9 from China and Germany possibly belong to two independent evolutionary lineages, whilst the CHN-MRSA-CC398 and the GER-MRSA-CC398 originated from the same ancestor [23]. Moreover, we speculated that the MRSA-CC398 might have been introduced into China through trading activities [23]. Another recent study also revealed a close evolutionary relationship be-

tween Chinese and European or Australian LA-MRSA ST398 [16]. The greater spatial distance of the CHN-MRSA-CC9 to the other three lineages during evolution of epidemic clones in swine might explain the more distinct metabolic profile of the MRSA-CC9 from China. The carriage of a larger number of antimicrobial resistance genes has possibly led to a higher fitness cost in the CHN-MRSA-CC9 than in other isolates [67], explaining the reduced metabolic capabilities regarding several substances and the throughout extended adaptation times before onset of cellular respiration. Increased metabolization of 2-deoxy-d-ribose and 3-0-β-D-galactopyranosyl-D-arabinose by the CHN-MRSA-CC9 in relation to the other groups may, however, represent a slight advantage for these isolates, but might as well result in notable disadvantages in case of harmful metabolite formation during metabolic processes.

In the previous study, we also suggested that the current epidemic MRSA-CC9 clone in China may be gradually replaced by the MRSA-CC398 lineage due to several advantageous features that allow MRSA-CC398 to persist in the pig host or hostile environment [23]. These included easier integration of foreign antimicrobial resistance genes, improved fitness and adaptation to varying environmental conditions, enhanced virulence in infection models, increased pathogenicity due to the *hysA*$^{\nu Sa\beta}$ gene, better adaptation to acidic and hyperosmotic environments and higher competitiveness compared to MRSA-CC9 [23]. The small disadvantages of the CHN-MRSA-CC9 in metabolic properties described in this study may support the replacement of the epidemic Chinese clone by MRSA-CC398 after further dissemination of isolates through livestock trade or human occupational exposure. The delayed onset of cellular respiration of the CHN-MRSA-CC9, combined with the decreased usage of many nutrients as well as possible disadvantages resulting from increased metabolization rates of some substrates might be sufficient for MRSA-CC398 to become established once extensively colonizing the pig host. Despite the metabolic robustness typical for *S. aureus* [61], the CHN-MRSA-CC9 may be less adaptable to environmental challenges than the MRSA-CC398 clone. The increasing reports of swine-associated MRSA-CC398 isolates from China support the hypothesis of the ongoing MRSA-CC9 replacement process [16,17]. It is important to note that in the future MRSA-CC398 may pose a greater public health threat than MRSA-CC9 due to their enhanced pathogenicity [23]. For example, results from Cui et al. recently suggested that ST398 was a frequent source of MRSA and MSSA infections in the Qinghai province [16]. Li et al. even implied a potential MRSA-ST398 transmission among different countries [17]. They reported an MRSA ST398 isolate obtained from an infected patient in Europe that differed by only 31 SNPs from the airborne dust-associated Chinese isolate in their study [17]. This highlights the need for comprehensive worldwide LA-MRSA monitoring.

3.2.3.3. Metabolic Diversity Based on the Assignment to a CC

Respiration curves resulting from the metabolization of D-serine and D-arabitol indicated a CC-dependent usage of these nutrients in accordance with the combined sPLS-DA variable selection. Considering D-serine, the MRSA-CC9 isolates displayed lower AUC, MH and slope values, but an extended PT in relation to the MRSA-CC398 isolates (Figure 6). In contrast, higher AUC, MH and PT values indicated an increased metabolic activity of the MRSA-CC9 compared to the MRSA-CC398 isolates regarding D-arabitol (Figure 6). The substrate was also included in the AUC sPLS-DA selection.

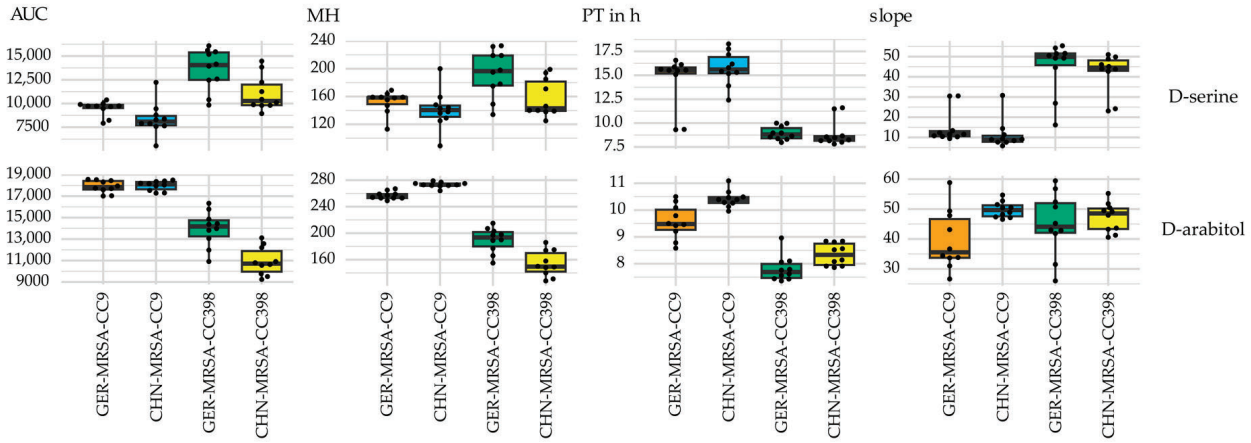

Figure 6. Box plots selected by combined sPLS-DA showing varying area under the curve (AUC), maximum height (MH), plateau time (PT) and slope values of the four isolate groups regarding the metabolization of D-serine and D-arabitol, indicating a CC-dependent nutrient usage. The PT is given in hours.

In a previous study [23] we identified several advantages of MRSA-CC398 isolates towards MRSA-CC9 as they revealed more diverse genome structures, higher tolerance to acids and high osmotic pressure and greater competitive fitness in co-culture experiments [23]. Moreover, a novel *hysA*$^{\nu Sa\beta}$ gene causing enhanced pathogenicity, which was present in CC398 isolates but absent in MRSA-CC9, might explain why MRSA-CC398 has been reported to be more likely to cause infections [6,23,68]. For this reason, we initially expected to detect multiple differences in the metabolic properties of MRSA-CC9 and MRSA-CC398. However, only single metabolic differences due to CC affiliation were recognized in this study overall.

3.2.3.4. Metabolic Diversity Attributed to the Country of Origin

Considering the carbon sources α-hydroxy glutaric acid-γ-lactone, glycolic acid, β-methyl-D-glucuronic acid, stachyose, γ-amino butyric acid, 4-hydroxy benzoic acid and oxalic acid in the combined sPLS-DA selection, differences in metabolic properties seemed to be associated with the isolates' origin. Throughout, the Chinese MRSA showed lower AUC and MH values as well as an extended LT in comparison with the German isolates, indicating a decreased nutrient usage (Figure 7a). Moreover, as shown by lower AUC values beyond the sPLS-DA selections, the CHN-MRSA-CC9 and CHN-MRSA-CC398 appeared to be less successful in metabolizing α-keto-butyric acid and especially 2-hydroxy benzoic acid compared to the German MRSA (Figure 7b). These findings may suggest an adaptation of the MRSA isolates to the unique character of the Chinese and German pig farm environments.

3.2.3.5. Other Metabolic Differences between the Groups

Some variations in the metabolic activity between the four groups CHN-MRSA-CC9, CHN-MRSA-CC398, GER-MRSA-CC9 and GER-MRSA-CC398 were individual observations and, thus, not discussed in detail. An example is given in Figure S1. According to the combined sPLS-DA, higher AUC and MH values and a shortened LT indicated a more successful usage of glycine by the GER-MRSA-CC398 compared to the other three groups.

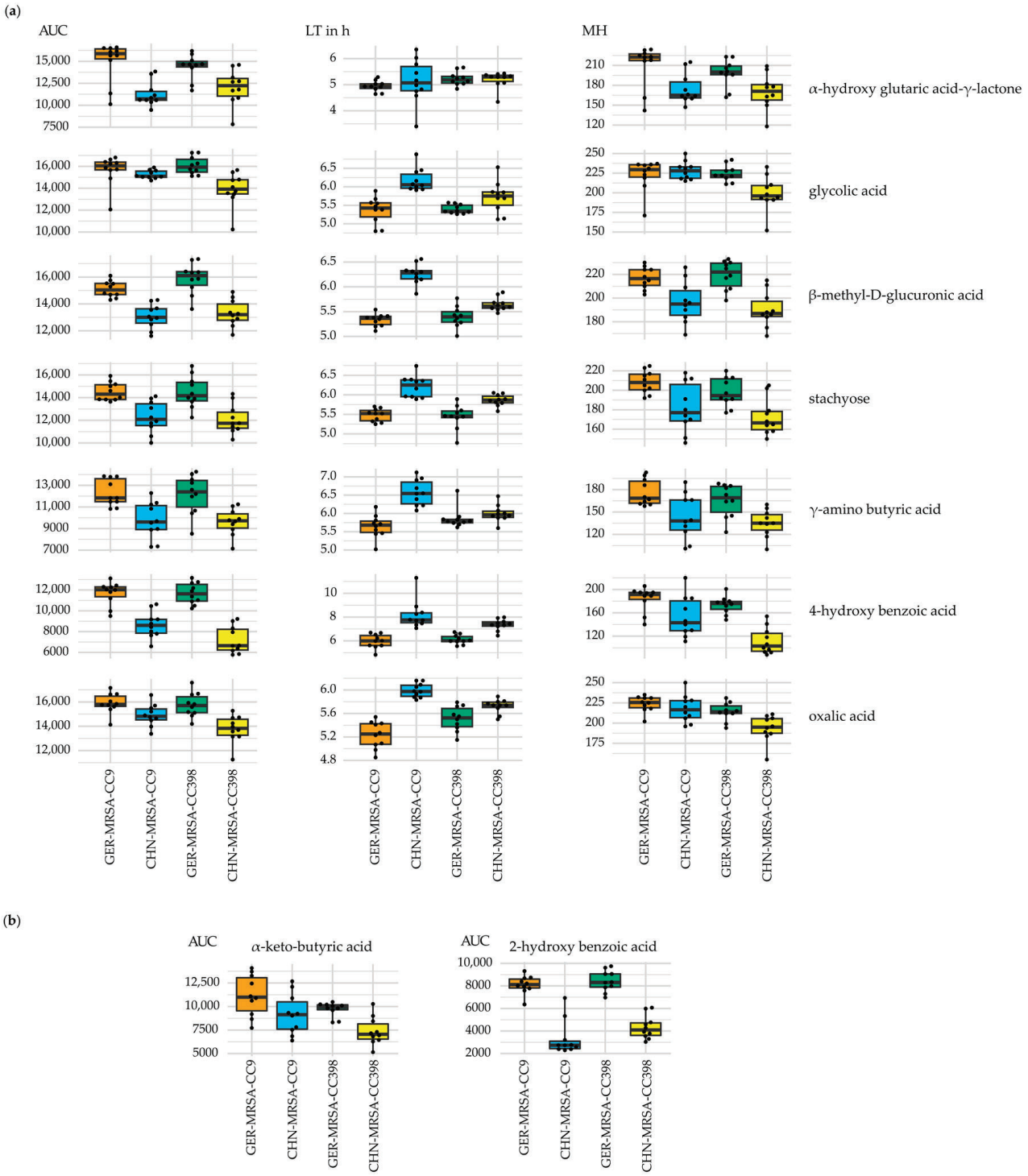

Figure 7. Metabolic differences associated with the isolates' origin. The box plots illustrate varying (**a**) area under the curve (AUC), lag time (LT) and maximum height (MH) values of the four isolate groups regarding the usage of the nutrients given on the right hand side of the individual diagrams with the LT given in hours and (**b**) AUC values of the four isolate groups regarding the usage of α-keto-butyric acid and 2-hydroxy benzoic acid.

In addition, variable results were detected for certain nutrients within each of the four groups and, therefore, not categorized further. Here, the metabolization of nutrients seemed to be more isolate-dependent. Selected examples displaying cellular respiration in the presence of dulcitol, tricarballylic acid, glycogen, a-methyl-D-glucoside, sodium lactate 3%

or sodium nitrite 40 mM are shown in Figure S2. Sodium lactate 3% is representative for sodium lactate 2% to 12% and sodium nitrite 40 mM is representative for sodium nitrite 10 mM to 100 mM.

3.2.3.6. Metabolic Variations Detectable Only after a 48 h Incubation Period

Some disparities in the isolates' metabolic activities were only detectable after an incubation period of 48 h. For the substance 3-0-β-D-galactopyranosyl-D-arabinose, this has already been mentioned in Section 3.2.3.2 (Figure 8). In addition, the metabolization of urea at a pH of 4.5 would have been interpreted differently, if only the 24 h interval would have been considered. The 24 h respiration curves suggested that only two GER-MRSA-CC9 were able to initiate metabolic activity. In contrast, the curves obtained after 48 h of incubation revealed that more isolates from several groups were able to utilize urea at a pH of 4.5 after an adaptation period. However, the CHN-MRSA-CC9 were unable to metabolize this substrate under these conditions (Figure 8).

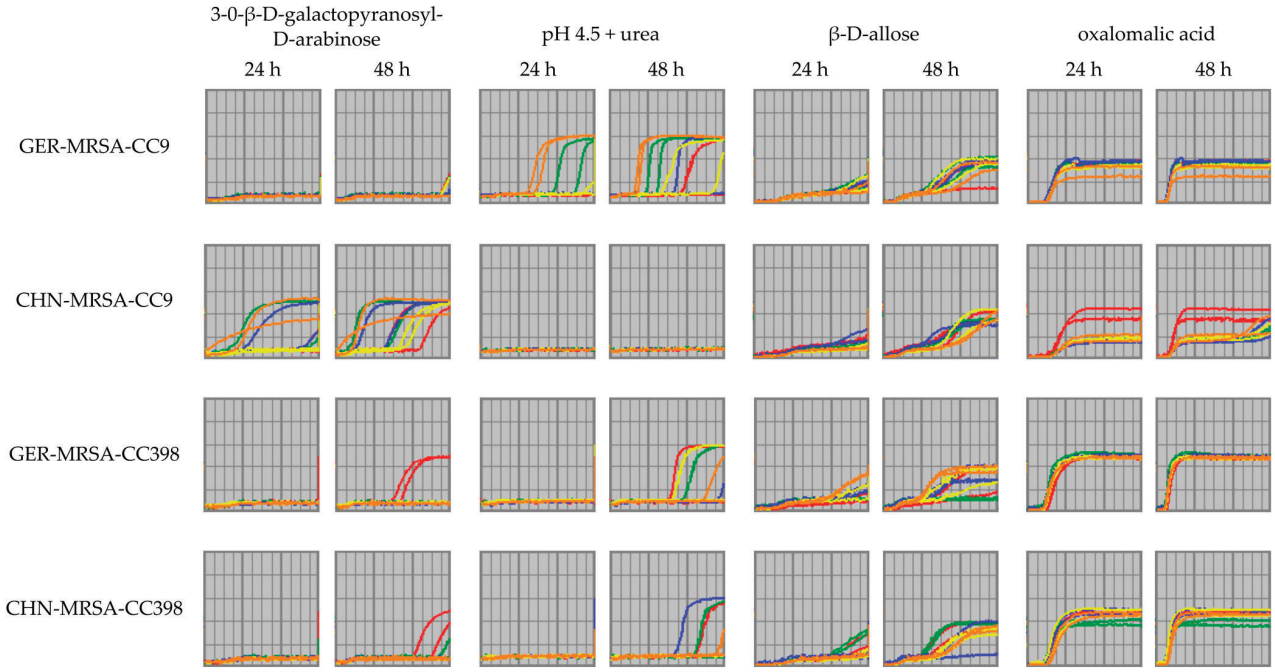

Figure 8. Curves displaying cellular respiration of the four isolate groups in the presence of 3-0-β-D-galactopyranosyl-D-arabinose, pH 4.5 + urea, β-D-allose and oxalomalic acid over an incubation period of 24 h and 48 h including two test runs. The isolates within each group are indicated in different colors. Here, interesting changes in curve progression are only detectable by investigating the 48 h respiration curves additionally.

The 48 h values also enabled the identification of changes in curve progression and thus of differences between the groups that otherwise would not have been recognized (Figure 8). According to the 24 h respiration curves, all four groups seemed to have difficulties in using the nutrient β-D-allose, but only the 48 h curves provided information on the further course. It was demonstrated for all groups that substrate utilization was possible after a period of adaptation. However, differing durations of adjustment indicated that the ability was not evenly distributed within the four groups. In general, the CHN-MRSA-CC9 required the longest adaptation time (Figure 8). The reduced usage of oxalomalic acid by the CHN-MRSA-CC9 has already been mentioned in Section 3.2.3.2. The CHN-MRSA-CC9 displayed a slightly delayed onset of cellular respiration and all isolates except one showed only very low levels of metabolic activity in relation to the other isolates. The 24 h respiration curves did not suggest changes in course progression at a later point in time. However, the 48 h

respiration curves of four CHN-MRSA-CC9 showed an increase towards the end of the incubation period almost up to the level of the other MRSA-CC9 isolates (Figure 8).

Finally, the examples referring to urea at pH 4.5, β-D-allose and oxalomalic acid also supported the conclusion that, compared to the other three groups, the CHN-MRSA-CC9 required a longer period of adaptation before the onset of cellular respiration and/or are not able to metabolize several substances at all or only to a lesser extent.

3.3. Potential Limitations of the Study

The aim of this study was to conduct a comparative evaluation of the metabolic properties of four MRSA groups with only five isolates per group and thus, 20 isolates in total. This is a relatively small sample size and might limit the transferability of the results for the represented groups. Considering the MRSA-CC398 from China, the isolates investigated were the only ones available to us during the study period. Due to limited resources, we decided to assemble comparable groups. Preliminary experiments confirmed, in general, a uniform metabolic behavior of the isolates within each group. For this reason, it was possible to draw the respective conclusions from the results obtained analyzing this limited isolate collection.

Only four Biolog PM microplate layouts out of ten possible metabolic phenotyping arrays available for bacteria and fungi provided by Biolog Inc. (Hayward, CA, USA) were used for the experiments. The microplate layouts PM1 (carbon utilization), PM2A (carbon utilization), PM9 (osmotic and ionic effects) and PM10 (pH effects) were chosen, because here we expected to observe relevant differences between the four MRSA groups. Cellular stress responses and a high osmotolerance allow *S. aureus* to persist in different environmental settings. In addition, despite its versatility in nutrient usage, *S. aureus* is known for typically metabolizing carbohydrates when they are available and the carbohydrate catabolism has been studied most extensively [61]. However, the application of additional Biolog PM layouts, e.g., phosphorus and sulfur sources, might be of interest for possible future studies. It should be noted that the PM5 (biosynthetic pathways) layout is not appropriate for testing staphylococci because of their polyauxotrophy [69].

Another potential limitation might be that no standardized statistical pipeline was available that would allow us to compare Biolog PM assay data not only between several bacterial isolates but also between the four isolate groups. For this reason, the AUC sPLS-DA and the combined sPLS-DA were carried out in an attempt to select the most relevant metabolic variations out of the extensive dataset. However, manual comparison of all respiration curves revealed substrates and/or conditions beyond the preselections as relevant due to notable metabolic variations between the four groups. Therefore, an automated, less work-intensive analysis of the data was not possible, although AUC sPLS-DA and combined sPLS-DA proved to be helpful.

Finally, it is worth noting that bacterial metabolism is a large and complex field. Biochemical processes and the regulatory interactions between them have been partially elucidated, but are still unknown to a large extent [70]. In order to generate insight into metabolic networks, integration of experimental results with published data, as well as accumulated observations and anecdotal tales exchanged among scientists working in this field is required [70]. However, a decline in research focusing on microbial metabolism would appear to make this more difficult [70]. Therefore, a limitation of this study may be that we were not able to discuss all substrates in detail that were mentioned in the context of relevant metabolic differences between the MRSA groups. Despite extensive literature research, which metabolic pathways of *S. aureus* involving these substrates play a role in fitness and niche competition in a given clinical or environmental setting is not known. It was also not possible to assign the respective substrates to specific groups, other than carbon sources, that would have allowed general conclusions to be drawn. Thus, further research integrating in silico, in vitro and in vivo approaches is required to better understand microbial metabolism in its details [70].

4. Conclusions

Applying Biolog PM technology, we characterized the metabolic profiles of rare as well as widespread porcine LA-MRSA from Germany and China. Overall, differences between the four LA-MRSA groups were rather small. In contrast to initial expectations, only a few metabolic differences were found between the dominant and rare lineages within the same CC. Therefore, unique metabolic profiles most likely did not play a key role in the formation of the dominant clones CHN-MRSA-CC9 and GER-MRSA-CC398, respectively. Surprisingly, the most frequent and substantial metabolic variations were detected between the CHN-MRSA-CC9 and the other three LA-MRSA groups. In addition to reduced or even absent usage of some substrates, and the increased metabolization of 2-deoxy-d-ribose and 3-0-β-D-galactopyranosyl-D-arabinose, especially the delayed onset of cellular respiration was often noticed. The AUC sPLS-DA heat map also suggested metabolic differences of the CHN-MRSA-CC9 compared to the other groups. These comparatively unfavorable metabolic properties might promote the suspected already ongoing gradual replacement of the current epidemic MRSA-CC9 clone in China by the superior MRSA-CC398 clone. Thus, the risk for public health posed by LA-MRSA originating from swine might rise alarmingly in the future due to the enhanced pathogenicity of the MRSA-CC398 lineage.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microorganisms10112116/s1, Table S1: Distance matrix of the core genome allelic profiles of the 20 representative porcine MRSA from Germany and China including 1749 of 1861 possible target genes by removing 112 columns with missing values from the comparison table created with the SeqSphere+ software, Table S2: Biolog PM microplate wells that showed abiotic "false-positive" reactions under the study's test conditions, Figure S1: Metabolization of glycine by the four isolate groups, and Figure S2: Selected Biolog PM assay results that were variable within the four isolate groups and, therefore, not categorized further.

