Molecular Evolution and Adaptation of Livestock-Associated Methicillin-Resistant \textit{Staphylococcus aureus} (LA-MRSA) Sequence Type 9

Fangyou Yu,\textsuperscript{a,h} Astrid V. Cienfuegos-Gallet,\textsuperscript{d} Marcus H. Cunningham,\textsuperscript{d} Ye Jin,\textsuperscript{c} Bingjie Wang,\textsuperscript{a,h} Barry N. Kreiswirth,\textsuperscript{d} Liang Chen\textsuperscript{e}\textsuperscript{*}

\textsuperscript{a}Department of Clinical Laboratory, Shanghai Pulmonary Hospital, Tongji University School of Medicine, Shanghai, China
\textsuperscript{b}Shanghai Key Laboratory of Tuberculosis, Shanghai Pulmonary Hospital, Tongji University School of Medicine, Shanghai, China
\textsuperscript{c}First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, Zhejiang, China
\textsuperscript{d}Center for Discovery and Innovation, Hackensack Meridian Health, Nutley, New Jersey, USA
\textsuperscript{e}Department of Medical Sciences, Hackensack Meridian School of Medicine, Nutley, New Jersey, USA

Fangyou Yu and Astrid V. Cienfuegos-Gallet contributed equally to this work. Author order was determined randomly.

ABSTRACT
Livestock-associated methicillin-resistant \textit{Staphylococcus aureus} (LA-MRSA) sequence type 9 (ST9) has emerged and disseminated in Asia. It is associated with colonization or infection in both humans and animal hosts; however, the genetic factors underpinning its adaptation to animal and human population remain to be determined. Here, we conducted a genomic analysis of 191 ST9 \textit{S. aureus} genomes collected from 12 different countries, including 174 genomes retrieved from public databases and 17 sequenced in this study. In \textit{silico} spa typing, staphylococcal cassette chromosome \textit{mec} (SCC\textit{mec}) typing, and antimicrobial resistance and virulence gene mining were conducted, and the temporal phylogenetic signal was assessed by Bayesian inference. Our results point toward a human methicillin-susceptible \textit{S. aureus} (MSSA) origin of ST9 that evolved approximately 2 centuries ago. Three major genetic events occurred during ST9 host shift from human to animals: the loss of the immune evasion cluster genes (\textit{scn}, \textit{chp}, and \textit{sak}), which were reported to contribute to virulence in human infections, the acquisition of the SaPIbov4-like element-encoding \textit{vwb} gene, which is an animal-specific virulent factor responsible for the clotting of animal plasma, and the acquisition of antibiotic resistance genes, including SCC\textit{mec}, quinolone resistance-determining region (QRDR) mutations, and a multidrug resistance genetic element (MDR\textsubscript{ST9}). Evidence of direct transmission of animal-adapted strains to human hosts also suggest that transmission could potentially reshape the resistance and virulence genetic pool in these isolates. The rapid clonal expansion of MDR ST9 strains in mainland China and Taiwan highlights the increasing need for effective surveillance of antibiotic consumption in animal husbandry to control antimicrobial resistance spread.

IMPORTANCE \textit{Staphylococcus aureus} sequence type 9 (ST9) is the main LA-MRSA clone spreading in the Asian continent. It can colonize and cause mild to severe infections both in animal and humans. Previous work described its genotypic characteristics; however, the molecular history of global spread of ST9 strains remains largely unclear. We conducted a detailed analysis of genomic evolution of global ST9 strains and identified key genetic changes associated with its adaptation to specific hosts. Our results suggest that the ST9 clone originated from human-adapted strains, which lost genes related to the evasion of the immune system. The introduction of ST9 strains in animal populations was aligned with the acquisition of animal-specific virulent factors and mobile elements harboring multiple antimicrobial resistance genes, especially in isolates from mainland China and Taiwan.
**KEYWORDS** ST9, evolution, genomic analysis, antimicrobial resistance, virulence factors, LA-MRSA, pathogenicity island