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Data Availability Statement: All data presented in this study are available in the text, figures and tables of the main article and in the supplementary material. Whole-genome sequences of the MRSA isolates included in this study are available at GenBank under the accession numbers JAMYYK000000000, JAMYYJ000000000, JAMYYI000000000, JAMYYH000000000, JAMYYG000000000, JAMYYF000000000, JAMYYE000000000, JAMYYD000000000, JAMYYC000000000, JAMYYB000000000, JAMYYA000000000, JAMYXZ000000000, JAMYXY000000000, JAMYXX000000000, JAMYXW000000000, JAMYXV000000000, JAMYXU000000000, JAMYXT000000000, JAMYXS000000000 and JAMYXR000000000.

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2.2 Publication II

Krüger-Haker H*, Ji X*, Hanke D, Fiedler S, Feßler AT, Jiang N, Kaspar H, Wang Y, Wu C, Schwarz S

Genomic Diversity of Methicillin-Resistant *Staphylococcus aureus* CC398 Isolates Collected from Diseased Swine in the German National Resistance Monitoring Program GE*RM*-Vet from 2007 to 2019

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* both authors contributed equally to this study

Authors' contributions:

1. **Conceptualization**, K-HH, FAT, SS B
2. **Methodology**, K-HH, JX, HD, FS, FAT, JN, KH, SS B
3. **Validation**, K-HH, JX, HD, FS, JN, SS B
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5. **Investigation**, K-HH, JX, HD, SS B
6. **Resources**, JX, FS, JN, KH, WY, WC, SS
7. **Data Curation**, K-HH, JX, HD B
8. **Writing – Original Draft Preparation**, K-HH, JX, SS C
9. **Writing – Review & Editing**, K-HH, JX, HD, FS, FAT, JN, KH, WY, WC, SS B
10. **Visualization**, K-HH, JX, HD, SS C
11. **Supervision**, FAT, WY, SS
12. **Project Administration**, WY, WC, SS
13. **Funding Acquisition**, WY, WC, SS

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Genomic Diversity of Methicillin-Resistant *Staphylococcus aureus* CC398 Isolates Collected from Diseased Swine in the German National Resistance Monitoring Program GE*RM*-Vet from 2007 to 2019

Henrike Krüger-Haker,[a,b] Xing Ji,[c] Dennis Hanke,[a,b] Stefan Fiedler,[d] Andrea T. Feßler,[a,b] Nansong Jiang,[e] Heike Kaspar,[d] Yang Wang,[e] Congming Wu,[e] Stefan Schwarz[a,b,e]

[a]Institute of Microbiology and Epizootics, Centre for Infection Medicine, School of Veterinary Medicine, Freie Universität Berlin, Berlin, Germany
[b]Veterinary Centre for Resistance Research (TZR), School of Veterinary Medicine, Freie Universität Berlin, Berlin, Germany
[c]Jiangsu Key Laboratory for Food Quality and Safety, State Key Laboratory, Cultivation Base of Ministry of Science and Technology, Institute of Food Safety and Nutrition, Jiangsu Academy of Agricultural Sciences, Nanjing, China
[d]Federal Office of Consumer Protection and Food Safety (BVL), Berlin, Germany
[e]Key Laboratory of Animal Antimicrobial Resistance Surveillance, MARA, College of Veterinary Medicine, China Agricultural University, Beijing, China

Henrike Krüger-Haker and Xing Ji contributed equally to this work. Author order was determined randomly.

ABSTRACT Livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) clonal complex 398 (CC398) isolates (n = 178) collected in the national resistance monitoring program GE*RM*-Vet from diseased swine in Germany from 2007 to 2019 were investigated for their genomic diversity with a focus on virulence and antimicrobial resistance (AMR) traits. Whole-genome sequencing was followed by molecular typing and sequence analysis. A minimum spanning tree based on core-genome multilocus sequence typing was constructed, and antimicrobial susceptibility testing was performed. Most isolates were assigned to nine clusters. They displayed close phylogenetic relationships but a wide molecular variety, including 13 *spa* types and 19 known and four novel *dru* types. Several toxin-encoding genes, including *eta*, *seb*, *sek*, *sep*, and *seq*, were detected. The isolates harbored a wide range of AMR properties mirroring the proportions of the classes of antimicrobial agents applied in veterinary medicine in Germany. Multiple novel or rare AMR genes were identified, including the phenicol-lincosamide-oxazolidinone-pleuromutilin-streptogramin A resistance gene *cfr*, the lincosamide-pleuromutilin-streptogramin A resistance gene *vga*(C), and the novel macrolide-lincosamide-streptogramin B resistance gene *erm*(54). Many AMR genes were part of small transposons or plasmids. Clonal and geographical correlations of molecular characteristics and resistance and virulence genes were more frequently observed than temporal relations. In conclusion, this study provides insight into population dynamics of the main epidemic porcine LA-MRSA lineage in Germany over a 13-year-period. The observed comprehensive AMR and virulence properties, most likely resulting from the exchange of genetic material between bacteria, highlighted the importance of LA-MRSA surveillance to prevent further dissemination among swine husbandry facilities and entry into the human community.

IMPORTANCE The LA-MRSA-CC398 lineage is known for its low host specificity and frequent multiresistance to antimicrobial agents. Colonized swine and their related surroundings represent a considerable risk of LA-MRSA-CC398 colonization or infection for occupationally exposed people through which such isolates might be further disseminated within the human community. This study provides insight into the diversity of the porcine LA-MRSA-CC398 lineage in Germany. Clonal and geographical correlations of molecular characteristics and resistance and virulence traits were detected and may be associated with the spread of specific isolates through livestock trade,

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Address correspondence to Stefan Schwarz, stefan.schwarz@fu-berlin.de.

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human occupational exposure, or dust emission. The demonstrated genetic variability underlines the lineage's ability to horizontally acquire foreign genetic material. Thus, LA-MRSA-CC398 isolates have the potential to become even more dangerous for various host species, including humans, due to increased virulence and/or limited therapeutic options for infection control. Full-scale LA-MRSA monitoring at the farm, community, and hospital level is therefore essential.

KEYWORDS LA-MRSA, evolution, antimicrobial resistance monitoring, whole-genome sequencing, cgMLST, antimicrobial susceptibility testing

Livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) has gained increasing attention as an important zoonotic pathogen since it was detected in Dutch swine in 2005 (1, 2). Clonal complex (CC) 398 is the major LA-MRSA lineage found in Europe, North and South America, and Australia (3–5). It has also been identified occasionally in Africa and Asia (6–8). CC398 comprises at least 43 sequence types (STs) (9), but among LA-MRSA, mainly ST398 isolates are described. Swine represent a major reservoir for LA-MRSA-CC398 (9). Such isolates are only occasionally reported as causes of diseases, such as pyoderma, pneumonia, or septicemia in swine, and mainly play a role as colonizers of the skin and the mucosal surfaces (3). However, LA-MRSA-CC398 has no particular host specificity. Thus, it can easily cross species barriers and colonize or infect other animals, such as cattle, sheep, goats, poultry, dogs, cats, horses, rabbits, rats, and mink, as well as humans (1, 10–16). In humans, LA-MRSA-CC398 isolates have mainly been reported from skin and soft tissue infections in the community and from nosocomial wound infections, pneumonia, and even septicemia when introduced into the hospital setting (1, 17). Persons in direct (occupational) contact with livestock are at particular risk of LA-MRSA-CC398 colonization or infection, and further human-to-human transmission cannot be excluded (1). It is important to note that the multiresistance to several classes of antimicrobial agents is typical for the LA-MRSA-CC398 lineage (9). Previous studies have shown that these isolates differ in their *spa* types, antimicrobial susceptibility profiles, and resistance and virulence gene patterns, highlighting the lineage's diversity (3, 4, 18–20).

In this study, LA-MRSA-CC398 collected from diseased swine all over Germany during 13 years on the basis of one isolate per herd were comparatively investigated for their genomic diversity, with an emphasis on virulence and antimicrobial resistance (AMR) properties. Phylogenetic relationships, temporal and/or geographical accumulations of virulence, and AMR gene patterns, as well as the occurrence of mobile genetic elements (MGEs), such as small transposons or plasmids, were explored to understand the population dynamics within the main epidemic LA-MRSA lineage in Germany over time.

RESULTS

Molecular typing of 178 LA-MRSA-CC398 isolates revealed the presence of four STs, 13 *spa* types, 23 *dru* types, and two staphylococcal cassette chromosome *mec* element (SCC*mec*) types. All STs detected were included in CC398, with the majority of isolates belonging to ST398 (*n* = 175; *arcC*_3, *aroE*_35, *glpF*_19, *gmk*_2, *pta*_20, *tpi*_26, *yqiL*_39). Three novel STs were submitted to the database, each differing in one allele from ST398 (ST6759, *n* = 1, *glpF*_45; ST7001, *n* = 1, *glpF*_884; ST7002, *n* = 1, *tpi*_779).

All *spa* and *dru* types identified are displayed in Table 1. The most common *spa* types were t011 (*n* = 120) and t034 (*n* = 38). Most *spa* types were closely related to each other, and t011 probably represented the original type from which the others have developed. In particular, single or multiple repeats were lost (t1451, t1456, t3423), and certain repeats were duplicated (t034, t2011, t2370) or exchanged (t1197). In other cases, most likely, an additional repeat was inserted (t2576), and repeats were deleted as well as duplicated (t1250) or lost and exchanged (t899, t2510, t6228). The most frequent *dru* types detected were dt11a (*n* = 117), dt6j (*n* = 13), and dt11af (*n* = 11). Four new *dru* types (dt8au, dt10ds, dt10dt, dt11dx) were submitted to the database. All *dru* types were closely related to one another, and dt11a most likely can be considered as

TABLE 1 The *spa* and *dru* types observed among the 178 LA-MRSA-CC398 investigated in this study

spa type	Repeat order	No. of isolates	*dru* type	Repeat order	No. of isolates
t011	08-16-02-25-34-24-25	120	dt3c	5a-2d-3e	1
t034	08-16-02-25-02-25-34-24-25	38	dt5e	5a-2d-4a-0-3e	5
t899	07-16-23-02-34	1	dt6j	5a-2d-4a-0-2d-3e	13
t1197	08-16-02-25-46-24-25	2	dt8ap	5a-2d-4a-0-2d-5b-2a-2f	1
t1250	08-16-02-25-02-25	1	dt8au[a]	5a-2d-4a-0-2d-5b-2a-3e	1
t1451	08-16-02-25-34-25	3	dt9v	5a-2d-4a-0-2d-2g-3b-4e-3e	1
t1456	08-16-02-25	1	dt10al	5a-2d-2d-2d-5b-3a-2g-4b-4e-3e	6
t2011	08-16-16-02-25-34-24-25	1	dt10an	4k-4a-0-2d-5b-3a-2g-3b-4e-3e	3
t2370	08-16-16-02-25-02-25-34-24-25	1	dt10ao	5a-4a-0-2d-5b-3a-2g-3b-4e-3e	1
t2510	08-17-25	7	dt10ds[a]	5a-2d-2d-2d-5b-3a-2g-3c-4e-3e	1
t2576	08-12-16-02-25-34-24-25	1	dt10dt[a]	5a-2d-4a-0-2d-6f-3a-2g-3b-4e	1
t3423	08-16-02-25-34-24	1	dt10q	5a-2d-4a-0-2d-5b-3a-2g-2c-4e	1
t6228	35-25-34-24-25	1	dt11a	5a-2d-4a-0-2d-5b-3a-2g-3b-4e-3e	117
			dt11ab	5a-2d-4a-0-2d-5b-3a-2g-3b-4e-2f	3
			dt11af	5a-2d-4a-0-2d-5b-2a-2g-3b-4e-3e	11
			dt11ag	5a-3c-4a-0-2d-5b-2a-2g-3b-4e-3e	2
			dt11ap	5a-2d-4a-0-2d-5b-3a-3o-3b-4e-3e	1
			dt11ax	5a-2d-4a-0-2d-6f-3a-2g-3b-4e-3e	2
			dt11c	5a-2d-4a-0-2d-5b-3a-2g-4b-4e-3e	3
			dt11ca	5a-3c-4a-0-2d-5b-3a-2g-3b-4e-3e	1
			dt11cv	5a-2d-4a-1b-2d-6f-3a-2g-3b-4e-3e	1
			dt11dx[a]	5a-2d-4a-2d-2d-5b-2a-2g-3b-4e-3e	1
			dt11v	5a-2d-4a-0-3c-5b-3a-2g-3b-4e-3e	1

[a]Novel *dru* types identified within this study and submitted to the *dru* typing database.

the founder from which the others developed. Mostly, single or multiple repeats were exchanged (dt11ab, dt11af, dt11ag, dt11ap, dt11ax, dt11c, dt11ca, dt11cv, dt11dx, dt11v) or lost (dt3c, dt5e, dt6j, dt9v, dt10ao). However, repeats also seemed to have been deleted as well as exchanged (dt8ap, dt8au, dt10an, dt10dt, dt10q) or maybe even deleted, duplicated, and exchanged (dt10al, dt10ds).

In 174 isolates, SCC*mec* type Vc(5C2&5) cassettes were detected. One isolate was not subtypeable [SCC*mec* type V(5C2&5)], and another harbored an SCC*mec* type IVa (2B) element. Two isolates carried a truncated SCC*mec* cassette.

The LA-MRSA-CC398 carried a uniform set of virulence genes typically associated with *S. aureus*. All individual virulence gene profiles are given in Data set S1 in the supplemental material. Most importantly, the Panton-Valentine leucocidin (PVL) genes *lukF-PV* and *lukS-PV* and the toxic shock syndrome (TSS) toxin 1 gene *tst* were not identified. Moreover, none of the isolates carried the genes *sak*, *chp*, and *scn*, which are indicative of β-hemolysin-converting phages. All isolates also lacked the exfoliative toxin genes *etb* and *etd*. In contrast, they harbored *eta* and the exotoxin genes *set1*, *set4*, and *set5* as well as several hemolysin genes. The staphylococcal enterotoxin type P gene *sep* was also detected in all LA-MRSA isolates. However, the enterotoxin type B gene *seb* was additionally detected in only four isolates and another four isolates were additionally positive for enterotoxin type K and Q genes *sek* and *seq*. Furthermore, the isolates were negative for the protease genes *splA*, *splB*, and *splE*, but *sspA* (n = 176) and *sspB* (n = 178) were detected almost uniformly among all isolates. The protease-associated genes *hysA*$^{\text{VSa}\beta}$, *paiB*, and *cfim* were also identified in all LA-MRSA. Minor differences were detected regarding genes coding for microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) such as *clfA* (n = 177), *clfB* (n = 171), *cna* (n = 154), *ebh* (n = 173), *efb* (n = 178), *fnbA* (n = 171), *fnbB* (n = 176), *sdrC* (n = 169), *sdrD* (n = 174), and *sdrE* (n = 176). All isolates carried a chromosomal *icaRADBC* operon for biofilm formation. Moreover, 34 isolates harbored an additional *ica*-like gene cluster, most likely on a plasmid. Seven types were identified with the majority of LA-MRSA (n = 20) harboring an *ica*-like operon that was described before on plasmid pAFS11 from a bovine MRSA-ST398 isolate (21). Plasmid pHKS3860 was identified in isolate 4 and harbored a singular type of *ica*-like gene cluster (22). Finally, the von Willebrand factor-binding protein gene *vwb* was identified (n = 177), and all isolates shared capsule type 5.

TABLE 2 Antimicrobial resistance genes harbored by the 178 LA-MRSA-CC398 included in the study

Resistance to:	Gene	No. of isolates	Gene combinations	No. of isolates
β-Lactams	*blaZ*	172[a]	*blaZ* + *mecA*	172
	mecA	178		
Tetracyclines	*tet*(K)	172	*tet*(K) + *tet*(M)	131
	tet(L)	43	*tet*(L) + *tet*(M)	3
	tet(M)	177	*tet*(K) + *tet*(L) + *tet*(M)	40
	tet(38)	178[b]		
Macrolides, lincosamides, streptogramin B	*erm*(A)	23	*erm*(A) + *erm*(B)	5
	erm(B)	39	*erm*(A) + *erm*(C)	4
	erm(C)	48	*erm*(A) + *erm*(54)	1
	erm(T)	10	*erm*(B) + *erm*(C)	2
	erm(54)	1	*erm*(C) + *erm*(T)	3
			erm(A) + *erm*(B) + *erm*(C)	1
Lincosamides	*lnu*(A)	3		
	lnu(B)	9		
Lincosamides, pleuromutilins, streptogramin A	$vga(A)_V$	25		
	$vga(A)_{LC}$	2		
	vga(C)	1		
	vga(E)	21		
	lsa(E)	9		
Aminoglycosides	*aacA-aphD* (gentamicin, kanamycin, tobramycin, amikacin)	15	*aacA-aphD* + *str*	1
			aacA-aphD + *aadD* + *str*	9
	aadD (kanamycin, neomycin, tobramycin)	42	*aadD* + *aadE*	5
			aadD + *str*	20
	aadE (streptomycin)	7	*aadD* + *aadE* + *str*	2
	str (streptomycin)	82		
Aminocyclitols	*spc* (spectinomycin)	24	*spc* + *apmA*	1
	spc_V (spectinomycin)	2	*spc* + *spw* + *apmA*	1
	spd (spectinomycin)	2		
	spw (spectinomycin)	7		
	apmA (apramycin, decreased gentamicin susceptibility)	20		
Trimethoprim	*dfrG*	47		
	dfrK	47		
Phenicols	*fexA*	16		
Phenicols, lincosamides, oxazolidinones, pleuromutilins, streptogramin A	*cfr*	1		

[a]One isolate harbored two *blaZ* copies.
[b]The *tet*(38) gene can be found in nearly every *S. aureus* genome, including phenotypically susceptible isolates, and may confer resistance to tetracycline only when overexpressed (23).

Numerous AMR genes were detected, many of which were carried by small transposons or plasmids. The AMR genes that were found are listed in Table 2 (23), and individual AMR gene profiles are shown in Data set S1. The LA-MRSA carried 3 to 15 different AMR genes, and 158 isolates showed multiresistance (24). All isolates harbored at least one β-lactam resistance gene (*mecA*) and one tetracycline resistance gene. A single isolate had two *blaZ* copies. In several isolates, combinations of genes conferring resistance to members of the same class (Table 2) or to different classes of antimicrobial agents were found. Interestingly, seven isolates harbored the genes *lnu*(B) plus *lsa*(E) plus *aadD* plus *aadE* plus *spw* in combination. The combination of *lnu*(B) plus *lsa*(E) also occurred in another two isolates. In all *apmA*-carrying isolates, *aadD* was also detected. Another isolate was positive for both *fexA* and *cfr*. Besides AMR genes, fluoroquinolone resistance-mediating point mutations in *gyrA* and *grlA* were detected, which resulted in the amino acid exchanges S84L/S80F (n = 8) and S84L/S80Y (n = 13), respectively.

Figure 1a shows that several AMR genes were part of small transposons or the multiresistance *spw* cluster, which is most likely of enterococcal origin (25–37). Overall, all *lnu*(B), $vga(A)_V$, *lsa*(E), *aadE*, spc_V, *spw*, *fexA*, and *cfr* genes identified here were found to be part of such an MGE. Only a fragmented Tn*916* was detected in two *tet*(M)-carrying isolates. One *vga*(E)-carrying isolate harbored a variant of Tn*6133*, which lacked *erm*(A)

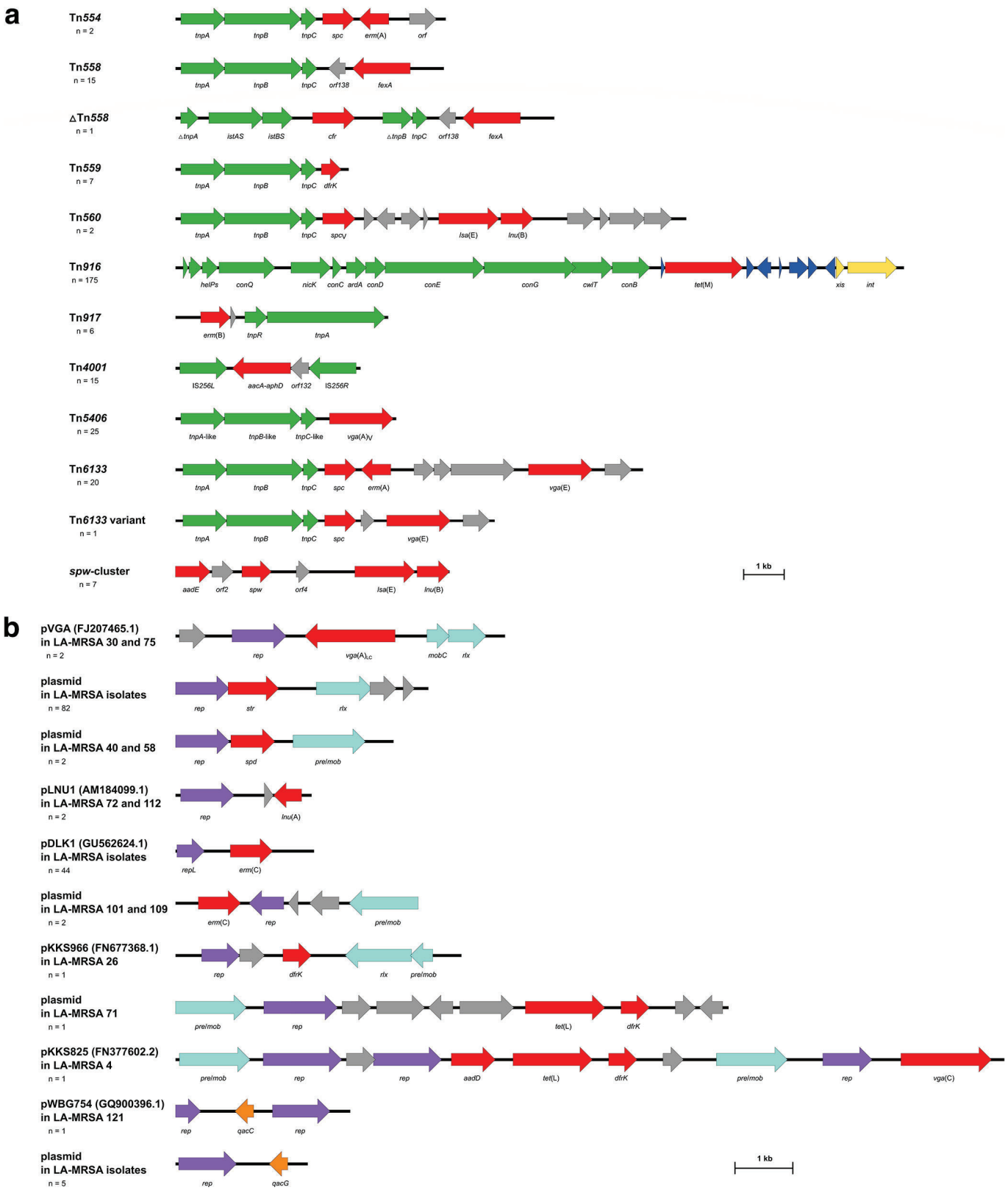

FIG 1 (a and b) Schematic comparison of (a) small transposons and the *spw*-cluster (25–37) and (b) small plasmids carried by the porcine LA-MRSA-CC398 from Germany. The number of isolates that harbored each mobile genetic element (MGE) is given below its designation. In cases where direct matches were found with plasmids from the database, the accession number is given in parentheses after the plasmid designation. The open reading frames are shown as arrows with the arrowhead indicating the direction of transcription. The transposase/conjugative transfer genes, recombination genes, plasmid recombination/mobilization genes, and replication genes are shown in green, yellow, turquoise, and purple, respectively. The antimicrobial resistance (AMR) genes, biocide resistance genes, transcriptional regulation genes, and genes encoding other functions are displayed in red, orange, dark blue, and gray, respectively. Truncated genes are indicated by a triangle, and size scales are given in the bottom-right corners.

and three further open reading frames. Tn*4001* is only 4.5 kb in size and comprises an *aacA-aphD* gene flanked by two IS*256* insertion sequence elements. Thus, the complete Tn*4001* could not be identified based on Illumina short-read sequences. Nevertheless, *aacA-aphD* was intact in all 15 isolates carrying this gene. However, the presence of the complete Tn*4001* in one isolate with a closed genome also suggested its presence in the remaining 14 isolates. Besides a complete *fexA*-carrying transposon, Tn*558*, detected in 15 isolates, a truncated ΔTn*558* in which the transposase genes Δ*tnpA* and Δ*tnpB* were partly deleted and replaced by the genes *istAS*, *istBS*, and *cfr*, was identified in a single isolate.

Several AMR genes were detected on small plasmids of 2,360 to 5,713 bp in size, including all *vga*(A)$_{LC}$, *str*, and *spd* genes as well as almost all *erm*(C) and *lnu*(A) genes of the LA-MRSA collection. In one and two isolate(s), the genetic environment of *lnu*(A) and *erm*(C), respectively, was fragmented. The *dfrK* gene was also identified on a small plasmid of 5,594 bp in one isolate. In addition, *dfrK* was found on a 9,585-bp plasmid with *tet*(L) in one isolate and on a 14,362-bp plasmid in combination with *aadD*, *vga*(C), and *tet*(L) in another isolate. A 36,929-bp plasmid harboring *erm*(54) next to an *ica* locus, the cadmium resistance genes *cadD* and *cadX*, the mercury resistance genes *merR* and *merA*, and the copper resistance genes *copB* and *mco* was described in one isolate (22). Moreover, six isolates harbored a small plasmid of 2,216 to 2,295 bp that carried a biocide resistance gene, either *qacC* (n = 1) or *qacG* (n = 5), both mediating elevated MICs to quaternary ammonium compounds such as benzalkonium chloride. In accordance with a previous study that described *qacC*-harboring *S. aureus* isolates from primates, which showed a slightly elevated MIC of 0.0004% for benzalkonium chloride (38), these isolates exhibited MIC values of 0.0005 to 0.001% for this biocide. Representative plasmid maps are shown in Fig. 1b.

The genotypic AMR profiles correlated almost completely with the phenotypic ones. Antimicrobial susceptibility testing results are displayed in Table 3. A total of 67 phenotypic AMR profiles were observed (Data set S1). For one *erm*(A)-positive isolate, inducible macrolide/lincosamide resistance was detected. MIC values of linezolid and quinupristin-dalfopristin were not available for all LA-MRSA, but in one isolate they confirmed expression of the *cfr* gene. Despite the presence of an AMR gene in the genome, the associated phenotype was not detected in one *fexA*-carrying, three *lnu*(A)-carrying, and eight *vga*(A)$_V$-carrying isolates. Changes in neither the nucleotide sequences of the respective resistance genes nor the associated promoter regions were found. In addition, four of 13 isolates harboring the S84L/S80Y exchanges in GyrA and GrlA showed MIC values lower than those considered resistant (Table 3). No resistance-mediating genes or point mutations were identified in two isolates exhibiting resistance to erythromycin and gentamicin, respectively.

The LA-MRSA-CC398 displayed close phylogenetic relationships. The minimum spanning tree based on core-genome multilocus sequence typing (cgMLST) revealed 177 allelic profiles comprising 9 clusters and 9 singletons (Fig. 2). Isolates 45 and 46 shared a profile. The profiles varied in 0 to 156 target genes of 1,569 alleles included (Data set S2, Fig. 2), and isolates were assigned to clusters with a maximum threshold of 29 allelic differences. Cluster 1 represented the main cluster comprising 119 isolates and started to emerge in 2007. Clusters 2 and 3 also developed starting in 2007 and finally consisted of seven and two isolates, respectively. Clusters 4, 5, and 6, including 5, 2, and 17 isolates, respectively, began to arise in 2008. Cluster 7 comprised 13 isolates and was first identified in 2009. Most recently, clusters 8 and 9 started to emerge in 2018, and both included two isolates. The relation between phylogenetic clustering and spatial distribution of the LA-MRSA-CC398 is demonstrated in Fig. 3 and Table 4.

Isolates within a cluster shared specific characteristics, and three clusters showed geographical correlations. The 119 isolates associated with the largest cluster, cluster 1, were found all over Germany during the whole study period. Remarkably, 114 of them displayed the dominant *spa* type, t011. Three isolates had *spa* type t1451, and one isolate each *spa* type t2011 or t2576, all present exclusively in cluster 1. The second most common *spa* type, t034 (n = 38), was not found within cluster 1, but was found in clusters 3 to 8 and among the singletons. Several AMR genes were exclusively observed within cluster 1, including *erm*(T), *vga*(A)$_{LC}$ on a plasmid, *aacA-aphD*, *apmA*,

TABLE 3 Distribution of MIC values for the 178 LA-MRSA-CC398 isolates identified in this study

	No. of isolates with MIC (mg/L) of:																	Isolates that are:					
																		Susceptible		Intermediate		Resistant[a]	
Antimicrobial agent(s)	**0.008**	**0.015**	**0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**512**	***n***	**%**	***n***	**%**	***n***	**%**
Oxacillin	X	-	-	-	-	-	-	-	-	13	71	*94*	X	X	X	X	X	-	**-**			178	**100**
Penicillin[b]	X	-	-	-	-	-	-	1	-	4	21	83	43	*26*	X	X	X	-	**-**			178	**100**
Ampicillin[b]	X	X	-	-	-	-	-	1	-	13	48	75	24	14	*3*	X	X	-	**-**			178	**100**
Amoxicillin/clavulanic acid[b,c]	X	X	-	-	-	-	-	1	21	100	46	9	1	-	X	X	X	-	**-**			178	**100**
Ceftiofur[b]	X	X	-	-	-	-	-	-	2	86	62	21	4	2	*1*	X	X	-	**-**			178	**100**
Cefquinome[b]	X	-	-	-	-	-	-	7	90	59	21	1	-	X	X	X	X	-	**-**			178	**100**
Cefotaxime[b]	X	-	-	-	-	-	-	-	-	2	92	62	19	*3*	X	X	X	-	**-**			178	**100**
Cefoperazone[b]	X	X	X	-	-	-	-	-	-	-	91	66	17	*4*	X	X	X	-	-			178	**100**
Erythromycin	X	-	-	-	-	23	47	1	1	1	-	1	-	*104*	X	X	X	70	**39**	3	**2**	105	**59**
Tylosin tartrate	X	X	X	-	-	-	-	58	26	1	1	1	-	-	-	*91*	X						
Tilmicosin	X	X	X	-	-	-	1	70	5	4	3	1	-	-	-	94	X						
Clindamycin	X	X	-	3	47	16	4	-	-	-	11	4	1	-	*92*	X	X	70	**39**	-	**-**	108	**61**
Pirlimycin	X	X	-	-	-	12	44	12	1	-	1	8	8	-	*92*	X	X						
Enrofloxacin		-	-	3	84	48	6	14	6	14	3	-	X	X	X	X	X	161	**90**	-	**-**	17	**10**
Gentamicin	X	X	X	X	-	51	75	14	1	11	10	-	5	8	3	-	X	152	**85**	10	**6**	16	**9**
Tetracycline	X	X	X	X	-	-	-	-	-	-	-	-	1	10	167	-	X	-	**-**	-	**-**	178	**100**
Sulfamethoxazole/trimethoprim[c]	X	-	2	65	17	16	39	22	9	8	-	-	-	X	X	X	X	170	**96**			8	**4**
Vancomycin	X	-	-	-	-	-	94	82	2	-	-	-	-	X	X	X	X	178	**100**	-	**-**	-	**-**

[a]Isolates were classified as susceptible, intermediate, or resistant if breakpoints were available in the CLSI document VET01S or M100 (97, 98). Despite the lack of breakpoints, isolates that showed high MIC values of enrofloxacin (≥4 mg/L) were considered resistant (3).

[b]Since oxacillin-resistant staphylococci are resistant to all currently available β-lactams, except for ceftaroline, resistance to these agents can be deduced from the oxacillin MIC values (97, 98).

[c]MIC values of amoxicillin/clavulanic acid (2:1) and sulfamethoxazole/trimethoprim (19:1) are given as amoxicillin and trimethoprim MIC values, respectively. Concentrations not included in the test panel are marked with an X or italic text. Isolates displayed in the lowest not-tested concentration showed growth in the highest test concentration, and the MIC is equal to or greater than the concentration following the highest test concentration. Black vertical lines indicate, if available, the CLSI breakpoints used to classify the isolates as susceptible, intermediate (if available), or resistant.

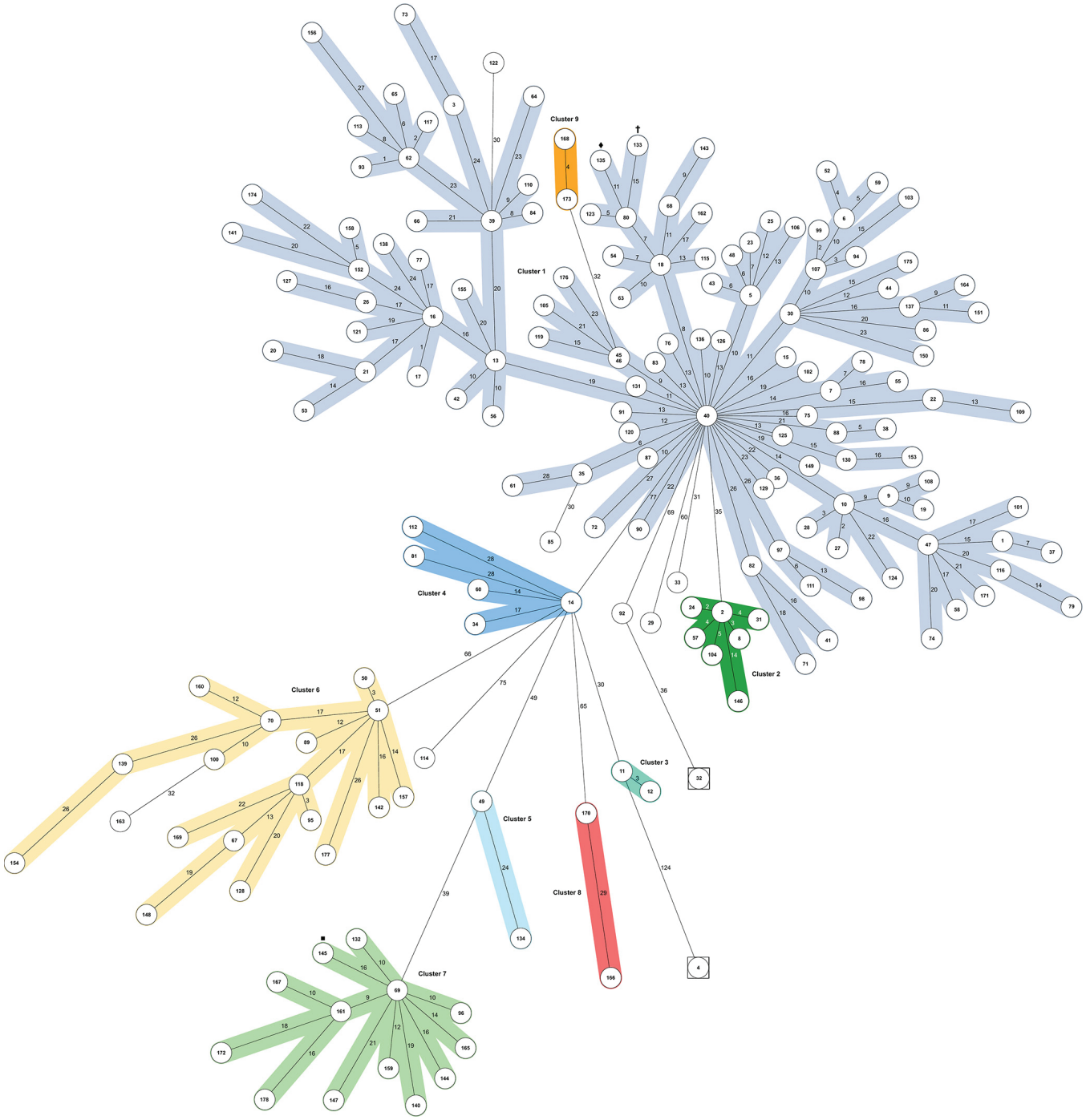

FIG 2 Minimum spanning tree displaying the phylogenetic relationship of the 178 LA-MRSA-CC398 isolates from Germany based on core-genome multilocus sequence typing (cgMLST) analysis with SeqSphere+, including 1,569 alleles. The circles represent different allelic profiles, and the count of varying target genes between them is shown next to the connecting lines. The nine clusters are illustrated in different colors: cluster 1 in blue-gray, cluster 2 in dark green, cluster 3 in turquoise, cluster 4 in dark blue, cluster 5 in light blue, cluster 6 in yellow, cluster 7 in light green, cluster 8 in red, and cluster 9 in orange. The two isolates carrying a truncated SCC*mec* cassette are framed by a box. The three isolates not belonging to sequence type (ST) 398 are marked by a rhombus (ST6759), square (ST7001), or cross (ST7002).

spd on a plasmid, and *cfr* as part of ΔTn*558* (see Table 2). Moreover, most of the *tet*(L)-, *erm*(B)-, *erm*(C)-, and *dfrK*-carrying isolates (36/43, 27/39, 31/48 [on plasmid], and 38/47, respectively) were found within cluster 1, while only four out of 23 *erm*(A)-harboring and five out of 47 *dfrG*-harboring isolates were detected here. Almost all *fexA*-carrying isolates (13/16) were also associated with this cluster, with 12 isolates harboring Tn*558*

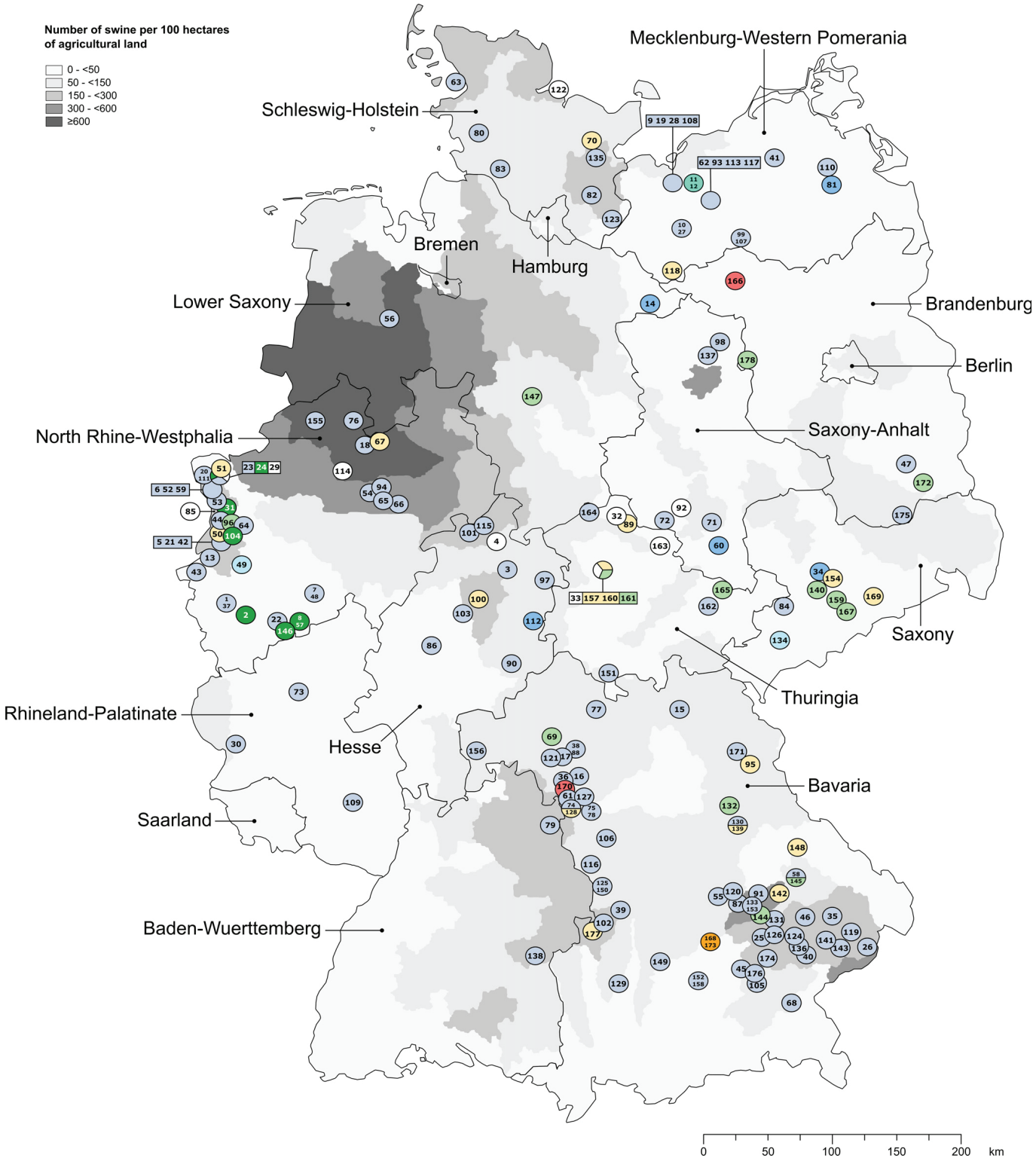

FIG 3 Swine density in Germany for the year 2020 and approximate locations of the farms from which the LA-MRSA-CC398 isolates originated based on zip code regions. The swine density is given as the number of swine per 100 ha of agricultural land according to the German federal and state statistical offices (https://agraratlas.statistikportal.de/#). A color legend is given in the top-left corner, and a distance scale is displayed in the bottom-right corner. The isolate IDs are given within the circles or rectangles, which contain more than one number in cases where multiple isolates were found within the same zip code area. The various colors of the circles and rectangles refer to the differentiation of isolate clusters shown by the minimum spanning tree in Fig. 2: cluster 1 in blue-gray, cluster 2 in dark green, cluster 3 in turquoise, cluster 4 in dark blue, cluster 5 in light blue, cluster 6 in yellow, cluster 7 in light green, cluster 8 in red, and cluster 9 in orange.