**M**ethicillin-resistant *Staphylococcus aureus* (MRSA) is a common nosocomial and community-associated pathogen that can colonize or cause infections in both human and animals. Livestock-associated MRSA (LA-MRSA) has recently emerged and disseminated in Europe and North America. Since the first report of a human case of LA-MRSA in a 6-month-old infant in a pig farming family in the Netherlands in 2005 (1), an increasing number of cases of LA-MRSA have been described globally. Molecular screening among people with close contact with pigs and calves before hospital admission in areas with a high density of pig farming has shown 78% LA-MRSA carriage among MRSA-positive patients (2). Currently, clonal complex 398 (CC398) is the most predominate LA-MRSA clone in North America and Europe. Recent studies have identified CC398 LA-MRSA in 24% of MRSA isolates from Netherlands (3) and more than 10% in Belgium, Denmark, Spain and Slovenia (4).

In Asia, in contrast, sequence type 9 (ST9) is the most frequent LA-MRSA detected in pig farms and food products of animal origin, including mainland China (5, 6), Taiwan (7), and Malaysia (8). The ST9 strains are typically multidrug resistant and display virulence profiles different from those of other LA-MRSA clones (5, 9). ST9 LA-MRSA strains have also been found colonizing both animal and human hosts (10), and causing mild to severe infections in humans (11, 12). Several studies reported that ST9 constituted 70% to 95% of MRSA isolates detected in pigs and pig farm workers in China (7, 13–15). In addition, previous studies have identified highly similar genomic profiles between ST9 MRSA isolates from swine and humans, suggesting a cross-species (animal-to-human or human-to-animal) transmission of this clone (10, 11, 16, 17).

Interestingly, strains of this clone have been reported to cause infections in patients without a history of livestock contact (11, 12). The predominance of this clone in animals and its detection in human cases raise the questions of its origin and its ability to spread between animal and human hosts. The aim of this study was to unveil the genomic evolution of ST9 LA-MRSA and to probe the genetic characteristics associated with its adaptation to different hosts and its antimicrobial resistance and virulence profiles.

**RESULTS**

**General characteristics of ST9 strains.** To probe the molecular evolution of ST9 strains, we analyzed publicly available genomes at the time of the study (January 2021) (n = 174) and 17 additional genomes from our collection, including ST9 strains collected globally between 1941 and 2019 from human and animal sources. These ST9 strains were isolated from swine (n = 110), human (n = 34), bovine (n = 12), meat (n = 11), other animal (n = 10), and unknown (n = 13) sources. The isolates were mostly collected in mainland China (n = 140), followed by Taiwan (n = 8), Ghana (n = 7), Germany (n = 6), the United States (n = 6), Czech Republic (n = 2), and Argentina, Colombia, Australia, Switzerland, and the United Kingdom (one isolate each). The 140 isolates from mainland China were collected from 13 of 31 different provinces, autonomous regions, or municipalities, with wide geographic distributions (see Fig. S1 in the supplemental material).

The 191 isolates belonged to 22 spa types, with spa t899 being the most frequent type (151/191, 79.1%). Eighty-one percent (122/151) spa t899 strains were identified in isolates from animal, while 16.5% (25/151) were from humans. Ninety-one percent (137/151) of spa t899 isolates were from mainland China or Taiwan. Other spa types identified were 1430 (7/191), t29922 (2/191), t4132 (2/191), t099, t100, t1334, t193, t2700, t464, t526, t587, t800, t13493, t2315, t3446, and t4794 (one of each).

Seventy-nine percent (151/191) of ST9 genomes harbored meCA, and the most predominant staphylococcal cassette chromosome mec (SCCmec) type was XII or XII-like (135/151). These SCCmec XII-like variants either harbored additional ccrA1/ccrB2 (n = 2)
or lacked meC class C2 and ccrC2 \((n = 11)\) (Fig. S2). SCCmeC\textsubscript{XII} or XII-like elements were detected in isolates from both animal (bovine, chicken, meat, and swine) and human sources. Eight isolates harbored SCCmeC\textsubscript{IV} (four from meat samples), and five contained hybrid SCCmeC\textsubscript{IV}\textsubscript{XII} (all from meat samples). Two strains harbored SCCmeC\textsubscript{V} \((2/191)\) and were of human origin. Selected genetic characteristics of human and animal isolates are presented in Table 1.