TABLE 4 Isolates identified by federal state and year, including the affiliation to a cluster

Federal state[a] (no. of isolates/state in total)	Yr[b]	Isolate ID(s)	Isolates within the same zip code regions	Cluster(s)
Baden Wuerttemberg (2)	2009	79		1
	2016	138		1
Bavaria (63)	2008	15, 16, 17, 25, 26, 35, 36, 38, 39, 40, 45, 46, 55, 58		1
	2009	61, 68, 69, 74, 75, 77, 78	75 and 78	1, 7
	2010	87, 88, 91, 95, 102, 105, 106, 116		1, 6
	2012	119, 120, 121		1
	2013	124, 125, 126, 127, 128, 129, 130, 131, 132, 133		1, 6, 7
	2015	136		1
	2016	139, 141, 142, 143, 144, 145, 148, 149, 150, 152, 153, 156, 158	152 and 158	1, 6, 7
	2018	168, 170, 171, 173, 174	168 and 173	1, 8, 9
	2019	176, 177		1, 6
Brandenburg (5)	2008	47		1
	2018	166, 172		7, 8
	2019	175, 178		1, 7
Hesse (10)	2007	3, 4		1, singleton
	2010	86, 90, 97, 100, 101, 103, 112, 115		1, 4, 6
Lower Saxony (3)	2008	14, 56		1, 4
	2016	147		7
Mecklenburg-Western Pomerania (18)	2007	9, 10, 11, 12, 19	9 and 19; 11 and 12	1, 3
	2008	27, 28, 41		1
	2009	62, 81		1, 4
	2010	93, 99, 107, 108, 110, 113	93 and 113; 99 and 107	1
	2011	117, 118		1, 6
North Rhine-Westphalia (41)	2007	1, 2, 5, 6, 7, 8		1, 2
	2008	13, 18, 20, 21, 22, 23, 24, 29, 31, 37, 42, 43, 44, 48, 49, 50, 51, 52, 53, 54, 57	21 and 42; 23, 24, and 29	1, 2, 5, 6, singleton
	2009	59, 64, 65, 66, 67, 76		1, 6
	2010	85, 94, 96, 104, 111, 114		1, 2, 7, singletons
	2016	146, 155		1, 2
Rhineland-Palatinate (3)	2008	30		1
	2009	73		1
	2010	109		1
Saxony (8)	2008	34		4
	2010	84		1
	2013	134		5
	2016	140, 154, 159		6, 7
	2018	167, 169		6, 7
Saxony-Anhalt (6)	2009	60, 71, 72		1, 4
	2010	92, 98		1, singleton
	2015	137		1
Schleswig-Holstein (8)	2009	63, 70, 80		1, 6
	2010	82, 83		1
	2012	122		Singleton
	2013	123		1
	2014	135		1
Thuringia (11)	2008	32, 33		Singletons
	2010	89		6
	2016	151, 157		1, 6
	2017	160, 161, 162	160 and 161	1, 6, 7
	2018	163, 164, 165		1, 7, singleton

[a]No isolates were detected in the federal states Berlin, Bremen, Hamburg, and Saarland.
[b]Numbers of isolates detected per year in total: 2007, *n* = 13; 2008, *n* = 45; 2009, *n* = 23; 2010, *n* = 35; 2011, *n* = 2; 2012, *n* = 4; 2013, *n* = 12; 2014, *n* = 1; 2015, *n* = 2; 2016, *n* = 22; 2017, *n* = 3; 2018, *n* = 12; 2019, *n* = 4.

and one harboring ΔTn*558*. Moreover, the *qacC*-positive isolate and three of five *qacG*-carrying LA-MRSA were part of cluster 1.

The seven cluster 2 isolates originated exclusively from North-Rhine Westphalia, near the Dutch border, with two isolates from the same zip code area. The *spa* (t2510) and *dru* (dt10al, dt10ds) types detected were unique within the LA-MRSA collection.

The *vga*(A)$_V$ gene as part of Tn*5406* was detected in all seven isolates. They showed a multiresistance profile harboring five to 10 AMR genes. Six of them carried *erm*(B), *aadD*, *tet*(K), and *tet*(L) as well as *tet*(M) on Tn*916*, which was, however, in one isolate only available fragmented. Five isolates harbored a *dfrK* gene. Moreover, three of four isolates positive for the enterotoxin genes *sek* and *seq* were associated with cluster 2.

Two isolates assigned to cluster 3 were collected in 2007 within the same zip code region in Mecklenburg-Western Pomerania. They were multiresistant and had an identical AMR gene profile, including *blaZ*, *mecA*, *tet*(K), *tet*(M) as part of Tn*916*, *erm*(C) on a plasmid, and *dfrG*.

All five cluster 4 isolates were collected relatively far apart from each other from 2008 to 2010 in central and northern Germany. They harbored AMR genes conferring resistance to three to five classes of antimicrobial agents and, thus, were classified as multiresistant. Three isolates harbored *vga*(A)$_V$ as part of Tn*5406*, and all isolates were positive for *dfrG*.

Although the two cluster 5 isolates originated from very different locations (North-Rhine Westphalia/Saxony) and years (2008/2013), they carried the same AMR genes (*blaZ*, *mecA*, *tet*(K), Tn*916* with *tet*(M), *str* on a plasmid, *dfrG*).

A wide distribution over Germany was also seen for the 17 cluster 6 isolates collected during almost the complete study period. Interestingly, all five isolates with *dru* type dt5e and 12 of 13 isolates with *dru* type dt6j were observed within this cluster. With eight to 11 AMR genes, the isolates harbored a particularly large number of resistance genes. The multiresistance profiles were almost identical [*blaZ*, *mecA*, *tet*(K), Tn*916* with *tet*(M)—fragmented in one isolate, *erm*(A), *erm*(B), *erm*(C), *vga*(E), *str* on a plasmid, *spc*, *dfrG*; individual losses of *erm*(B) and/or *erm*(C) and/or *str*]. In these isolates, 17 of 23 *erm*(A) genes were detected as part of Tn*6133* with *vga*(E) and *spc*, alone or in combination with either *erm*(B) found on Tn*917* (6/6 isolates) or *erm*(C) on a plasmid. Considering *vga*(E) and *spc*, 17 out of 21 and 24 positive isolates, respectively, were identified within this cluster.

Cluster 7 comprised 13 isolates identified at greater distances to each other mainly in central and southern Germany. Interestingly, 10 of 11 *dru* type dt11af isolates were part of this cluster. Seven isolates carried *erm*(C) on a plasmid, and three isolates carried *fexA* as part of Tn*558*. The same six isolates harbored, next to *aadD*, the *spw*-cluster that also comprised *lnu*(B), *lsa*(E), and *aadE*. This combination was otherwise only identified in a single cluster 1 isolate. Moreover, all cluster 7 isolates were positive for *dfrG* and the same kind of variations in GyrA and GrlA (S84L/S80Y), which were not detected in other isolates.

The two cluster 8 isolates from 2018 differed not only in their origin (Brandenburg/Bavaria), but also in their AMR gene profiles. However, both isolates exhibited a multiresistance profile, including *dfrG* and Tn*560* comprising *lnu*(B), *lsa*(E), and *spc*$_V$. The *spc*$_V$ gene was not found in other isolates.

The two cluster 9 isolates from 2018 originated from the same zip code region in Bavaria. They carried only the AMR genes *blaZ*, *mecA*, *tet*(K), and, as part of Tn*916*, *tet*(M) and were resistant only to β-lactams and tetracyclines.

Eight of the remaining nine singletons were isolated in central Germany, while one isolate came from northern Schleswig-Holstein. The only isolate of the collection harboring *vga*(C) [on a plasmid with *aadD*, *dfrK*, and *tet*(L)] and *erm*(54) (on another plasmid with heavy metal resistance genes) was found among these singletons. Considering trimethoprim resistance, *dfrG* and *dfrK* were detected here. Three of eight isolates positive for one or two enterotoxin gene(s) in addition to *sep* also belonged to the singletons.

Locations of the farms from which the LA-MRSA-CC398 isolates originated largely reflected the pork production density in Germany, and further geographical correlations were revealed. Most of the 178 isolates were collected in southern Germany (Bavaria, $n = 63$) and western Germany near the Dutch border (North-Rhine Westphalia, $n = 41$) (Fig. 3, Table 4).

The *spa* type t1197 was detected in two isolates found relatively close to each other (32 and 92, Fig. 3). Moreover, two of three *spa* type t1451 isolates were collected close to each other in Bavaria (16 and 17, Fig. 3). All seven *spa* type t2510 isolates were identified exclusively in North-Rhine Westphalia grouped within cluster 2 as mentioned

above. Six of these isolates displayed *dru* type dt10al, which was not observed more often. Two of three *dru* type dt11ab isolates originated from relatively close locations on the border between Baden-Württemberg and Bavaria (79 and 116, Fig. 3).

One isolate carrying the enterotoxin gene *seb* and all four isolates positive for the enterotoxin genes *sek* and *seq* were detected in proximity to each other in North-Rhine Westphalia (155/29, 31, 57, and 104, Fig. 3).

The most frequently detected AMR genes within our collection were spread throughout Germany. However, isolates harboring specific genes still aggregated partly in particular regions. Furthermore, some genes were found in nearby isolates but also in single LA-MRSA isolates from more distant areas. AMR genes that occurred less frequently were often found in isolates of more distant geographical origin. Interestingly, *aacA-aphD* was mainly identified in isolates from Mecklenburg-Western Pomerania (9, 10, 19, 27, 28, 108) and Bavaria (58, 74, 61, 116, 124, 171) (Fig. 3). Four and two isolates, respectively, from Mecklenburg-Western Pomerania even originated from the same zip code region. Out of 20 *apmA*- and *aadD*-positive isolates, five (63, 80, 83, 123, 135; Fig. 3) originated from Schleswig-Holstein, and six of these 20 isolates (44, 52, 54, 59, 76, 94; Fig. 3) originated from North-Rhine Westphalia. Only two isolates (40 and 58) harbored *spd* on a plasmid, but both were identified relatively nearby in Bavaria (Fig. 3). Out of 16 isolates carrying *fexA* on Tn*558*, 11 aggregated in two locations in Bavaria (74, 75, 127, 45, 46, 141, 144, 145, 152, 158, 174; Fig. 3). In addition, several isolates that were collected in nearby locations showed identical resistance profiles. Out of five isolates carrying the biocide resistance gene *qacG* on a plasmid, two isolates each were found in Thuringia (151 and 164) and Brandenburg (166 and 178) (Fig. 3).

Temporal correlations of typing results, virulence, and resistance properties were observed less often. The total number of isolates per year varied from one (2014) to 45 (2008) (Table 4). From 2007 to 2019, most of the isolates investigated were obtained by 2010 ($n = 116$). In years with higher isolate numbers, a distribution all over Germany or at least over certain regions was observed (Table 4). Some isolates were even identified in the same zip code region in the same year. They also belonged to the same respective clusters, except for some isolates in North-Rhine Westphalia (23, 24, 29) and Thuringia (160, 161) (Table 4, Fig. 3).

Certain *spa* (t1197, t1451) and *dru* (dt10al, dt11ag, dt11ax, dt11c) types occurred only during the early years of our study period until 2010.

One isolate from 2007 and 22 isolates from 2008 were negative for the collagen adhesion-encoding gene *cna*. Considering later years, this was recognized only in 2018 for two LA-MRSA isolates. Furthermore, six of the eight isolates harboring either *seb* or *sek* and *seq* were from 2008 (*seb*, $n = 1$; *sek*/*seq*, $n = 3$) or 2010 (*seb*, $n = 1$; *sek*/*seq*, $n = 1$) and, thus, were identified only in the early years of our study period.

The presence of specific AMR determinants was overall not limited to certain years. Exceptions were the genes *vga*$(A)_{LC}$ on a plasmid (2008 to 2009), *spc*$_V$ as part of Tn*560* together with *lnu*(B) and *lsa*(E) (2018), and *spd* on a plasmid (2008). However, in each case only two LA-MRSA isolates carrying the respective gene(s) were found. Isolates harboring a *dfr* gene were detected during the whole study period. Until 2015, mainly *dfrK* was identified. However, since 2016 the dominant variant apparently changed so that more *dfrG*-positive than *dfrK*-positive LA-MRSA isolates were found. In 2018 and 2019, *dfrK* was not detected. The isolates carrying *qacG* on a plasmid from nearby locations in Thuringia and Brandenburg were collected in later years of the study period (2016 to 2019).

DISCUSSION

Conventional systems still dominate among swine farms in Germany (https://www.giscloud.nrw.de/arcgis/apps/storymaps/stories/5e62a2b3316a45e18a356d7d6a6afeae). They have shown high percentages of LA-MRSA-positive samples, with CC398 dominating by far, while LA-MRSA was reported to be nearly absent in the more rarely occurring organic farms (20, 39). More precisely, LA-MRSA-CC398 has been reported to colonize the animals asymptomatically in almost half of German swine farms (20, 40) and up to 86% of

humans occupationally exposed to them (17, 40). Fortunately, extended human-to-human transmission happened rarely, and nasal LA-MRSA carriage was found in only 0.08 to 0.2% of humans at hospital admission in Germany as a whole (17). Nonetheless, numbers are notably higher in areas of high livestock density (17, 41). In this study, LA-MRSA-CC398 was also recognized to be widespread all over Germany, largely reflecting the pork production density (https://agraratlas.statistikportal.de/#). However, isolate submission to the national resistance monitoring program GE*RM*-Vet occurs on a voluntary basis and includes only isolates from diseased animals; hence, the program does not necessarily reflect the true prevalence of monitored pathogens.

The cgMLST analysis revealed close relationships between the LA-MRSA-CC398 isolates, as their allelic profiles varied in only 0 to 9.9% of 1,569 alleles included. The assignment of isolates to a cluster was based on a previous study stating that *S. aureus* isolates displaying ≥30 allelic variations should be considered unrelated (42). Next to the main cluster (cluster 1) comprising most of the LA-MRSA, new side clusters and singletons emerged over time, which points toward an ongoing diversification within the porcine LA-MRSA-CC398 lineage resulting in novel variants. Our discovery of new STs and *dru* types underlines this assumption. All *spa* types detected were related and typically associated with LA-MRSA-CC398 (20, 43–45). The *dru* types identified were also related, and many have already been known to occur in LA-MRSA-CC398 (dt3c, dt5e, dt6j, dt10q, dt11a, dt11ab, dt11af, dt11ap, dt11c, dt11v) (46–49). However, *dru* type dt9v was previously described only in MRSA-CC5 (48, 49) and the types dt10ao, dt11ax, and dt11ca only in methicillin-resistant *Staphylococcus pseudintermedius* (50–52). In accordance with previous literature (53), exclusively SCC*mec* type IV and V elements were found in the LA-MRSA-CC398 besides two truncated cassettes. The multitude of *spa* and *dru* types identified emphasizes the molecular diversity within CC398 that has already been reported and is still increasing (9, 20).

LA-MRSA-CC398 typically lacked major virulence-associated factors often found in community- or hospital-associated MRSA, such as TSS toxin or PVL (17, 54). Nevertheless, they harbored genes encoding hemolysins, exotoxins, enterotoxins, and an exfoliative toxin. Exfoliative toxin-producing *S. aureus* strains can induce greasy pig disease along with the usual causative agent *Staphylococcus hyicus* (55). This is of clinical relevance for LA-MRSA-positive swine farms, and one isolate of our collection originated from an animal with this medical history. The exfoliative toxin gene *eta* was also detected in an MRSA-ST398 from a hospitalized Chinese child (56). Interestingly, *eta* and enterotoxin genes were previously described as less frequent in LA-MRSA-CC398 (54, 57). All LA-MRSA carried the protease-associated genes $hysA^{VSa\beta}$, *paiB*, and *cfim*, whose prevalence in *S. aureus* was suggested to result in enhanced proteolytic activity (58). Consequently, these genes may be part of virulence-associated genomic traits that might account for the success of epidemic clones (58). However, so far, only the enhanced pathogenicity of LA-MRSA-CC398 due to $hysA^{VSa\beta}$ has been functionally confirmed (59).

LA-MRSA-CC398 typically exhibited a wide range of AMR to different classes of antimicrobial agents. In agreement with the literature (57), part of the isolates showed only resistance to β-lactams and tetracyclines (n = 20), while more comprehensive phenotypes were also recognized. The AMR profiles mirrored the amounts of antimicrobial agents dispensed in veterinary medicine in Germany (https://www.bvl.bund.de/SharedDocs/Pressemitteilungen/05_tierarzneimittel/2022/2022_PM_Abgabemengen_Antibiotika_Tiermedizin.html). Penicillins and tetracyclines account for the largest shares, followed by sulfonamides, polypeptides, and macrolides. These classes of antimicrobial agents are also commonly used on German swine farms (60, 61).

Next to AMR genes commonly found among staphylococci of animals (29, 57, 62), the novel *erm*(54) gene (22) and several other resistance genes were found in ≤10 isolates, including *erm*(T), *lnu*(A), *lnu*(B), $vga(A)_{LC}$, *vga*(C), *lsa*(E), *aadE*, spc_V, *spd*, *spw*, and *cfr*. Since *cfr* confers transferable resistance also to oxazolidinones, which are last-resort antimicrobial agents in human medicine, there is particular interest in the dissemination of this gene (63). As is known for LA-MRSA (62), several AMR genes mediating the

same resistance phenotype were detected here, and many isolates harbored two or three AMR genes accounting for the same resistance property (Table 2). This might be due to an acquisition at different times and/or their location on MGEs carrying additional AMR genes (62). In fact, a large proportion of AMR genes was found to be part of small transposons or plasmids and, as such, might be transferred easily by horizontal gene transfer across strain, species, or genus boundaries. In addition to persistence of AMR genes in bacterial populations directly linked to the usage of certain antimicrobial agents, coselection processes due to more than one AMR gene or additional biocide and heavy metal resistance genes located on the same MGE need to be taken into account (62). MGEs harboring more than one AMR gene were also identified here. The characterization of SCC*mec* cassettes and possible, larger (multiresistance) plasmids among the 178 LA-MRSA isolates should be the subject of a follow-up study, because these elements could not be characterized completely here, as data from hybrid genome assembly were only available for a limited number of isolates.

Several characteristics of the LA-MRSA-CC398 were related to their phylogenetic affiliation to a cluster and/or their geographical origin. For example, many *spa* or *dru* types and AMR or biocide resistance genes were identified to a large extent or exclusively in isolates within the same cluster. The isolates within a cluster also often displayed similar or even identical AMR gene combinations. The main cluster 1, comprising most of the isolates, showed the greatest variety in molecular characteristics and AMR properties. Regional accumulations were most likely to be seen in clusters 2, 3, and 9. However, the latter two only comprised two isolates each. Isolates originating from the same zip code areas mainly belonged to the same cluster and/or were often identified in the same year. Certain *spa* and *dru* types were associated with specific areas in Germany. A spatial connection was also revealed for the detection of the biocide resistance gene *qacG* on a plasmid as well as the enterotoxin genes *seb*, *sek*, and *seq*. Regarding *sek* and *seq*, an association with cluster 2 was observed. Frequently detected AMR genes were spread all over Germany, but aggregation was also recognized in individual regions. More rarely occurring genes were observed in isolates from distant regions, although accumulation in individual areas was sometimes also revealed. In addition, similar or even identical AMR gene profiles were recognized in isolates originating from nearby regions. Temporal correlations of certain characteristics were recognized less often. Some *spa* or *dru* types were only seen in isolates identified during the early years. Isolates negative for *cna* or harboring one or two additional enterotoxin gene(s) besides *sep* tended to occur at the beginning of the study period. Next to a cluster-associated distribution, the AMR genes *dfrG* and *dfrK* most likely showed a time-dependent dissemination. The presence during limited periods was observed only for AMR or biocide resistance genes detected in small numbers. Finally, the exchange of genetic material between bacteria and their clonal dissemination enhanced by livestock trading activities, human occupational exposure, or emission of dust might be reasons for the isolates' close phylogenetic relationships and the clonal and geographical correlations of characteristics outweighing temporal relations.

Studies of porcine LA-MRSA-CC398 have also been performed in other European countries (18, 64–69). The available information suggests that the AMR and virulence properties of those isolates are comparable to the characteristics of the LA-MRSA-CC398 isolates from Germany investigated in the present study.

Germany has established instruments providing antibiotic consumption data from human and veterinary medicine; however, region-specific data are needed (70) to evaluate the influence of possibly preferred agents on the distribution of individual resistance properties in specific areas and/or at certain times. Moreover, regarding all observed correlations here, it is worth noting that samples were not collected evenly over time and across the federal states in the GE*RM*-Vet program. Especially in the early years, the northern German federal states were overrepresented, and for Lower Saxony—the state with the highest swine density—too few isolates were included to get a coherent picture for all of Germany. Considering the lineage's high diversification and transmission ratios and the intensity of livestock trade (9), a large-scale monitoring of

LA-MRSA is needed so that intervention is possible in case novel, possibly more virulent and/or resistant variants are emerging. The prevalence of LA-MRSA will most likely not decrease (9), and the latest data available, covering part of our study period, even indicated an increase in the proportion of LA-MRSA in the German MRSA population, with up to 20% in "swine-dense" regions (71). If decolonization programs are targeted, a combination of measures concerning different husbandry factors needs to be considered (39).

In summary, this study uncovered diversity and population dynamics of the epidemic porcine LA-MRSA lineage in Germany over a 13-year-period. Clonal and geographical correlations of molecular characteristics, virulence, and resistance properties were identified. Full-scale LA-MRSA monitoring is needed to detect new emerging, possibly more dangerous clones and to prevent LA-MRSA transmission between livestock farms and introduction into human community or health care settings.

MATERIALS AND METHODS

Bacterial isolates. In total, 178 MRSA-CC398 isolates from diseased swine were investigated in this study. The isolates originated from local diagnostic facilities in Germany and were included in the national resistance monitoring program G*ERM*-Vet during the years 2007 to 2019 on the basis of one isolate per swine herd. According to the background information from the diagnostic laboratories, 92 of the 178 isolates were presumably causative of the respective diseases (skin infections [$n = 53$], gastritis/enteritis [$n = 9$], musculoskeletal system infections [$n = 7$], septicemia [$n = 6$], central nervous system infections [$n = 5$], urinary-genital tract infections [$n = 4$], abscess/mammary lump [$n = 2$], staphylococcal infection [$n = 2$], abortion [$n = 1$], enterotoxemia [$n = 1$], greasy pig disease [$n = 1$], and polyserositis [$n = 1$]). One animal suffered from skin as well as central nervous system infection. Another 57 isolates were obtained from swine with respiratory tract infections and most likely represented colonizers of the respiratory mucosa, which were coisolated with other respiratory tract pathogens, such as *Pasteurella multocida*. For 25 isolates no background data were available except that the isolates originated from deceased animals. Finally, three isolates were probably only incidental findings and not causative agents of the respective diseases (streptococcal sepsis [$n = 1$], vitamin E and selenium deficiency [$n = 1$], and volvulus [$n = 1$]).

Short-read and long-read sequencing. All 178 LA-MRSA-CC398 isolates were subjected to short-read sequencing. Genomic DNA of 152 isolates was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) with adaptions for staphylococci as described previously (72). The libraries were prepared using the Nextera XT DNA Library Preparation Kit (Illumina, Inc., San Diego, CA, USA) according to the manufacturer's recommendations, followed by 2 × 300-bp paired-end sequencing in 40-fold multiplexes on the MiSeq platform with the MiSeq reagent kit v3 (Illumina, Inc.). Genomic DNA of the remaining 26 isolates was extracted using the HiPure Bacterial DNA Kit (Magen, Guangzhou, China). Here, the libraries were prepared with the KAPA Hyper Prep Kit (Kapa Biosystems, Boston, MA, USA). Sequencing was performed on the Illumina HiSeq X-Ten system (Annoroad Genomics Co., Beijing, China) and 2 × 150-bp paired-end reads with a minimum of 250-fold coverage were obtained for each isolate.

In addition, 12 LA-MRSA-CC398 isolates showing interesting characteristics were selected for long-read sequencing in order to generate complete, closed genomes. These isolates either held central positions within the different clusters of the cgMLST minimum spanning tree and/or harbored uncommon AMR genes/SCC*mec* cassettes and/or showed a—with the short-read sequences alone—not clearly identifiable SCC*mec* type. High-molecular-weight DNA was extracted with QIAGEN Genomic Tips 100G (Qiagen, Hilden, Germany) and sheared using g-TUBES (Covaris, Woburn, MA, USA) to an average of 10 to 15 kb. Libraries were prepared using the SMRTbell Express Template Prep Kit 2.0 (Pacific Biosciences of California, Inc., Menlo Park, CA, USA) according to the manufacturer's protocol, "Preparing Multiplexed Microbial Libraries" (v07). Barcoded adapters were prepared with the Barcoded Overhang Adapter Kits 8A and 8B (Pacific Biosciences of California, Inc.). Equimolar pooling was calculated with the PacBio Express Microbial Multiplexing Calculator, followed by size selection with the BluePippin system (Biozym Scientific GmbH, Hessisch Oldendorf, Germany) using the high-pass option at 7 kb. Primer annealing and polymerase binding were done with sequencing primer V4 according to the SMRT Link Sample Setup (Pacific Biosciences of California, Inc.). Circular consensus sequencing reads were then generated by diffusion loading with 85 pM and an estimated insert size of 12 kb using one single-molecule real-time (SMRT) Cell (SMRT Cell 8M Tray, Pacific Biosciences of California, Inc.) and a Sequel II Sequencing Kit v2.0 (Pacific Biosciences of California, Inc.) on a Sequel II platform (2-h preextension, 30-h movie time; Pacific Biosciences of California, Inc.).

Sequence assembly and annotation. The 152 short-read sequences generated on the Illumina MiSeq platform were quality checked using FastQC v0.11.9 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and trimmed with Trim Galore v0.6.5 (RRID:SCR_011847). *De novo* assembly into contigs was carried out with Unicycler v0.4.9 (73). The 26 short-read sequences obtained from the Illumina HiSeq X-Ten system were assembled with SPAdes v3.12.0 (74) after quality control processing via FastQC v0.11.5 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and trimming with Trimmomatic v0.39 (75). In order to generate complete genomes of the 12 isolates subjected to long-read sequencing, the PacBio-HiFi long reads and Illumina short reads were hybrid assembled with Unicycler v0.4.9 (73) and the Flye algorithm in MaSuRCA v3.4.2 (76). In addition, a third assembly was performed with Canu

v2.2 (77) using the PacBio-HiFi long reads. The generated genomes from MaSuRCA and Canu were polished using Pilon v1.2.3 (78) using the Illumina short reads. Subsequently, for all three generated genomes a consensus sequence was generated with Trycycler v0.5.3 (79). All assembled sequences were checked for errors with Geneious v11.1.4 (Biomatters, Ltd., Auckland, New Zealand) and annotated with Prokka v1.14.5 (80) as well as a subsystem technology server (RAST) (81) for further investigation.

Molecular typing and phylogenetic analysis. For all 178 LA-MRSA-CC398 whole-genome sequences, MLST, *spa* typing, and SCC*mec* typing were carried out using the MLST v2.0 (82), *spa*Typer v1.0 (83), and SCC*mec*Finder v1.2 (https://cge.food.dtu.dk/services/SCCmecFinder/) online analysis tools from the Center for Genomic Epidemiology (CGE). The *dru* types were identified according to the drutyping.org database (84) using the basic local alignment search tool (BLAST) function in Geneious v11.1.4 (Biomatters, Ltd., Auckland, New Zealand). Similar to *spa* typing, *dru* typing is a single-locus sequence-based typing method that, however, targets a region between the *mecA* gene and the adjacent IS*431* element in SCC*mec* cassettes of methicillin-resistant staphylococci. This region consists of mostly 40-bp direct repeat units (*dru*) which differ slightly in their sequences. The number and order of the different *dru* repeats define the *dru* type. As of 3 April 2023, 103 *dru* repeats and 579 *dru* types, comprising 1 to 23 repeats, had been assigned.

The 178 LA-MRSA-CC398 genomes were also subjected to phylogenetic analysis in Ridom SeqSphere+ v7.5.5 (85) using the *S. aureus* cgMLST approach (86). A minimum spanning tree was built based on a distance matrix of the core genome allelic profiles in order to illustrate the clonal relationships between the isolates. Overall, 1,569 of 1,861 potential target genes were included in the analysis by removing 292 columns with missing values from the comparison table. Isolates were assigned to clusters with a maximum threshold of 29 allelic differences (42).

Investigation of virulence and AMR properties and MGEs. In the 178 LA-MRSA-CC398 genomes, common virulence genes were identified with ABRicate v1.0.1 (https://github.com/tseemann/abricate) using the VFDB database (87) and with CGE VirulenceFinder v2.0.3 (88). Known AMR genes were located with ABRicate v1.0.1 using the NCBI AMRFinder tool (89) and the CGE ResFinder (90) databases. In individual cases of fragmented genes, the presence of AMR determinants was confirmed by PCR. Chromosomal point mutations conferring AMR were detected by applying PointFinder (91) as part of the CGE ResFinder v4.1 tool (90). The CGE PlasmidFinder v2.0.1 tool (92, 93) and MobileElementFinder v1.0.3 (94) were used to determine MGEs. All results were checked for errors with Geneious v11.1.4 (Biomatters, Ltd., Auckland, New Zealand), BLAST (95), and the UniProt knowledgebase (96).

Antimicrobial susceptibility testing (AST). AST by broth microdilution was performed in the GE*RM*-Vet program according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI) for 29 antimicrobial agents using commercially available microtiter plates (Sensititre, Thermo Fisher Scientific, Waltham, MA, USA) (97, 98). Due to plate layout changes over the years, MICs for all 178 LA-MRSA-CC398 isolates are available for 18 antimicrobial compounds, including beta-lactams (oxacillin, amoxicillin/clavulanic acid 2:1, ampicillin, penicillin, cefoperazone, cefotaxime, ceftiofur, and cefquinome), tetracycline, macrolides (erythromycin, tilmicosin, and tulathromycin), lincosamides (clindamycin and pirlimycin), an aminoglycoside (gentamicin), a fluoroquinolone (enrofloxacin), folate pathway inhibitors (trimethoprim/sulfamethoxazole 1:19), and a glycopeptide (vancomycin). Isolates were classified as susceptible, intermediate, or resistant if breakpoints were available in the CLSI documents VET01S or M100 (97, 98) or in accordance with a previous study (3) (Table 3). Since several antimicrobial compounds were not included in the commercial microtiter plate layouts, the functionality of certain detected AMR genes was verified testing the growth of the corresponding isolates on Mueller-Hinton agar plates containing the respective antimicrobial agents at a specific threshold concentration. Despite the lack of CLSI-approved breakpoints, the functionality of the associated AMR gene was considered confirmed, in accordance with the sequencing data, for isolates that showed growth on plates with high concentrations of the antimicrobial agent (tiamulin, 2 to 8 mg/L [48]; kanamycin, 16 to 32 mg/L (99); streptomycin, 16 mg/L; spectinomycin, 128 to 256 mg/L [3]; apramycin, 16 mg/L [21]; trimethoprim, 8 mg/L [98]; florfenicol, 8 to 16 mg/L [99]). If available, CLSI-approved breakpoints or MIC values from previous studies were considered for the determination of threshold concentrations. In certain cases, up to two dilution steps lower than the information from the literature had to be tested. Moreover, screening for inducible macrolide/lincosamide resistance by agar disc diffusion (D-zone test) was conducted according to CLSI standards using discs containing erythromycin (15 μg; BBL Sensi-Disc, BD, Franklin Lakes, NJ, USA) and clindamycin (2 μg; Oxoid, Thermo Fisher Scientific, Waltham, MA, USA).

Biocide susceptibility testing (BST). BST for benzalkonium chloride was carried out by broth microdilution using commercial microtiter plates (Sifin Diagnostics GmbH, Berlin, Germany), which contained benzalkonium chloride in 12 2-fold dilution steps (0.000008 to 0.016%). BST performance followed a published protocol (100) that was modified as described previously (101).

Data availability. The whole-genome shotgun project comprising the genome sequences of 174 of the LA-MRSA-CC398 isolates included in this study has been deposited in GenBank under accession number PRJNA814867. The genome sequences of the remaining four LA-MRSA-CC398 isolates can be found within a previous project under accession number PRJNA842861. Isolate IDs and individual accession numbers are given in Data set S1.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 0.1 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.

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We have no conflict of interest to declare.

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2.3 Publication III

Ji X*, Krüger H*, Feßler AT, Liu J, Zeng Z, Wang Y, Wu C, Schwarz S

A novel SCC*mec* type V variant in porcine MRSA ST398 from China

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* both authors contributed equally to this study

Authors' contributions:

1. **Conceptualization**, JX, KH, WY, WC, SS	A
2. **Methodology**, JX, KH, WY, SS	B
3. **Validation**, JX, KH, JL, ZZ, WY, SS	B
4. **Formal Analysis**, JX, KH, WY, SS	B
5. **Investigation**, JX, KH, WY, SS	B
6. **Resources**, JX, JL, ZZ, WY, WC, SS	
7. **Data Curation**, JX, KH, WY, SS	B
8. **Writing – Original Draft Preparation**, JX, KH, WY, SS	B
9. **Writing – Review & Editing**, JX, KH, FAT, JL, ZZ, WY, WC, SS	B
10. **Visualization**, XJ, KH, SS	B
11. **Supervision**, FAT, WY, SS	
12. **Project Administration**, WY, WC, SS	
13. **Funding Acquisition**, WY, WC, SS	

All authors have read and agreed to the published version of the manuscript.

2.4 Publication IV

Krüger H*, Ji X*, Wang Y†, Feßler AT, Wang Y, Wu C, Schwarz S

Identification of Tn*553*, a novel Tn*554*-related transposon that carries a complete *blaZ-blaR1-blaI* β-lactamase operon in *Staphylococcus aureus*

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* both authors contributed equally to this study

Authors' contributions:

1. **Conceptualization**, KH, JX, WY, WC, SS	A
2. **Methodology**, KH, JX, WY†, WY, SS	B
3. **Validation**, KH, JX, WY†, WY, SS	B
4. **Formal Analysis**, KH, JX, SS	C
5. **Investigation**, KH, JX, SS	C
6. **Resources**, JX, WY†, WY, WC, SS	
7. **Data Curation**, KH, JX, WY, SS	B
8. **Writing – Original Draft Preparation**, KH, JX, FAT, SS	C
9. **Writing – Review & Editing**, KH, JX, WY†, FAT, WY, WC, SS	B
10. **Visualization**, KH, JX, SS	C
11. **Supervision**, FAT, WY, SS	
12. **Project Administration**, WY, WC, SS	
13. **Funding Acquisition**, WY, WC, SS	

All authors have read and agreed to the published version of the manuscript.

2.5 Publication V

Ji X*, Krüger H*, Wang Y†, Feßler AT, Wang Y, Schwarz S, Wu C

Tn*560*, a Novel Tn*554* Family Transposon from Porcine Methicillin-Resistant *Staphylococcus aureus* ST398, Carries a Multiresistance Gene Cluster Comprising a Novel *spc* Gene Variant and the Genes *lsa*(E) and *lnu*(B)

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* both authors contributed equally to this study

Authors' contributions:

1. **Conceptualization**, JX, KH, WY, SS, WC A
2. **Methodology**, JX, KH, WY†, WY, SS B
3. **Validation**, JX, KH, WY†, WY, SS, WC B
4. **Formal Analysis**, JX, KH, WY†, FAT, SS B
5. **Investigation**, JX, KH, SS B
6. **Resources**, JX, WY†, WY, SS, WC
7. **Data Curation**, JX, KH, WY†, WY, SS, WC B
8. **Writing – Original Draft Preparation**, JX, KH, WY†, WY, SS B
9. **Writing – Review & Editing**, JX, KH, WY†, FAT, WY, SS, WC B
10. **Visualization**, JX, KH, SS B
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LETTER TO THE EDITOR

Tn*560*, a Novel Tn*554* Family Transposon from Porcine Methicillin-Resistant *Staphylococcus aureus* ST398, Carries a Multiresistance Gene Cluster Comprising a Novel *spc* Gene Variant and the Genes *lsa*(E) and *lnu*(B)

Xing Ji,[a,b] Henrike Krüger,[c,d] Yaxin Wang,[a] Andrea T. Feßler,[c,d] Yang Wang,[a] Stefan Schwarz,[a,c,d] Congming Wu[a]

[a]Beijing Key Laboratory of Detection Technology for Animal-Derived Food Safety, College of Veterinary Medicine, China Agricultural University, Beijing, China
[b]Jiangsu Key Laboratory for Food Quality and Safety—State Key Laboratory Cultivation Base of Ministry of Science and Technology, Institute of Food Safety and Nutrition, Jiangsu Academy of Agricultural Sciences, Nanjing, China
[c]Institute of Microbiology and Epizootics, Centre for Infection Medicine, Department of Veterinary Medicine, Freie Universität Berlin, Berlin, Germany
[d]Veterinary Centre for Resistance Research (TZR), Department of Veterinary Medicine, Freie Universität Berlin, Berlin, Germany

Xing Ji and Henrike Krüger contributed equally to this article. Author order was determined both alphabetically and with regard to the geographical origin of the investigated strain.

KEYWORDS PLS_A resistance, *lsa*(E), transposon, *Staphylococcus aureus*

Combined resistance to pleuromutilins, lincosamides, and streptogramin A antibiotics (PLS_A) in staphylococci is often conferred by ABC-F genes, including *lsa*(E) (1, 2). The *lsa*(E) gene is commonly part of multiresistance gene clusters, in which the region comprising the streptomycin resistance gene *aadE*, the spectinomycin resistance gene *spw*, the lincosamide resistance gene *lnu*(B), and the PLS_A resistance gene *lsa*(E) is conserved (3–7). During the analysis of the whole-genome sequence of the porcine methicillin-resistant *Staphylococcus aureus* (MRSA) sequence type 398 (ST398) strain GDC6P096P from China, obtained by hybrid assembly of Illumina short-read and MinION long-read data, we identified a novel 12,518-bp Tn*554*-like transposon harboring part of the *lsa*(E) gene cluster. This transposon, designated Tn*560*, differed from all previously reported transposons of the Tn*554* family in its structure and organization (8–10).

Tn*560* was inserted into the chromosomal *radC* gene. It displayed two 6-bp sequences, 5′-GATGTA-3′ at the left-end junction and 5′-TGATAA-3′ at the right-end junction. While the transposase genes *tnpA*, *tnpB*, and *tnpC* of Tn*560* were identical to those of Tn*554* and the *lnu*(B) and *lsa*(E) genes of Tn*560* shared 99.88% and 99.93% nucleotide sequence identities with the previously reported prototype genes (11, 12), the Tn*560*-borne "*spw*" gene showed only 78.38% nucleotide sequence identity with the original *spw* gene (13). However, this gene showed 93.74% nucleotide sequence identity with the Tn*554*-borne *spc* gene. The deduced amino acid sequence revealed that the N-terminal 200 amino acids (aa) were identical to those of Spc, whereas the C-terminal 68 aa were identical to those of Spw, suggesting that the novel *spc* gene variant, designated spc_V, resulted from the in-frame *in vivo* recombination of the two related genes *spc* and *spw*. A similar finding has been reported for the macrolide-lincosamide-streptogramin B resistance gene *erm*(33), which resulted from the *in vivo* recombination of the genes *erm*(A) and *erm*(C) (14).

Tn*560* most likely originated from recombination of a Tn*554* transposon with the *spw-lsa*(E)-*lnu*(B)-carrying, 74,612-bp multiresistance plasmid pE508-2 from porcine *Enterococcus faecalis* (GenBank accession number MK784778.1). A 7,588-bp segment of Tn*560*, which comprised part of the *spw* gene and reached until the region downstream of *lnu*(B), exhibited 99.95% nucleotide sequence identity with the corresponding region in plasmid pE508-2 (Fig. 1a). Two potential recombination sites were identified (Fig. 1b). Recombination site A is located within the *spc* gene of Tn*554* and includes a stretch of 14 bp of 100% identity

Address correspondence to Congming Wu, wucm@cau.edu.cn, or Stefan Schwarz, stefan.schwarz@fu-berlin.de.

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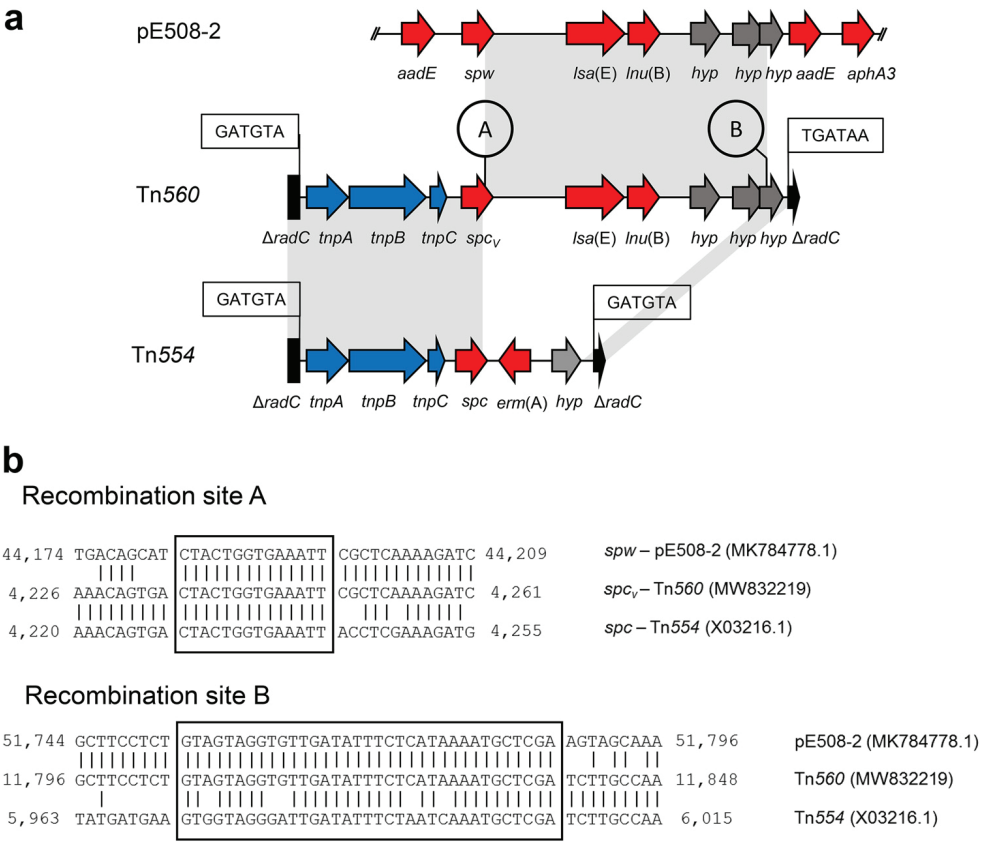

FIG 1 (a) Comparison of Tn*560*, Tn*554*, and a part of plasmid pE508-2. The areas of >99% identity are shaded in gray. Antimicrobial resistance genes are shown in red, transposase genes are in blue, and hypothetical proteins are in gray. The positions of the two recombination sites A and B are indicated by circles. (b) Detailed structures of the two recombination sites A and B. Vertical bars indicate identical bases with respect to the Tn*560* sequence. The positions refer to the corresponding database entries (GenBank accession numbers are in parentheses). The boxed areas indicate the areas where the crossover most likely took place.

between the *spc* and *spw* genes. Upstream of recombination site A, the sequence of Tn*560* corresponds to *spc* of Tn*554*, downstream of it to *spw* of pE508-2. Recombination site B was located downstream of the gene *lnu*(B) within an open reading frame (ORF) for a hypothetical protein and comprised a stretch of 35 bp, with 100% and 85.71% identities to the corresponding regions of pE508-2 and Tn*554*, respectively. Upstream of recombination site B, the sequence of Tn*560* corresponded to the sequence of pE508-2, downstream of it to Tn*554*.

The detection of the circular Tn*560* by PCR (primers Tn560-fw [5′-CGGCTTATTCTCCACTTCT-3′] and Tn560-rev [5′-CCTGGATGCCAATTCCATA-3′], with an annealing temperature of 50°C and an amplicon size of 460 bp) indicated that this transposon is functionally active in this strain (Fig. 2). Sequence analysis of the amplicon showed that it comprised 175 bp from the left terminus and 285 bp from the right terminus of Tn*560*.

Data availability. The data have been deposited in GenBank under accession number MW832219.