**Virulence factor-encoding genes in ST9 genomes.** We also investigated the presence of several virulence factor genes, including those for capsule, adhesins, secreted enzymes, toxins, and immune evasion, to analyze the presence of particular virulence profile in ST9 strains. All ST9 genomes harbored 12 genes of the capsular serotype 8 \((capB)\) cluster, the adhesin gene \(ebp\), and the polysaccharide intercellular adhesion locus genes \(icaA\), \(icaB\), \(icaC\), \(icaD\), and \(icaR\) (except that two were negative for \(icaB\) and \(icaC\)) (Data Set S1). Only a subset of isolates harbored the adhesion genes \(clfA\) \((71.7\%; n = 137)\) and \(clfB\) \((58.1\%; n = 111)\), most of which were from animal sources \((109/137\) and \(91/111\), respectively). Genes encoding secreted enzymes were identified in nearly all isolates, including those for aureolysin \((aur)\) \((100\%)\), serine proteases \((sspA, sspB,\) and \(sspC)\) \((100\%)\), lipase \((geh)\) \((99.5\%; n = 190)\), and hyaluronidase \((hys)\) \((95.3\%; n = 182)\).

Most ST9 genomes carried hemolysin genes, including \(hle\) \((91.1\%; n = 174)\), \(hld\) \((99.5\%; n = 190)\), \(hlgA\) \((100\%; n = 191)\), \(hlgB\) \((100\%; n = 191)\), \(hlgC\) \((100\%; n = 191)\), and \(hly/hla\) \((95.5\%; n = 190)\), which have been shown to play an important role in skin colonization and infection (18). ST9 genomes also encoded a pivotal virulence factor, the type 7 secretion system \((T7SS)\), which has been found to contribute to membrane integrity and homeostasis in the presence of antimicrobial fatty acids (19). Identified genes of \(T7SS\) corresponded to four membrane proteins \((esaA, esaA, essB,\) and \(essC)\), one cytosolic protein \((esaB)\), and two effector proteins \((esxA\) and \(esxB)\). All ST9 genomes also harbored the iron uptake protein genes \(isdA\), \(isdB\), \(isdC\), and \(isdC\), while most isolates lacked the enterotoxin genes \(sea, sec, selk, self, selq\) \((0.5\%; 1/191)\), and \(seb\) \((2.6\%, 5/186)\). Likewise, a gene encoding exfoliatin \((eta)\) was found in only five isolates,
including one from human and four from unknown sources. All isolates were negative for the Panton-Valentine leukocidin (PVL) genes lukS-PV and lukF-PV.

We also evaluated the presence of the immune evasion cluster (IEC) genes encoding staphyloccocal complement inhibitor (scn), chemotaxis-inhibiting protein (chp), and staphylokinase (sak). These genes were found in φSa3 prophages and were reported to be a major mechanism of human-specific adaptation contributing to the increased virulence of ST398 (20, 21). Importantly, we found that the IEC genes were located in an ~42-kb prophage similar to phage P282 sequence, which truncated the hlb gene, the common insertion site for φSa3 (Fig. S3). In total, 15 isolates were found to harbor IEC, nine of human origin and six of unknown origin.

Staphyloccocal type Vα genomic island in ST9 genomes. A previous study showed that ST9 strains harbor a VαSaα genomic island, which carries a SaPIbov4-like element, in addition to the staphyloccocal superantigen-like (ssl1 to ssl11) and lpl tandem genes (22). This VαSaα was designated as a type Vα genomic island based on the structure comparison with previously described type I to IV genomic islands and six additional novel VαSaα genomic islands (type VI to XI) (22). The SaPIbov4-like element encodes a von Willebrand binding protein (vwb), an important animal-related virulence factor that can cause bovine and caprine plasma clotting (22). To investigate whether the type Vα genomic island was conserved in different ST9 strains, we analyzed its presence and sequence variation in the 191 genomes. We found that all isolates carry a VαSaα structure, and 86.4% (165/191) of genomes contain the SaPIbov4-like element in VαSaα. The sequence alignment showed that an ~14-kb SaPIbov4-like element was inserted downstream of the glutamate synthase gene (guaA) and upstream of the amnoglycoside transferase gene (aadE) (Fig. 1). This SaPIbov4-like element had high frequencies (>90%) in swine (102/110), bovine (11/12), chicken (2/2), livestock farm (7/7), and meat (11/11) samples but a lower frequency (25/34; 73.5%) in human samples.

Antimicrobial resistance genes in ST9 genomes. To understand how the ST9 evolution could be driven by antibiotic selection pressure, we investigated the presence and colocalization of antibiotic resistance genes in ST9 genomes. Most ST9 isolates harbor multiple antimicrobial resistance genes, encoding resistance to β-lactams, aminoglycosides, lincosamides, chloramphenicol, tetracyclines, and quinolones. β-Lactam resistance was mainly mediated by mecA (79.1%; n = 151), and 83.8% of isolates also harbored a penicillin-hydrolyzing class A β-lactamase (blaZ) (83.8%; n = 160). Other common resistance genes included Inu(B) (75.4%; n = 144; encoding resistance to lincosamide), Isa(E) (75.9%; n = 145; lincosamide/streptogramin resistance) and erm(C) (60.7%; n = 116; macrolide resistance). Additional genes found in CC398 LA-MRSA isolates were also frequently found in the ST9 genomes, such as fexA (encoding a chloramphenicol/florfenicol efflux major facilitator superfamily [MFS] transporter) (69.1%; n = 132) (23). Most ST9 isolates carried the tetracycline resistance gene tet(L) (79.6%; n = 152), and one isolate (1/191) carried tet(M), which is also found in CC398 isolates (23). Seventy-six percent of ST9 isolates (n = 147) also harbored dfrG, a major determinant of trimethoprim resistance in human S. aureus infections (24). Only five isolates contained dfrK, an additional trimethoprim resistance gene.

Mutations associated with quinolone resistance in S. aureus were also commonly found in ST9 genomes, with 89.0% (n = 170) presenting the double mutation gyrA_S84A/S84L/S84V and parC_S80F in the quinolone resistance-determining regions (QRDRs). A small number of isolates carried rpoB mutations, including H481N (4.71%; n = 9), which was reported to promote the emergence of stable rifampin-resistant small-colony variant (SCV) subpopulations with reduced susceptibility to vancomycin and daptomycin (25). Other rare rpoB mutations (I527M and S529L) were detected in two isolates.

Further genome mining revealed that most antimicrobial resistance genes were chromosome borne. Besides the SCCmec region, the fexA transporter gene harboring Tn558 was located downstream of a JAB domain-containing protein gene (locus tag D1G35_05855) on the chromosome. Interestingly, most of the resistance genes, including aac(6′)-Ie-aph(2′)-la, blaRT, blaZ, tet(L), Inu(B), and Isa(E), were found to be colocized within an ~38- to 45-kb MDR region (tentatively named MDRST9α), inserted into a...
l-lactate permease gene (llp; locus tag E3T15_09260) with a 15-bp invert repeat (TGTCAGTTTTGGAGT) and a 7-bp target sequence duplicate (ATTATTA) on both termini. This region was separated into two fragments in some genomes (e.g., strains S57 and NX-T55), presumably due to transposase mediated recombination. We also detected ST9 isolates harboring resistance plasmids (blaI, blaRI, blaZ, arsR, arsB, arsC, and mco genes), which could potentially be the origin of chromosome-borne resistant genes through recombination. 

The antimicrobial resistance profile of the 17 isolates available from our collection is presented in Data Set S2. These isolates were intermediate or resistant to methicillin (71.4%; n = 15), gentamicin (71.4%; n = 15), levofloxacin (76.2%; n = 16), moxifloxacin (76.2%; n = 16), erythromycin (52.4%; n = 11), and clindamycin (81.0%; n = 17). All 17 isolates were susceptible to ceftaroline, linezolid, daptomycin, vancomycin, and rifampin. The susceptibility testing results were in accordance with the results of the genome analysis, and most isolates were MDR (81.0%).