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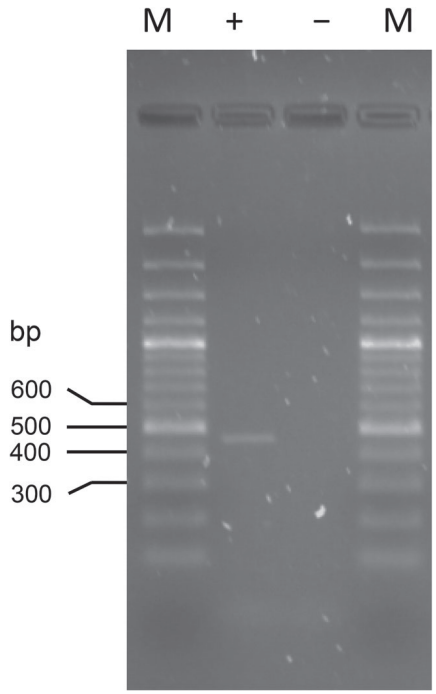

FIG 2 Detection of the 460-bp amplicon obtained from the circular intermediate of Tn*560* that has been formed after the excision of Tn*560* from the chromosomal DNA. Lanes M contain the GeneRuler 100-bp Plus DNA ladder (Thermo Fisher Scientific, Dreieich, Germany), lane + contains the PCR amplicon obtained from *S. aureus* GDC6P096P, and lane − is the negative control, i.e., demineralized water. The sizes of selected fragments of the DNA ladder are indicated in base pairs.

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2.6 Publication VI

Krüger H, Ji X, Hanke D, Schink A-K, Fiedler S, Kaspar H, Wang Y, Schwarz S, Wu C, Feßler AT

Novel macrolide-lincosamide-streptogramin B resistance gene *erm*(54) in MRSA ST398 from Germany

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Authors' contributions:

1. **Conceptualization**, KH, SA-K, SS, FAT	B
2. **Methodology**, KH, HD, SA-K, FS, KH, SS	C
3. **Validation**, KH, HD, SA-K, SS	B
4. **Formal Analysis**, KH, HD, SS, FAT	C
5. **Investigation**, KH, HD, SS, FAT	C
6. **Resources**, HD, FS, KH, SS	
7. **Data Curation**, KH, JX, HD, SS	B
8. **Writing – Original Draft Preparation**, KH, SS, FAT	C
9. **Writing – Review & Editing**, KH, JX, HD, SA-K, FS, KH, WY, SS, WC, FAT	B
10. **Visualization**, KH, HD, SS	C
11. **Supervision**, WY, SS, FAT	
12. **Project Administration**, WY, WC, SS	
13. **Funding Acquisition**, WY, WC, SS	

All authors have read and agreed to the published version of the manuscript.

3 Discussion

3.1 Different epidemic LA-MRSA lineages in pigs in Germany and China

Multiple studies have expanded our knowledge on epidemiological, genotypic and/or pathogenic characteristics of epidemiologically successful LA-MRSA CC398 and CC9 isolates (Yu et al. 2021; Monecke et al. 2018; Ballhausen et al. 2017; Butaye et al. 2016; Price et al. 2012; Monecke et al. 2011; Kadlec et al. 2009). However, features of the non-successful lineages and the specific factors that promote the dominance of the LA-MRSA CC9 lineage in pigs in China, and that of the LA-MRSA CC398 lineage in pigs in Germany as well as in most other parts of the world, remained uncovered. Moreover, it was not known why LA-MRSA CC398 caused infections of humans and animals more frequently than other lineages. In order to clarify these issues, different objectives were pursued in joint approaches by the project partners (**Publication I** and **II**; see list of publications, **Jiang et al. 2021**, **Ji et al. 2021**). Genomic characteristics, fitness, metabolic properties, AMR and virulence traits of successful and non-successful LA-MRSA CC398 and CC9 isolates from Germany and China were comparatively investigated. Besides bacteria-associated factors, also host-associated factors allowing a clone to become a successful pig colonizer were studied.

3.1.1 Pig farming systems in Germany and China

China is the world's largest producer of pork, followed by the USA and Germany (Federal Ministry of Food and Agriculture (BMEL) 2023). Accordingly, Germany is Europe's largest pork producer (Federal Ministry of Food and Agriculture (BMEL) 2023).

In Germany, pigs are the most important source of meat and a supporting pillar of the national agriculture (Federal Ministry of Food and Agriculture (BMEL) 2023). Most recent data states an annual production of approximately 5.1 million tons of meat (Federal Ministry of Food and Agriculture (BMEL) 2023). Pig farming has changed rapidly in recent decades: Highly specialized farms with large numbers of animals characterize the sector at increasing frequencies during recent years (Federal Ministry of Food and Agriculture (BMEL) 2023). The number of pig farmers decreased by around 50% from 2007 to 2016, with a slight increase in the number of animals (Federal Ministry of Food and Agriculture (BMEL) 2023). Modern systems aim at hygienic, efficient and cost-effective production (Federal Ministry of Food and Agriculture (BMEL) 2023). Most farms focus on individual production stages, such as piglet production or fattening (Federal Ministry of Food and Agriculture (BMEL) 2023). However, there is also a rising number of farms carrying out all steps of production (Federal Ministry of Food and Agriculture (BMEL) 2023). Computer-controlled feeding and ventilation systems as well as special barns for each stage of husbandry are standard these days (Federal Ministry of Food and Agriculture (BMEL) 2023). In general, three housing systems can be

distinguished: the conventional (closed building, forced ventilation, partially or fully slatted floor), alternative (free ventilation, straw bedding and/or outdoor climate) and organic (free ventilation, straw bedding and/or outdoor climate, according to guidelines of certain associations) systems (Kobusch et al. 2022). With 96% of all husbandries, conventional systems dominate by far in Germany, whereas alternative and organic pig farming is still rather rare (German federal and state statistical offices 2023). Traditional breeds, such as the Swabian-Hall Swine or the German Saddleback, are used on smaller scale, usually in organic farms, as purebred or cross-bred with conventional lines (Leenhouwers and Merks 2013). For conventional pork production, high-performance animals are used, which are usually crosses of Duroc or Pietrain sire lines with German Landrace and/or German Large White and/or German Edelschwein (Leenhouwers and Merks 2013; Mörlein et al. 2007).

Since China is not only the largest pork producing, but also pork consuming country of the world, the pork sector is also vital for the Chinese agricultural industry (Wang et al. 2021). Already in 2010, the total annual pork production exceeded 50 million tons and it is predicted to be higher than 60 million tons by 2029 (Wang et al. 2021). Rural backyard farming is still common in China, where the pigs are normally fed waste products and, thus, the farmer has very low input costs. In addition, there are small and large commercial pig husbandries in China (McOrist et al. 2011). As in Germany, large-scale intensive pork production systems have increasingly replaced small family-based systems and intensification, specialization and standardization of the sector is expected to proceed (Wang et al. 2021). Modern breeding and management technologies are widely used (Wang et al. 2021). However, small-scale (family-based) production, which usually means less than 500 slaughtered pigs annually, still accounts for nearly 50% of all pork producers (Wang et al. 2021). The structure of modern pork production, comprising feed production and processing, sow and nursery pig production, growing and finishing pigs, slaughterhouse operations, as well as waste and manure management, is the same in China as all over the world (Wang et al. 2021). At the initiation of industrial pig farming, technologies have been widely imported from Western countries (Wang et al. 2021). Moreover, leaner breeds, such as Landrace, Large White and Duroc, were introduced to improve production parameters relying on high-performance crossbreeds (Wang et al. 2021; McOrist et al. 2011). Native pig breeds, such as the Jinhua pig and the TaiHu valley breeds Meishan or Fengjing, are also seen and may be cross-bred with Western breeds to form new production lines (McOrist et al. 2011).

Certainly, the unique character of the pig farming systems in Germany and China, respectively, has influence on the development of persisting bacterial clones. However, since the pork production systems of the two nations are in general very similar regarding their structure, used breeds and technologies, as well as the proceeding development towards large

and highly specialized farms, other factors than the system itself were probably more crucial for the development of two different epidemic porcine LA-MRSA clones.

3.1.2 Reasons for the success of two different clones in Germany and China

Publication I reports the comparative investigation of rare and widespread porcine LA-MRSA CC398 and CC9 isolates from Germany and China for metabolic variations that could have played a role in the development of the two clones. Prior to this study, the Biolog PM technology has not been applied to characterize LA-MRSA isolates. It allowed an extensive analysis of the LA-MRSA phenotypes. Overall, the porcine LA-MRSA from Germany and China were able to use a wide range of substrates, in particular many different carbon sources. In fact, it is known that *S. aureus* prefers to metabolize carbohydrates, when they are available, but can use various nutrients, for example also nucleic and amino acids (Somerville 2016a). Only few substrates could not be used by the LA-MRSA, which highlights the metabolic versatility of this species (Somerville 2016a). The results obtained via Biolog PM also underline the microbe's pronounced osmotolerance, which allows the colonization of body sites, such as the skin and mucosal surfaces (Frees and Ingmer 2016). It was also apparent that acidic pH values limit the metabolic activity of LA-MRSA, which is commonly utilized to control *S. aureus* growth and toxin-production in food (Lanciotti et al. 2001) or needs to be considered to maintain a balanced skin microbiome (Iyer et al. 2021).

Initially, it seemed likely that the dominant LA-MRSA lineages could have an advantage in host colonization due to exclusive metabolic properties, because the metabolic state of *S. aureus* is connected with its pathogenesis via impact on virulence factor regulators and the survival at different body sites of a host requires unique metabolic pathways to use a varying range of nutrients (Richardson 2018). However, since only few relevant differences were detected, there was no evidence that metabolic variations between the dominant and rare lineages within the same CC were critical in the development of the two epidemic LA-MRSA clones in pigs. Individual metabolic advantages of the dominant lineages in environments with limited substrate availability seem negligible considering the pathogen's versatility in nutrient usage and the fact that multiple substances can be used in identical essential pathways (Somerville 2016a). Certainly, the selection pressure imposed by the application of antimicrobial agents is a key factor leading to the development of dominant LA-MRSA lineages in Germany and China. Another study of the joint project (see list of publications, **Ji et al. 2021**) showed that the CC398 as well as CC9 isolates from Germany and China harbored several AMR genes, but their numbers and distributions differed. The dominant LA-MRSA lineages showed higher resistance rates than their respective counterpart, which indicates that the dominant clones have in fact adapted to the most commonly used

antimicrobial agents used in pig industry in the respective country: The Chinese LA-MRSA CC9 displayed more complex AMR profiles and were frequently resistant to tetracycline, tiamulin, florfenicol and MLS_B antibiotics, whereas the German LA-MRSA CC398 were all resistant to tetracycline (Larsen et al. 2016; Krishnasamy et al. 2015; Zhang et al. 2015; Merle et al. 2012). The further demonstrated increased biofilm formation ability and enhanced tolerance to desiccation (see list of publications, **Ji et al. 2021**) could have been supportive to the survival of the dominant LA-MRSA variants, enabling them to outcompete the non-successful clones under limiting environmental conditions (Tahir et al. 2019; Moormeier and Bayles 2017). The newly described pathogenicity islands SaPIpig1 and SaPIpig2, carrying the von-Willebrand-factor-binding protein-encoding gene *vwb*$^{vSa\alpha}$ that mediates coagulation activity for ruminant and porcine plasma (see list of publications, **Ji et al. 2021**), may promote the colonization of the dominant lineages in pigs as well as their transmission between individuals (Viana et al. 2010). In accordance with a previous study (Tulinski et al. 2014), *vwb*$^{vSa\alpha}$ might play an essential role during adhesion to the pig nose tissue in dominant LA-MRSA clones.

Surprisingly, **Publication I** revealed a unique metabolic profile for the LA-MRSA CC9 from China. It differed distinctly from the more similar metabolic properties of the CC398 isolates from Germany as well as China and the CC9 isolates from Germany. The Chinese LA-MRSA CC9 isolates were in part more sensitive to increasing osmolyte concentrations, leading either to a delayed, delayed and reduced, or absent metabolism. In addition, they seemed to require a longer adaptation time to their environmental conditions before bacterial cell respiration started. This was especially evident for the utilization of amino acids at alkaline pH values. It is important to note, that S. *aureus* is, in general, dependent on the external availability of amino acids and certain amino acids are considered critical for the growth in niches where preferred nutrients are limited, for example within an abscess (Halsey et al. 2017; Somerville 2016a). Moreover, the Chinese LA-MRSA CC9 showed a decreased or absent usage compared to the other LA-MRSA regarding several nutrients. However, the LA-MRSA CC9 from China could also use two substances more efficiently. In summary, the observed metabolic variations suggested that the Chinese LA-MRSA CC9 clone may be at disadvantage in niche adaptation compared to the other LA-MRSA lineages. The increased utilization of two substrates may also be unfavorable, imagining the formation of noxious metabolic products. Therefore, in the long term the LA-MRSA CC9 lineage might be replaced in China by a superior clone. In another study of the joint project, we discovered various beneficial characteristics of the LA-MRSA CC398 lineage that may enable survival under hostile environmental conditions or in the host (see list of publications, **Ji et al. 2021**). Compared to LA-MRSA CC9, this clone showed greater tolerance to acids and high osmotic pressure. Thus, CC398 isolates are more likely to survive and colonize acidic or hypertonic surroundings, such as the nasal cavity or the

skin (Proksch 2018; Jantsch et al. 2015; Summerfield et al. 2015; England et al. 1999). In addition, LA-MRSA CC398 showed more diverse genome structures, harboring a larger accessory genome mainly comprising MGEs, including SCC*mec* cassettes, AMR genes and prophages (see list of publications, **Ji et al. 2021**). On one hand, this suggests that an increased number of mutations and recombinations allows these isolates better adaptation to varying environments (Uhlemann et al. 2017; Lindsay 2014). On the other hand, the CC398 lineage seems to easier integrate foreign genetic material, such as AMR genes. This is probably due to missing genes of a type 1 restriction-modification (RM) system, which offers, when complete, protection against foreign DNA, for example carried by bacteriophages (Waldron and Lindsay 2006). Moreover, the Chinese LA-MRSA CC9 showed a lower growth rate in co-cultivation experiments compared with the other groups (see list of publications, **Ji et al. 2021**). The presence of a greater number of AMR genes compared to other isolates might have led to a higher fitness cost (Vogwill and MacLean 2015). This may also be the reason for the decreased nutrient usage and delayed onset of metabolic activity of the Chinese CC9 isolates. As a consequence, the LA-MRSA CC398 lineage might gradually replace the contemporary Chinese LA-MRSA CC9 clone once it gets disseminated further through human occupational exposure as well as trading activities, and adapts to the differing pig breeding environment due to an improved fitness, competitiveness and integration of foreign DNA. Before there were restrictions because of the current outbreaks of African swine fever, communications from China Customs (http://english.customs.gov.cn/home/index) indicated that pork imports from European Union countries, in particular from Spain, Germany and Denmark, accounted for about 60% of the total Chinese pork import volume. Although the precise transmission routes are not clear, this suggests that the current occasional presence of LA-MRSA CC398 in China might be the result of an introduction from European countries. In support of this, we showed that LA-MRSA CC398 from China and Germany shared close phylogenetic relationships with CC398 isolates from other European countries, Australia and the USA, which indicates that the CC398 lineages from Germany and China might originate from one ancestor (see list of publications, **Ji et al. 2021**). Cui et al. also observed a close evolutionary relationship between LA-MRSA ST398 from China and Europe or Australia (Cui et al. 2022). In contrast, the LA-MRSA CC9 from Germany and China seem to have developed independently from each other (see list of publications, **Ji et al. 2021**). The development of unique metabolic characteristics in Chinese LA-MRSA CC9 might be explained by a larger spatial distance of this lineage to the other three clones during evolution of epidemic lineages in pigs. At the start of the joint project in 2019, only five porcine LA-MRSA CC398 isolates from China were available to us. According to the literature, LA-MRSA CC9 in Europe and LA-MRSA CC398 in China were only occasionally found in pigs (Li et al. 2018b; Monecke et al. 2011). However, during the last years, studies reporting LA-MRSA CC398 isolates from China have

increased (Cui et al. 2022; Li et al. 2022; Cui et al. 2020), which substantiates the assumption of an already ongoing replacement of the Chinese LA-MRSA CC9 lineage by LA-MRSA CC398. It is important to note that we observed an increased virulence and pathogenicity for LA-MRSA CC398 in relation to LA MRSA CC9 due to the *hysA*$^{vSa\beta}$ gene on the genomic island vSaβ (see list of publications, **Ji et al. 2021**). This gene is homologous to the chromosomal *hysA*, which is crucial for the virulence of *S. aureus* encoding a hyaluronate lyase that decomposes the host's hyaluronic acid and, thus, allows spreading and proliferation of the bacterium (Makris et al. 2004). A *hysA* homologue on vSaβ has also been described in an LA-MRSA ST398 isolate originating from a case of human endocarditis (Schijffelen et al. 2010). However, the regulatory and immunological processes involving *hysA*$^{vSa\beta}$ enhancing the virulence of LA-MRSA-ST398 need to be investigated further. Nevertheless, LA-MRSA originating from pigs might pose a greater threat to public health in the future due to the carriage of *hysA*$^{vSa\beta}$. In fact, LA-MRSA CC398 have been reported to cause infections more likely than, for example, the LA-MRSA CC9 lineage (Jin et al. 2020; Cuny et al. 2013a). Moreover, studies on LA-MRSA ST398 from China have already suggested this strain as a common source of infections (Cui et al. 2022) and have additionally pointed out the potential of isolate transmission between different countries (Li et al. 2022).

3.1.3 Characteristics of the epidemic LA-MRSA lineage in pigs in Germany

Publication II provided insight into genomic diversity and population dynamics of the in Germany dominating porcine LA-MRSA CC398 lineage over a 13-year-period, in a similar way as another study coordinated by the Chinese project partners about the LA-MRSA CC9 lineage dominating among pigs in China (see list of publications, **Jiang et al. 2021**). The LA-MRSA CC398 isolates were widespread within Germany and reflected the pig density to a large extent (German federal and state statistical offices 2020). It is worth noting that in both studies (**Publication II**; see list of publications, **Jiang et al. 2021**), the data set was uneven regarding the number of collected samples, because the isolate availability from the different Chinese provinces or German federal states was limited. In Germany, isolate submission to the national resistance monitoring program GE*RM*-Vet occurs voluntarily according to varying sampling plans and only isolates from diseased individuals are included. Therefore, the program might not mirror the true prevalence of porcine LA-MRSA CC398 in Germany. The isolates included in **Publication II** were not obtained evenly across the federal states and over time: The northern federal states were overrepresented especially in the early years of the study and for Lower Saxony as the German state with the highest pig density, probably too few isolates were collected. In previous studies, LA-MRSA CC398 has been described to colonize healthy animals in almost half of the pig farms in Germany (Cuny et al. 2015b; European Food Safety Authority 2009a). In contrast, the LA-MRSA CC9 prevalence in healthy

pigs in the provinces of China differed from 3.6% to 47%, with an average of 11.2% (Li et al. 2017; Cui et al. 2009). Kobusch et al. have shown that pig husbandry conditions affect the LA-MRSA colonization (Kobusch et al. 2022). Compared with the commonly and consistently LA-MRSA-positive conventional farms, alternative housing systems displayed a more dynamic colonization during the fattening period, and LA-MRSA was almost absent in the rarer organic farms in Germany (Kobusch et al. 2022). It also needs to be mentioned that several of the 178 isolates from diseased pigs investigated in **Publication II** were presumably not causative of the reported illnesses, because they might have been co-isolated with other respiratory tract pathogens as colonizers of the respiratory mucosa (n = 57) or background data indicated only an incidental detection (n = 3). For 25 LA-MRSA CC398, no background information was available, except for the decease of the animals. In Germany, LA-MRSA CC398 has been reported to colonize up to 86% of humans regularly occupationally exposed to colonized pigs (Cuny et al. 2015b; Cuny et al. 2013a). However, further human-to-human transmission seems to happen rarely (Cuny et al. 2013a). Consequently, LA-MRSA colonization was noticed only in 0.08 to 0.2% of persons admitted to German hospitals, however, the prevalence is substantially increased in livestock-dense regions (van Alen et al. 2017; Cuny et al. 2013a). During the absence of livestock exposure, the carriage of LA-MRSA CC398 has been on one hand demonstrated to decrease strongly, on the other hand, there is evidence that regular contact to pigs results in a rather persistent colonization (Köck et al. 2012). In addition, further LA-MRSA dissemination routes need to be considered, such as via exhaust air from farms or contaminated food products (Cuny et al. 2013a). However, the previous literature does not imply that LA-MRSA is a typical food-borne pathogen (Wendlandt et al. 2013c). In China, the prevalence of nasal LA-MRSA carriage among pig farmers has been reported to be 15% (Chuang and Huang 2015). This comparatively low number might rather be the result of a lower data availability from resource-limited regions presumably because of lacking diagnostic laboratories, than of a real low prevalence (Chuang and Huang 2015). Our study on porcine LA-MRSA CC9 from China (see list of publications, **Jiang et al. 2021**) suggested that pigs und humans may have exchanged isolates during evolution of this clone, because isolates originating from pigs and humans showed close relationships. Moreover, other reports from Asia indicated that LA-MRSA isolates from humans occupationally exposed to pigs were mainly the same as the LA-MRSA isolates from pigs, suggesting a cross-species transfer (Chuang and Huang 2015).