**Phylogeographical context and comparative genomics of ST9 strains.** Next, we implemented a Bayesian phylogenetic inference to decipher the global evolutionary history of ST9 LA-MRSA and to identify key genetic changes associated to its adaptation to human and animal populations. Core genome analysis identified 6,955 core SNPs across 191 ST9 strains. The BactDating model of temporal phylogenetic signal showed convergence and was significantly better than the randomized dates model, with effective
population sizes of greater than 200 ($\alpha$, $\mu$, and $\sigma$ were >200). BactDating estimates that the most recent common ancestor (MRCA) of ST9 strains was around 1826 (95% confidence interval [CI], 1588 to 1912), approximately 200 years ago. The estimated mutation rate is 4.7 (95% CI, 3.6 to 5.9) single nucleotide polymorphisms (SNP)/genome/year, which is similar to the rates in other $S$. aureus lineages (26).

Our analysis divided the 191 genomes into two main clades (I and II) (Fig. 3). The ancestral clade I ($n=7$) originated around 1894 and included isolates from human (3/7) and unknown (4/7) sources collected in Taiwan ($n=2$), Australia ($n=1$), and the United States ($n=1$). Most isolates (5/7) were MSSA and belonged to diverse spa types: t2700, t4358, and t4522. Two isolates harbored SCCmec type V.

The clade II strains can be divided into three subclades (IIa, IIb, and IIc) and a few singletons ($n<2$), with cluster IIc containing the largest number of genomes ($n=147$) from mainland China and Taiwan. Clade IIa comprised only MSSA isolates, which were of human origin (5/6), collected in several countries around the world, including Argentina, the United States, and the United Kingdom. The spa types identified were t099, t100, t193, t464, and t587. Interestingly, most clade I and IIa (93.3%; 14/15) strains harbored the aforementioned IEC genes, $scn$, $chp$, and $sak$. The fact that we detected human MSSA isolates harboring the human complement evasion-associated IEC genes in the ancestral clades suggests a possible MSSA human origin of ST9 LA-MRSA clones and the change of human-specific virulence factors during the host shift to animals.

The separation of clade IIb/IIc from clade IIa correlated with the loss of the IEC genes ($scn$, $chp$, and $sak$), followed by the acquisition of the SaPIbov4-like element in the backbone of $C23a$ genomic island (type V) (Fig. 1) and the acquisition of QRDR mutations ($parC_{S80F}$) in ~1956 (Fig. 3).

Clade IIb ($n=28$) included isolates from animal sources, i.e., livestock farms ($n=7$), meat ($n=10$), swine ($n=4$), and unknown origin ($n=7$), collected in Europe (Czech Republic, Germany, and Poland), Africa (Ghana), and the Americas (United States and Colombia), and included both MSSA (14/28) and MRSA (14/28) isolates. The SaPIbov4-like element was present in 23/28 isolates, which were mostly from animal (17/23) sources. SCCmec was later acquired in the background of the SaPIbov4-like isolates.

---

**FIG 2** Structure of mobile genetic element $MDR_{ST9}$ carrying the resistance genes $blaI$, $blaRI$, $blaZ$, $aa(6')-le/aph(2')-Ia$, $tet(L)$, $aaD1$, $spw$, $lsa(E)$, and $lnu(B)$. Light blue shading represents regions of homology; green shading denotes inversely displayed regions of homology. The $MDR_{ST9}$ genetic element is inserted in $llp$ (l-lactate permease) gene. Orange arrows, hypothetical proteins; gray arrows, integrase; yellow arrows, transposases; red arrows, resistance genes; olive arrows, recombinases; green arrows, arsenite detoxification; teal arrows, copper detoxification. The direct repeats are in bold while the invert repeats are in italics and underlined. The $MDR_{ST9}$ in strain NX-T55 ([CP031839](https://www.ncbi.nlm.nih.gov)) was separated into two regions separated by >210 kb, and the region between $int$ and $Tn552$ was inverted.
Molecular Evolution of LA-MRSA ST9

MRSA isolates (14/28) carried SCCmec IV (8/14), IV+XII (5/14), and XII (1/14), belonged to spa type t899 (11/14), and were mainly from European countries (10/14). MSSA isolates within this clade (14/28) belonged to seven different spa types, with t1430 (7/14) being the most common. These isolates were collected from Asia, Ghana (n = 7), the United States (n = 3), and Colombia (n = 1).