In **Publication II**, the LA-MRSA CC398 isolates from Germany displayed a relatively small number of variations in their core genome, since the affiliation to a CC already presupposes a certain similarity. Particularly similar isolates were assigned to different clusters. The LA-MRSA CC9 isolates from China also showed generally close relationships, although isolates from distinct provinces displayed variable differentiation grades (see list of

publications, **Jiang et al. 2021**). The emergence of new clusters and singletons over time points towards an ongoing diversification in the sense of a microevolution within CC398, resulting in new variants (**Publication II**). The novel STs and *dru* types we reported confirm this assumption. Consequently, as previously reported, the molecular diversity of CC398 increases (Butaye et al. 2016; European Food Safety Authority 2009a). All *spa* types identified here were related and in accordance with previous studies on LA-MRSA CC398 (Nemeghaire et al. 2014; Köck et al. 2013; Verhegghe et al. 2013; European Food Safety Authority 2009a). The *dru* types were also related, but, in addition to the four newly described types, the *dru* types dt9v, dt10ao, dt11ax, and dt11ca have not been found in LA-MRSA CC398 prior to this study. The detected SCC*mec* type IV and V cassettes were consistent with the literature (Monecke et al. 2018). The Chinese LA-MRSA CC9 isolates (see list of publications, **Jiang et al. 2021**) were assigned to the common *spa* types t899 or t4358 (Li et al. 2017; Cui et al. 2009; Neela et al. 2009; Wagenaar et al. 2009). However, one isolate displayed *spa* type t4132, which has been reported from LA-MRSA ST398 (Tegegne et al. 2021). The carriage of a SCC*mec* XII or pseudo-SCC*mec* XII element was consistent with other studies (Yu et al. 2021; Jiang et al. 2019a). Phylogenetic and molecular clock analysis indicated an emergence of CC9 to the dominant LA-MRSA clone in China from its proposed most recent common ancestor around 1987 (see list of publications, **Jiang et al. 2021**). This is 15 years later than the first presumable description of LA-MRSA in Europe from cases of bovine mastitis, which was an occasional finding prior to the first confirmed LA-MRSA case (Butaye et al. 2016; Devriese et al. 1972). Nonetheless, our study suggested a faster evolution rate of CC9 in relation to CC398 (see list of publications, **Jiang et al. 2021**), which might be the result of a more extensive usage of antimicrobials in the Chinese pig production and the growing pig trading activities (Wang et al. 2021). Interestingly, one study has revealed that the CC9 lineage was present in Europe before CC398 and it is one of the most frequent MSSA clones in pigs (Espinosa-Gongora et al. 2014).

For the most part, the German LA-MRSA CC398 and the Chinese CC9 lineages displayed similar virulence profiles commonly associated with the respective clone. Most importantly, the German LA-MRSA CC398 (**Publication II**) typically lacked genes coding for the major virulence factors TSST-1 and PVL (Wendlandt et al. 2013c). The Chinese LA-MRSA CC9 isolates (see list of publications, **Jiang et al. 2021**) were also negative for these genes. However, in the literature, only the absence of PVL-encoding genes has been described as usual for LA-MRSA CC9 (Chuang and Huang 2015). Although exfoliative and enterotoxin genes have been reported to be less common in LA-MRSA CC398 (Becker et al. 2017; Wendlandt et al. 2013c), all CC398 isolates harbored the exfoliative toxin gene *eta* and the enterotoxin type P gene *sep*. Eight isolates even carried one or two additional enterotoxin encoding gene(s). Considering the toxin-associated greasy pig disease and food poisoning

(Park et al. 2013; Argudín et al. 2010), respectively, this finding might be of clinical relevance as the virulence potential of porcine LA-MRSA CC398 seemed to have increased in relation to earlier reports. The *eta* gene was also identified in China in an MRSA ST398 from a hospitalized child (Wu et al. 2011). In comparison, the Chinese LA-MRSA CC9, for the most part, also harbored *eta* and typically the *egc* enterotoxin gene cluster (Monecke et al. 2011). Moreover, both clones carried multiple hemolysin genes, which have an important role in colonization and infection (Katayama et al. 2013), and the von-Willebrand-factor-binding protein gene *vwb* that is discussed as essential for host adaptation (Viana et al. 2010) (see also section *3.1.2*). However, the German LA-MRSA CC398 shared capsule type 5, whereas Yu et al. reported capsule type 8 for LA-MRSA CC9 (Yu et al. 2021). The IEC genes *sak*, *chp* and *scn* were absent in both lineages. Since they were described to be key factors in the human-specific adaptation of ST398 (Kashif et al. 2019; Uhlemann et al. 2012), this observation indicates that the German CC398 as well as the Chinese CC9 clone are more adapted to animals than to humans. The carriage of an *icaRADBC* operon for biofilm formation was a shared characteristic of the German CC398 isolates and the CC9 lineage (Yu et al. 2021). However, 34 of the German LA-MRSA CC398 harbored an additional *ica*-like gene cluster, probably on a plasmid. The *ica* operon is usually located in the chromosomal DNA of *S. aureus* (Marincola et al. 2021). Seven varying types of this cluster could be distinguished and 20 of these isolates carried an operon that has already been detected on a multiresistance plasmid in a bovine LA-MRSA ST398 (Feßler et al. 2011). The *ica*-like-positive plasmid from one of the 34 isolates carrying two biofilm operons is characterized in **Publication VI** (see section **3.2** for further discussion).

The German LA-MRSA CC398 clone typically exhibited a great variety of AMR properties, which reflected the amounts of the different classes of antimicrobial agents dispensed in veterinary medicine in Germany (Federal Office of Consumer Protection and Food Safety (BVL) 2022). Penicillins and tetracyclines are most widely used, followed by sulfonamides, polypeptides and macrolides. These groups are also commonly applied in German pig farming (Seitz et al. 2016; Merle et al. 2012). The isolates harbored three to 15 different AMR genes and with 158 out of 178, most of them were multiresistant. At least one β-lactam resistance gene (*mecA*) and one tetracycline resistance gene (*tet*(K)/*tet*(L)/*tet*(M)) was detected in all isolates. Common for LA-MRSA, several isolates harbored multiple genes mediating the same resistance phenotype (Schwarz et al. 2018). However, combinations of genes conferring resistance to varying classes of antimicrobial agents were also identified in numerous isolates. Many of the found AMR genes (Table 2 in **Publication II**) are commonly detected among LA-MRSA (see section **1.2**) or staphylococci of animal origin (Schwarz et al. 2018; Wendlandt et al. 2013c) (**Figure 2**). Several rarer resistance genes were identified in up to 10 isolates, including *aadE*, *spd*, *spw*, *erm*(T), *lnu*(A), *lnu*(B), *lsa*(E), *vga*(A)$_{LC}$, *vga*(C) and

cfr. Two isolates carried the novel *spc* variant spc_V on Tn*560*, which is reported in **Publication V**. Moreover, the study collection also comprised the isolate that harbored the novel plasmid-borne MLS_B resistance gene *erm*(54) characterized in **Publication VI**. The detection of the multiresistance gene *cfr* is of great concern (see section *1.2.7*). Two studies from Spain also identified *cfr* in a different Tn*558* variant together with *fexA* in a LA-MRSA CC398 isolate from a pig farmer and a diseased pig, respectively (Ruiz-Ripa et al. 2021a; Ruiz-Ripa et al. 2021b). There is particular interest in the spread of this gene because it also confers transferable resistance to last-resort antimicrobial agents, the oxazolidinones (Schwarz et al. 2018). In order to still be able to treat infections caused by multiresistant Gram-positive bacteria in the future, it is of prime importance to retain their effectiveness. Taking up on this, we prepared a comprehensive review on mobile oxazolidinone resistance genes in Gram-positive and Gram-negative bacteria within the framework of the joint project (see list of publications, **Schwarz et al. 2021**). Oxazolidinone resistance genes have been identified occasionally in various bacterial species from humans, animals, food and the environment in different countries of the world. These genes are mainly part of plasmids, but also of other MGEs, which plays an important role in their spread via HGT across strain up to order boundaries. Since these MGEs commonly also carry other AMR genes, biocide or heavy metal resistance genes, co-selection processes are probably a critical factor in the persistence of oxazolidinone resistance genes. This needs also to be taken into account considering the further dissemination of the entirety of AMR genes found among the German LA-MRSA CC398 collection, because many of these genes were also part of MGEs, such as transposons and plasmids (Figure 1 in **Publication II**). In comparison, the Chinese LA-MRSA CC9 lineage displayed more extensive AMR properties, probably reflecting a more diverse usage of different antimicrobial classes in Chinese pig production in relation to Germany (see list of publications, **Jiang et al. 2021**, **Ji et al. 2021**). The resistance profiles reflected the sales and consumption figures of antimicrobial drugs applied in the Chinese pig production (Kirchhelle 2018). For example, these isolates had distinctly higher resistance rates considering aminoglycosides, aminocyclitols, lincosamides, phenicols, trimethoprim, and fluoroquinolones. Due to the application of antimicrobial agents critical for human health and the total volume, China's antimicrobial usage is of global concern (Yang et al. 2019). The European Union and China have made great efforts on improving the rational and responsible application of antimicrobial agents in the sense of antimicrobial stewardship in the past decades (Lim and Grohn 2021). However, they are in different stages of this progress: The ban of antimicrobial agents used in animal feed for growth promotion came into effect in the European Union in 2006, in China in 2020 (Lim and Grohn 2021). The future will show whether the entities' different policies to decrease the usage of antimicrobial drugs in human and veterinary medicine will have a positive impact on the occurrence and persistence of AMR in LA-MRSA and other bacteria. In China for example, there has been

implemented a pilot project from 2018 to 2021 aiming at maintaining zero increase in the application of antimicrobial agents in livestock (Chinese Ministry of Agriculture and Rural Affairs (CMARA) 2018). In Germany, the sales figures of veterinary antimicrobial drugs have dropped by 68% between 2011 and 2022 (Federal Office of Consumer Protection and Food Safety (BVL) 2023). Similar to the German CC398 clone, the Chinese CC9 lineage harbored several AMR mediating MGEs (see list of publications, **Jiang et al. 2021**). This is not surprising since MGEs have been reported to constitute 15 to 20% of the *S. aureus* genome, thereby providing an evolutionary advantage, especially if resistance genes are present (Lindsay 2010). The chromosomal integration of MGEs stabilizes the presence of such genes compared to a location on plasmids (see list of publications, **Jiang et al. 2021**). For example, Tn*4001*, Tn*558* and the *spw* multiresistance cluster were detected in both lineages. However, the *spw* cluster of the CC9 isolates was more complex, also compared with previous studies that have shown the wide spread of the *spw* cluster among LA-MRSA ST9 isolates (Li et al. 2017; Li et al. 2014; Li et al. 2013; Lozano et al. 2012). The clusters comprised in part more AMR genes, and the numerous insertion sequences suggest that HGT might have led to the formation of these variants. Nonetheless, the German LA-MRSA CC398 clone displayed overall a greater variety of distinct MGEs, such as transposons and plasmids, than the Chinese CC9 isolates. This finding is also supported by results from Yu et al. (Yu et al. 2021) and is in accordance with our deduction of the CC398 lineage seeming to easier integrate foreign genetic material probably due to an incomplete type 1 RM system (see section *3.1.2*).

Furthermore, several correlations were observed among the German LA-MRSA CC398 collection (**Publication II**). For example, isolates of the same cluster often showed similar characteristics, such as the more frequent or exclusive display of certain *spa* and *dru* types or AMR and biocide resistance genes as well as the exhibition of similar or even identical resistance profiles. Moreover, some isolate clusters, *spa* and *dru* types or AMR and biocide resistance genes were associated with specific regions of Germany. Assorted isolates originating from nearby locations displayed similar or identical resistance profiles. Some *spa* and *dru* types, virulence and AMR genes were identified only during the early years of the study period. However, this might be due to an overrepresentation of this period, considering the higher isolate numbers until 2010. In addition, our study possibly suggested a change of the dominant *dfr* gene variant from 2016 from *dfrK* to *dfrG*. Notably, another study on the LA-MRSA ST9 lineage also reported *dfrG* as the main trimethoprim resistance determinant (Yu et al. 2021), which is also the globally predominant variant in human *S. aureus* infections (Nurjadi et al. 2015). Overall, clonal and geographical correlations of molecular characteristics, virulence and resistance properties were observed more frequently than temporal relations. Although Germany has implemented and continues to refine instruments monitoring antimicrobial consumption in human and veterinary medicine, region-specific data are not

available yet to evaluate whether the observed correlations might be related to the application of potentially preferred drugs (Noll et al. 2018). In addition, the uneven data set must also be taken into account.

Finally, studies on LA-MRSA CC398 in pigs from other European countries (Ruiz-Ripa et al. 2021a; Pirolo et al. 2019; Sieber et al. 2018; Mroczkowska et al. 2017; Sharma et al. 2016; Verhegghe et al. 2016; Karpíšková et al. 2015; Peeters et al. 2015) have revealed AMR and virulence characteristics that are comparable to the findings of our study, which was, nevertheless, the most comprehensive approach.

3.2 Detection of novel MGEs and AMR genes

Bacterial populations undergo genetic changes over time. The more comprehensive an isolate collection is investigated, the greater is the probability of finding interesting and/or novel genetic features. Whole-genome sequencing (WGS) allows deep insight into clonal relationships, the genetic basis of AMR and possible dissemination routes (Juraschek et al. 2021). However, for certain purposes long-read or short-read sequencing alone might be insufficient (Juraschek et al. 2021). Long-read assembly might lead to missing information or the incorporation of errors, whereas short-read assembly does not allow closing of certain DNA fragments and is therefore not appropriate for drawing complex conclusions (Juraschek et al. 2021). For this reason, a hybrid sequencing approach providing closed genomes was chosen for the detailed characterization of certain LA-MRSA (Juraschek et al. 2021). These isolates carried interesting MGEs that could be characterized thoroughly this way.

Publication III reports the identification of a novel SCC*mec* type V variant in a porcine LA-MRSA ST398 isolate from China. This isolate displayed *spa* type t571, which is not very common in MRSA ST398 (Cuny et al. 2013b; van Belkum et al. 2008), but in MSSA ST398 associated with the environment, pigs and humans (Li et al. 2022), including human infections reported from China and several other countries (Bonnet et al. 2018; Wardyn et al. 2018; Cuny et al. 2013b; Jimenez et al. 2011; Vandendriessche et al. 2011; van der Mee-Marquet et al. 2011; Chen et al. 2010; van Belkum et al. 2008). Prior to infection, many of the infected patients had no or only indirect livestock contact. However, one study has recently shown that *spa* type t571 was predominant among pig-associated LA-MRSA ST398 circulating in a Chinese slaughterhouse (Li et al. 2022). In comparison to *spa* type t034, which is frequently identified in LA-MRSA CC398, *spa* type t571 is lacking only one repeat, r24. Moreover, the ST398 isolate displayed a novel *dru* type, designated dt9bw. The novel SCC*mec* variant was 79,947 bp in size (GenBank accession number MN220716) and represented a composite of a type V SCC*mec* element including the methicillin resistance determinant, a novel pseudo-SCC element and a remnant of a SCC*mec* XII cassette (Figure 1 in **Publication III**). It was classified

as SCC*mec* type V variant in accordance with the nomenclature of the IWG-SCC (International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC) 2009). The element harbored a novel *ccrA* allotype, tentatively designated as *ccrA**. An IS*431* element flanked the *mec* gene complex, which is commonly observed to integrate into different SCC*mec* cassette types at varying positions (Wang et al. 2022). In addition, two type 1 RM systems have been detected within the element, which protect the respective isolate from invading foreign genetic material and may play an essential role in the stabilization of the SCC*mec* cassette in the chromosomal DNA (Ito et al. 2004). The shared homologies of the novel SCC*mec* type V variant with other type V as well as type XII elements point towards a continuing evolution of SCC*mec* cassettes in LA-MRSA CC398 from pigs (Monecke et al. 2018). Together with the detection of a rather uncommon *spa* type and a novel *dru* type, this emphasizes the ongoing diversification within CC398 discussed in section *3.1.3*. The SCC*mec* element plays a crucial role in the molecular evolution of MRSA and the dissemination of AMR, thus a detailed characterization of its structure and prevalence is a key element in the surveillance of MRSA isolates as requirement for taking appropriate preventive or therapeutic measures. Moreover, SCC*mec* typing is meant to improve the understanding of phylogenetic relationships between isolates as well as their epidemiological origin (International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC) 2009). Novel SCC*mec* types and variants have been commonly identified in isolates of human or animal origin and more rarely in food-associated MRSA (Wang et al. 2022). It is worth noting that SCC*mec* elements are not only carried by *S. aureus*, but even more often by coagulase-negative staphylococci (CoNS) (Garza-González et al. 2010). The same SCC*mec* types can exist in *S. aureus* and CoNS, but specific new variants might also be found exclusively among CoNS (Garza-González et al. 2010). However, the CoNS SCC*mec* cassettes seem to be more diverse and CoNS are suspected as a reservoir from which MSSA can obtain SCC*mec* resulting in the development of novel MRSA clones (Partridge et al. 2018).

Publication IV describes the detection of Tn*553*, a new 9,050-bp member of the Tn*554* family, in a porcine LA-MRSA ST9 from China (GenBank accession number CP031838). Various Tn*554*-related transposons have been reported during recent years, all sharing a rather conserved transposition unit consisting of the transposase genes *tnpA*, *tnpB* and *tnpC*, but varying in the carried AMR and biocide resistance determinants (Figure 1 (a) in **Publication IV**). Although Tn*553* showed a typical organization of the transposase genes, the corresponding Tnp proteins differed notably, as did the remaining part of the element. On its 5’ end, it harbored a complete *blaZ-blaR1-blaI* β-lactamase operon, which displayed high similarity to the equivalent operon on Tn*552* from *S. aureus* (Rowland and Dyke 1990). The Tn*554*-related transposons move by a unique mechanism (Murphy et al. 1985), but Tn*553*

prefers a different integration site than the former members of the family, namely the chromosomal *yolD* gene (Figure 1 (b) in **Publication IV**). This may be due to a differing target site specificity of the distantly related Tn*553* transposases. The mobility of Tn*553* in this isolate was confirmed by detection of its circular form via PCR. In accordance with earlier reports of the detection of Tn*554*-like transposons or other Tn*554*-related transposons, such as Tn*558* and Tn*5406*, on plasmids, a Tn*553*-like transposon was also found on a conjugative *S. aureus* plasmid inserted into a *yolD*-like gene (Kehrenberg and Schwarz 2005; Haroche et al. 2002; Townsend et al. 1986). Moreover, database searches revealed a wide dissemination of Tn*553* and closely related variants, integrated into the chromosomal *yolD* of isolates of many staphylococcal species from animals, humans and food in various parts of the world.

A new member of the Tn*554* family was also characterized in **Publication V**. Tn*560* was 12,518 bp in size (GenBank accession number MW832219). It was identified in a porcine LA-MRSA ST398 from China, typically inserted into the chromosomal *radC* gene (Murphy et al. 1985). In contrast to Tn*553* from **Publication IV**, the three transposase genes of Tn*560* were identical to those of Tn*554* (Figure 1 (a) in **Publication V**). Furthermore, Tn*560* carried a multiresistance gene cluster comprising a novel *spc* variant, designated *spc*$_V$, as well as *lsa*(E) and *lnu*(B). Figure 1 (a) and (b) in **Publication V** show that Tn*560* is most likely the result from a recombination of Tn*554* with a *spw*-*lsa*(E)-*lnu*(B)-carrying multiresistance plasmid from porcine *Enterococcus faecalis*. Similar to the *erm*(33) gene, an *in vivo* recombination product between *erm*(A) and *erm*(C) (Schwarz et al. 2002), the novel *spc*$_V$ is probably the result of an *in vivo* recombination between the related genes *spw* and *spc* (Figure 1 (a) and (b) in **Publication V**). The functional activity of Tn*560* in this isolate was confirmed via identification of its circular form by PCR.

Publication VI deals with the identification and characterization of a novel *erm* gene, designated *erm*(54), on the non-conjugative 36,929-bp plasmid pHKS3860 (GenBank accession number OM362497) in a porcine LA-MRSA ST398 isolate from Germany. The *erm* genes code for methylation of the A2058 residue in the 23S rRNA of the 50S ribosomal subunit, what confers cross-resistance to macrolides, lincosamides and streptogramin B compounds due to the agents' overlapping binding sites (Schwarz et al. 2018). Macrolides and lincosamides are largely used in pigs for the treatment of various infections, whereas streptogramins are not approved for the use in veterinary medicine in the European Union (Giguère 2013a; Giguère 2013b; European Medicines Agency 2011). However, MLS$_B$ compounds are also important broad-spectrum antimicrobial agents in human medicine. Macrolides are classified with regard to the size of their lactone ring: Erythromycin is, for example, a 14-membered ring macrolide, the 15-membered ring group includes compounds such as gamithromycin and azithromycin, while tilmicosin or tildipirosin belong to the 16-membered ring macrolides (Giguère 2013b). Including *erm*(54), 48 *erm* genes along with

numerous subclasses have been characterized so far and they are mainly carried by plasmids and transposons (Roberts 2022). In order to clarify the nomenclature of the *erm* genes, a nomenclature system has been established (Roberts 1999). A novel gene must have ≤79% aa identity in its deduced sequence with all previously characterized MLS proteins before receiving a unique name (Roberts 1999). The Erm(54) protein was closest related to Erm(B) identified in *Streptococcus suis* with an aa identity of 72.2% (Figure 1 (b) in **Publication VI**). Database searches revealed the presence of Erm(54) also in *E. faecalis* isolates from poultry in the USA and in MRSA ST398 isolates from a pig and a human, respectively, from the Netherlands. However, the corresponding genes were not further characterized and no confirmation of functionality or transferability was carried out. In our study, the transferability of *erm*(54) was confirmed via electrotransformation of plasmid pHKS3860 and its functionality was proven by AST of the transformants. The *erm*(54) gene distinctly increased the MICs of all macrolides and lincosamides tested, whereas streptogramin B could not be tested individually because test substances are not commercially available. The 30:70 streptogramin B/A mixture quinupristin/dalfopristin was tested instead, but since the proportion of the B compound is too low in the mixture, only a two-fold increase of the MIC was detected, which is commonly considered to be within the acceptable error rate of MIC determination. However, it is very likely that the MIC for streptogramin B would also be elevated, if the agent would be available individually due to the cross-resistance typically mediated by the *erm* genes. The expression of MLS_B resistance can be either constitutive or inducible (Leclercq 2002). In inducible MLS_B resistance, inactive messenger RNA (mRNA) is produced, which only becomes active posttranscriptionally under the influence of an inducer compound (Leclercq 2002; Weisblum 1995). This is the consequence of a regulatory region known as translational attenuator located upstream of the *erm* gene. This translational attenuator controls the gene expression and determines the pattern of inducer molecules through its structure (Leclercq 2002). Therefore, inducible MLS_B resistance can be very diverse and is possibly associated with rather complex phenotypes (Leclercq 2002). An mRNA is transcribed from the *erm* gene and the translational attenuator. The translational attenuators of the different *erm* genes comprise one or more reading frames for regulatory peptides and several different pairs of IRs, which are reverse complementary sequences. In the presence or absence of an inducer, the IRs form different mRNA secondary structures, which then allow or prevent translation of the *erm* gene (Leclercq 2002; Weisblum 1995). The isolates carrying an inducible *erm* gene are phenotypically resistant to the inducers but susceptible to the non-inducers. Considering for example inducibly expressed *erm*(A) and *erm*(C) genes, very common *erm* genes in staphylococci (Schwarz et al. 2018), 14- and 15-membered ring macrolides are inducers, whereas 16-membered ring macrolides, all lincosamides and all streptogramin B agents are non-inducers (Leclercq 2002). In contrast, constitutive expression is associated with structural

modifications in the translational attenuator, such as deletions, duplications or point mutations, and typically leads to a characteristic high-level MLS_B-cross-resistance phenotype (Feßler et al. 2018; Leclercq 2002). The phenomenon of inducible MLS_B resistance has clinical relevance. In the presence of non-inducers, constitutive mutants can be generated *in vitro* overnight and this selection has also been demonstrated in the clinical setting (Feßler et al. 2018; Leclercq 2002). Therefore, the treatment of an infection caused by an inducibly resistant strain with a non-inducer MLS_B compound is not without risk (Leclercq 2002) and should be avoided. The *erm*(54) found in **Publication VI** was expressed constitutively. The translational attenuator identified upstream of *erm*(54) was in fact more complex in relation to the corresponding segments of other common staphylococcal *erm* genes (Feßler et al. 2018; Schmitz et al. 2002; Weisblum 1995). Furthermore, a newly developed *erm*(54)-specific PCR assay could detect *erm*(54) in the original strain and the pHKS3860-carrying transformants, but none of the next-related *erm* genes available to us. Although plasmid pHKS3860 did not harbor additional AMR genes, it carried several heavy metal resistance genes. Besides *cadD* and *cadX* or *copB* and *mco* known among LA-MRSA (Schwarz et al. 2018), also the mercury resistance genes *merR* and *merA* were detected, which are normally part of the *mer* operon together with additional genes, such as *merT*, *merP* and/or *merC*, *merF* (Nascimento and Chartone-Souza 2003). The *merR* and *merA* genes encode the functional proteins for regulation and mercury reduction, respectively, and are alone most likely not sufficient for the full expression of mercury resistance (Nascimento and Chartone-Souza 2003). However, susceptibility to heavy metals was not investigated because there are no standardized testing methods or interpretive criteria available (Rensing et al. 2018) and at least cadmium and mercury are toxic compounds that cannot be handled easily in every laboratory. Due to the co-location of *erm*(54) on the same plasmid with various heavy metal resistance genes, co-selection of *erm*(54) may also occur under the selection pressure imposed by metal compounds, such as copper, in animal feed (Rensing et al. 2018). Mercury and cadmium occur naturally in the environment or may accumulate, for example in animal feed, due to industrial contamination (Adamse et al. 2017). Furthermore, an *ica*-like gene cluster was detected on pHKS3860. The LA-MRSA isolate was among the 34 isolates of the German LA-MRSA CC398 collection discussed in **Publication II** carrying two biofilm operons (see section *3.1.3*). In comparison, plasmid pHKS3860 harbored a singular type of *ica*-like operon. The bacterial biofilm is understood as a structured bacterial community, which adheres to a surface encased by a self-produced extracellular matrix mainly consisting of polysaccharides, proteins and extracellular DNA (Schilcher and Horswill 2020; Costerton et al. 1978). It protects the individual bacteria from harmful environmental influences, such as desiccation, antimicrobial agents, disinfectants or the host immune system (Schilcher and Horswill 2020). The ability to produce robust biofilms on biotic and abiotic surfaces represents an important pathomechanism, also

contributing to the emergence of staphylococcal nosocomial infections (Schilcher and Horswill 2020). Normally, the *ica* cluster is present in the chromosomal DNA of *S. aureus* (Marincola et al. 2021). Carriage of a chromosomal *ica* locus and a differing plasmid-associated *ica* operon has already been reported previously from a bovine LA-MRSA ST398 isolate (Feßler et al. 2017). Similar to pHKS3860, several AMR and heavy metal resistance genes were found to be co-located with the *ica* cluster on the same plasmid in that study (Feßler et al. 2017). The carriage of two differing *ica* operons in the same isolate was probably the result of several recombination events leading to the formation of the plasmid that has subsequently been acquired via HGT (Marincola et al. 2021). This may also have been the course of events considering the 34 LA-MRSA CC398 harboring two biofilm loci discussed in **Publication II**. However, despite two *ica*-cluster the bovine LA-MRSA ST398 from the previous study did not produce biofilm when tested in routine assays (Feßler et al. 2017). The reason for this is a regulatory crosstalk between plasmid-encoded and chromosomally encoded factors inhibiting the *ica*-mediated biofilm formation, most likely to prevent high metabolic costs in a situation where the biofilm is not essential for survival (Marincola et al. 2021). Lastly, plasmid pHKS3860 harbored several different complete or truncated transposase genes (Figure 1 (a) in **Publication VI**), which points towards the possibility of genetic rearrangements.

Finally, the detection of novel MGEs and AMR determinants underlines the ongoing diversification of LA-MRSA isolates within CC398. Considering the formation mechanisms and the dissemination of the MGEs, not only intraspecies, but also interspecies and intergenus genetic shifts in staphylococci and other Gram-positive bacteria need to be taken into account. Further monitoring of the novel MGEs and AMR genes is necessary to early recognize future public health problems.

3.3 Concluding remarks

LA-MRSA CC398 has become the most common LA-MRSA lineage worldwide dominating in Europe, Australia and the Americas, while LA-MRSA CC9 prevails in Asia (Butaye et al. 2016). In the context of our German-Chinese joint project, we identified the extensive acquisition of AMR properties, an increased biofilm formation ability and an enhanced tolerance to desiccation as factors that could have contributed to the development of two different epidemic clones in Germany and China, respectively. Metabolic variations did most likely not play a crucial role in that process. However, we hypothesize that LA-MRSA CC398 might gradually replace the CC9 lineage in China because of several advantageous features that render CC398 superior. Metabolic disadvantages and a lower growth rate of the CC9 clone may support this process. Due to an increased pathogenicity of the CC398 lineage, LA-MRSA originating from pigs might then pose a greater public health risk.

In addition, we gained understanding of the common characteristics of the dominating Chinese LA-MRSA CC9 and German LA-MRSA CC398 lineages. Both clones exhibited attributes typical for them, but also some unusual traits, such as the carriage of *eta* by all CC398 isolates or the lack of *tst* in the LA-MRSA CC9. Comparison of the two clones revealed similarities, such as the high detection rate of AMR and MGEs, but also differences when investigating in detail, such as the differing AMR profiles or variety of MGEs. Deeper insight into the population dynamics of the German CC398 lineage disclosed a wide molecular diversity, close phylogenetic relationships and clonal, geographical and temporal correlations of molecular characteristics, AMR and virulence traits.

Furthermore, this thesis made clear that the pathogenicity of LA-MRSA has been demonstrated in animals as well as humans (Butaye et al. 2016; Cuny et al. 2015b). In addition, their common multiresistance to various different classes of antimicrobial agents poses a global health risk (Butaye et al. 2016). Having in mind that AMR has been predicted to become the greatest threat to healthcare by 2050 (O'Neill 2014), LA-MRSA represent a considerable AMR repository. They can act as AMR gene donors for other bacteria across genus boundaries, including those being present exclusively in the environment (Schwarz et al. 2018). In this regard, HGT is a major driver of the rapid global spread of AMR (Lerminiaux and Cameron 2018). We characterized multiple novel MGEs and AMR genes in LA-MRSA within the project, which contributes to the understanding of how bacteria exchange AMR properties. Such novel MGEs and AMR gene variants should be closely monitored in order to early detect potential future problems for public health. Notably, we showed once again that AMR, heavy metal resistance and virulence properties may be transmitted via MGEs in a single transfer event. Therefore, co-selection processes need to be considered when measures, such as a ban of certain antimicrobial and metal compounds, are taken in order to reduce AMR (Rensing et al. 2018; Schwarz et al. 2018).

In view of the increasing diversity of LA-MRSA and ongoing genetic rearrangements, there will always be a lot to learn about the associated MGEs. Even between members of the same MGE family or modules sharing a function, there is an extensive sequence divergence, which points towards a continuing co-evolution with their host (Partridge et al. 2018). Further research of these elements in a broad environment is needed, including improved WGS approaches to obtain the most comprehensive understanding as possible (Partridge et al. 2018).

Finally, livestock trading activities, human occupational exposure, and environmental emissions favor the spread of LA-MRSA and subsequently the exchange of genetic material including AMR, heavy metal resistance, biocide resistance and virulence determinants. The prevalence of LA-MRSA will most likely not decrease, but diversification will proceed (Butaye et al. 2016). The type of pig husbandry plays a crucial role in the persistence of LA-MRSA in

pigs (Kobusch et al. 2022). Thus, decolonization programs need to target multiple husbandry factors (Kobusch et al. 2022). However, since an extensive implementation of this remains uncertain, a large-scale LA-MRSA surveillance including the farm, community and healthcare level is essential to prevent new emerging and possibly more virulent and/or resistant clones from further dissemination among pig husbandries and entry into human community.

4 Summary

Comparative phenotypic and genotypic studies on LA-MRSA CC398 and CC9 of pigs from Germany and China

Livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) are opportunistic zoonotic pathogens that have been globally recognized as a health threat in human and veterinary medicine. They are known for their common multiresistance to antimicrobial agents and may cause severe diseases although normally lacking major virulence factors. Colonized pigs and their related surroundings represent a considerable LA-MRSA reservoir. Due to their low host specificity, LA-MRSA isolates can easily cross species barriers. Therefore, persons with frequent (occupational) exposure to pigs have an increased risk of LA-MRSA colonization and/or infection and they may further transmit these isolates to contact persons and animals. The LA-MRSA prevalence differs geographically: Clonal Complex (CC) 398 dominates among pigs in Europe, Australia and the Americas, whereas CC9 is the most common lineage among pigs in Asia.

This thesis revealed that differences in metabolic properties did most likely not play a key role in the development of the aforementioned two different epidemic porcine LA-MRSA clones in Germany and China, respectively. However, it was hypothesized that LA-MRSA CC398 might gradually replace the currently dominating CC9 lineage in China in the future because of many beneficial features that may enable preferential survival of the CC398 lineage under hostile environmental conditions or in the host. Possibly unfavorable metabolic variations and a lower growth rate of the Chinese LA-MRSA CC9 clone may be disadvantageous in niche adaptation and, thus, may support the replacement process. Consequently, the public health risk originating from porcine LA-MRSA might rise because of the increased pathogenicity of the CC398 lineage.

Moreover, this thesis provided insight into population dynamics and molecular diversity of the epidemic porcine LA-MRSA CC398 lineage in Germany over 13 years in comparison with the porcine LA-MRSA CC9 lineage dominating in China. In general, both clones exhibited typical individual characteristics, but also some novel features. During comparison, similarities as well as distinct differences were recognized between the two lineages. The German CC398 isolates were widespread within Germany and largely reflected the pig density of the different regions. They displayed a wide molecular diversity and rather close phylogenetic relationships. An ongoing diversification was recognized over time. The CC398 and CC9 lineage both displayed a clear virulence potential, although genes coding for the major virulence factors Panton-Valentine leucocidin and toxic shock syndrome toxin-1 were not detected. Moreover, both lineages exhibited a great variety of AMR properties that mirrored

the respective sales figures of veterinary antimicrobial agents in the two countries. The AMR profiles of the Chinese isolates were more extensive. In both lineages, AMR was largely associated with mobile genetic elements (MGEs), which can be transferred easily across strain, species or even genus boundaries via horizontal gene transfer (HGT). However, the German CC398 isolates displayed a greater variety of different MGEs, which points towards the easier integration of foreign genetic material. In addition, clonal and geographical correlations of molecular characteristics, virulence and resistance properties were more frequently observed among the LA-MRSA CC398 from Germany than temporal correlations.

Furthermore, multiple novel MGEs were characterized in this thesis. A novel type V variant of the methicillin-resistance mediating SCC*mec* cassette was identified in a porcine LA-MRSA CC398 isolate from China. Homologies with other type V and XII elements indicate an ongoing evolution of SCC*mec* elements in LA-MRSA CC398 from pigs. Tn*553* and Tn*560* represent two new members of the Tn*554* transposon family detected in Chinese LA-MRSA CC9 and CC398 isolates, respectively. Tn*553* harbored a complete β-lactam resistance mediating *blaZ-blaR1-blaI* β-lactamase operon and preferred a different integration site than former Tn*554*-related transposons. Tn*560* carried a multiresistance gene cluster comprising the spectinomycin resistance gene spc_V, a novel *spc* variant that is most likely the product of a recombination between the related *spc* and *spw* genes, the pleuromutilin-lincosamide-streptogramin A resistance gene *lsa*(E) and the lincosamide resistance gene *lnu*(B). Plasmid pHKS3860 from a German porcine LA-MRSA CC398 isolate harbored the novel macrolide-lincosamide-streptogramin B resistance gene *erm*(54), cadmium, mercury and copper resistance genes, as well as an *ica* gene cluster for biofilm formation. As a consequence of such co-localizations on the same MGE, multiple resistance and/or virulence properties can be transferred in a single event. Even in the absence of a direct selection pressure, the risk for co-selection processes is increased.

Finally, this thesis expanded the knowledge on the epidemic porcine LA-MRSA lineages in Germany and China. They represent a considerable AMR repository adding to the global health threat posed by AMR with HGT as major driver of rapid dissemination. Moreover, the understanding of genetic rearrangements in LA-MRSA and how these bacteria exchange genetic material has been improved. The diversity of LA-MRSA further increases and new emerging and possibly more virulent and/or resistant variants should be closely monitored. Due to the zoonotic potential and the risk of further spread among pig husbandries and into the human community, a full-scale LA-MRSA surveillance is vitally important.

5 Zusammenfassung

Vergleichende phänotypische und genotypische Untersuchungen von LA-MRSA CC398 und CC9 bei Schweinen aus Deutschland und China

Nutztierassoziierte Methicillin-resistente *Staphylococcus aureus* (LA-MRSA) Isolate zählen zu den opportunistischen Zoonoseerregern, die weltweit in der Human- und Veterinärmedizin als Gesundheitsgefahr erkannt wurden. Diese Isolate sind für ihre häufige Multiresistenz gegenüber antimikrobiellen Wirkstoffen bekannt und können schwere Erkrankungen verursachen, obwohl ihnen normalerweise wesentliche Virulenzfaktoren fehlen. Kolonisierte Schweine und deren Umgebung stellen ein bedeutsames LA-MRSA-Reservoir dar. Aufgrund ihrer geringen Wirtsspezifität können LA-MRSA-Isolate leicht Speziesbarrieren überwinden. Daher haben Personen mit häufigem (beruflichen) Kontakt zu Schweinen ein erhöhtes Risiko für eine LA-MRSA-Besiedlung und/oder -Infektion und sie können diese Isolate weiter an Kontaktpersonen und -tiere übertragen. Die LA-MRSA-Prävalenz unterscheidet sich geografisch: Bei Schweinen in Europa, Australien und Nord- und Südamerika dominiert der klonale Komplex (CC) 398, während bei Schweinen in Asien CC9 die häufigste klonale Linie darstellt.

In dieser These wurde gezeigt, dass unterschiedliche Stoffwechseleigenschaften höchstwahrscheinlich keine entscheidende Rolle bei der Entwicklung der beiden unterschiedlichen epidemischen LA-MRSA-Klone bei Schweinen in Deutschland beziehungsweise China spielten. Es wurde jedoch die Hypothese aufgestellt, dass LA-MRSA CC398 die derzeit in China dominierende CC9 Linie in Zukunft schrittweise ersetzen könnte, da viele vorteilhafte Eigenschaften der CC398 Linie ein bevorzugtes Überleben unter feindlichen Umweltbedingungen oder im Wirt ermöglichen könnten. Möglicherweise nachteilige Stoffwechselunterschiede und eine geringere Wachstumsrate des chinesischen LA-MRSA CC9 Klons könnten bei der Anpassung an ökologische Nischen von Nachteil sein und somit den Verdrängungsprozess unterstützen. Infolgedessen könnte das von porzinen LA-MRSA-Isolaten ausgehende Risiko für die öffentliche Gesundheit aufgrund der erhöhten Pathogenität der CC398 Linie ansteigen.

Darüber hinaus lieferte diese These einen Einblick in die Populationsdynamik und die molekulare Diversität der epidemischen porzinen LA-MRSA CC398 Linie in Deutschland über einen Zeitraum von 13 Jahren im Vergleich zur porzinen LA-MRSA CC9 Linie, die in China dominiert. Im Allgemeinen wiesen beide Klone typische individuelle Merkmale, aber auch einige neuartige Eigenschaften auf. Beim Vergleich wurden sowohl Gemeinsamkeiten als auch deutliche Unterschiede zwischen den beiden Linien festgestellt. Die deutschen CC398 Isolate waren innerhalb Deutschlands weit verbreitet und spiegelten zu einem großen Teil die

Schweinedichte der verschiedenen Regionen wider. Sie zeigten eine große molekulare Vielfalt und eher enge verwandtschaftliche Beziehungen. Über die Zeit wurde eine fortschreitende Diversifizierung festgestellt. Sowohl die CC398 als auch die CC9 Linie zeigten eindeutiges Virulenzpotenzial, obwohl Gene, die die wesentlichen Virulenzfaktoren Panton-Valentine-Leukozidin und Toxic-Shock-Syndrome-Toxin-1 kodieren, nicht nachgewiesen wurden. Außerdem besaßen beide Linien eine große Vielfalt an antimikrobiellen Resistenzeigenschaften, die die jeweiligen Verkaufszahlen von tiermedizinisch genutzten antimikrobiellen Wirkstoffen in den beiden Ländern widerspiegelten. Die antimikrobiellen Resistenzprofile der chinesischen Isolate waren ausgeprägter. Bei beiden Linien war die antimikrobielle Resistenz größtenteils mit mobilen genetischen Elementen assoziiert, die durch horizontalen Gentransfer leicht über Stamm-, Spezies- oder sogar Gattungsgrenzen hinweg übertragen werden können. Allerdings wiesen die deutschen CC398 Isolate eine größere Vielfalt an unterschiedlichen mobilen genetischen Elementen auf, was auf eine erleichterte Aufnahme fremden genetischen Materials hindeutet. Weiterhin wurden klonale und geografische Korrelationen von molekularen Merkmalen, Virulenz- und Resistenzeigenschaften bei den LA-MRSA CC398 Isolaten aus Deutschland häufiger beobachtet als zeitliche Zusammenhänge.

Außerdem wurden im Rahmen dieser These mehrere neue mobile genetische Elemente charakterisiert. Eine neue Typ-V-Variante der Methicillin-Resistenz vermittelnden SCC*mec*-Kassette wurde in einem porzinen LA-MRSA CC398 Isolat aus China identifiziert. Homologien mit anderen Typ-V- und -XII-Elementen deuten auf eine fortlaufende Evolution von SCC*mec*-Elementen in LA-MRSA CC398 Isolaten von Schweinen hin. Tn*553* und Tn*560* sind zwei neue Mitglieder der Tn*554*-Transposonfamilie, die in chinesischen LA-MRSA CC9 beziehungsweise CC398 Isolaten nachgewiesen wurden. Tn*553* trug ein vollständiges β-Laktam-Resistenz vermittelndes *blaZ-blaR1-blaI* β-Laktamase-Operon und bevorzugte eine andere Integrationsstelle als vorherige Tn*554*-verwandte Transposons. Tn*560* enthielt ein Multiresistenz-Gencluster bestehend aus dem Spectinomycin-Resistenzgen spc_V, einer neuen *spc*-Variante, die wahrscheinlich das Produkt einer Rekombination zwischen den verwandten Genen *spc* und *spw* ist, dem Pleuromutilin-Lincosamid-Streptogramin-A-Resistenzgen *lsa*(E) und dem Lincosamid-Resistenzgen *lnu*(B). Das Plasmid pHKS3860 von einem deutschen porzinen LA-MRSA CC398 Isolat trug das neue Makrolid-Lincosamid-Streptogramin-B-Resistenzgen *erm*(54), Cadmium-, Quecksilber- und Kupferresistenzgene sowie ein *ica*-Gencluster für Biofilmbildung. Als Folge solcher Co-Lokalisationen auf demselben mobilen genetischen Element können mehrere Resistenz- und/oder Virulenzeigenschaften in einem einzigen Vorgang übertragen werden. Selbst wenn kein direkter Selektionsdruck besteht, ist das Risiko für Co-Selektionsprozesse erhöht.

Abschließend hat diese These das Wissen über die epidemischen LA-MRSA-Linien bei Schweinen in Deutschland und China erweitert. Beide Linien stellen ein bedeutsames Repositorium antimikrobieller Resistenzgene dar, das zur globalen Gesundheitsbedrohung durch diese beiträgt, wobei der horizontale Gentransfer der wesentliche Faktor für eine schnelle Verbreitung ist. Darüber hinaus wurde das Verständnis der genetischen Umgestaltungen bei LA-MRSA-Isolaten und darüber wie diese Bakterien genetisches Material austauschen verbessert. Die Diversität von LA-MRSA-Isolaten nimmt weiter zu und neu auftretende, möglicherweise virulentere und/oder resistentere Varianten sollten genau beobachtet werden. Aufgrund des zoonotischen Potenzials und des Risikos einer weiteren Verbreitung innerhalb der Schweinehaltung und in die menschliche Gemeinschaft ist eine umfassende LA-MRSA-Überwachung von entscheidender Bedeutung.

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Selbstständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, den 04.12.2023 Henrike Krüger-Haker