The largest subclade, clade IIC, emerged in ~1986 and contained 147 genomes, exclusively from mainland China and Taiwan. The estimate of divergence time is consistent with the result (around 1987) of a recent ST9 genomic study in China (16). The isolates were mostly MDR strains, carrying aminoglycoside, β-lactam, macrolide, lincomamide, streptogramin, quinolone, chloramphenicol, tetracycline, and trimethoprim resistance genes (Fig. S4). In addition, genomes from clade IIC showed a highly similar resistance profile, and all carried the above-described MDR<sub>ST9</sub> genetic element(s), supporting the hypothesis of clonal expansion. Almost all isolates from clade IIC carried an
SaPlbov4-like element (98.7%; 145/147) and were recovered from both animal (118/145) and human (26/145) sources. This clade, clade Ilc, further diverged in two subclades, clades Iic-1 and Iic-2. The clade Iic-1 (n = 11) strains originated around 2002; all belonged to spa t899 (11/11) and were from both human (6/11) and swine (4/11) sources, including both MSSA (7/11) and MRSA (4/11, all SCCmec XII) isolates. Clade Iic-2 is the major sublineage currently disseminating in mainland China. This subclade originated around 1994 and rapidly disseminated into multiple provinces in China. No apparent introducing time could be determined for individual provinces. Genomes of isolates from different provinces frequently clustered together, despite the presence of some small clusters of closely related strains originating from the same provinces. The results suggested extensive ST9 isolate exchange in different regions in China, presumably due to frequent animal trade, as suggested in a previous study (16). Clade Iic-2 includes isolates from swine (n = 102), bovine (n = 11), chicken (n = 2) and human (n = 20) sources. Most clade Iic-2 strains contained a unique QRDR GyrA S84A mutation. Several genomes from animal and human sources were phylogenetically close and separated by <20 core SNPs and contained the same resistance and virulence genes, suggesting the likelihood of animal-to-human (or human-to-animal) transmissions (Fig. 3). Clade Iic-2 is primarily composed of MRSA isolates (131/136), belonging to spa t899 (122/131). However, in contrast to the SCCmec IV-spa t899 European isolates in clade IIb, isolates from clade Iic were SCCmec XII-spa t899 (121/122). These results suggested that the spa t899 strains in clades Iib and Iic each independently acquired SCCmec IV and XII. The presence of SaPlbov4-like is also common within this subclade (133/136), and the isolates with the broad MDR profile are found exclusively in this clade (Fig. S4).

DISCUSSION

We detected three major genetic events along the evolutionary history of ST9: the loss of the IEC genes (scn, chp, and sak), which were reported to contribute to virulence in human infections, the acquisition of the SaPlbov4-like element-encoding vwb gene, which is an animal-specific virulence factor responsible for the clotting of animal plasma, and the acquisition of antibiotic resistance genes, including SCCmec, QRDR mutations and the MDRST9 genetic elements.

First, all human MSSA isolates from the ancestral clade carried a φSa3 β-hemolysin-converting prophage, harboring the genes implicated in immune evasion (scn, chp, and sak). In contrast, isolates from clade Iib/Iic carried an intact β-hemolysin gene and were negative for φSa3, supporting the role of scn, chp, and sak as specific mechanisms of human adaptation. These three genes have a major role in complement evasion: the staphylococcal complement inhibitor (scn) binds to C3 convertases, preventing the activation of all three complement pathways (27); the chemotaxis inhibitory protein (chp) binds to C5aR1 and FPR1, thereby blocking the recognition of C5a and fMLF chemoattractants (28); and the staphylokinase (sak) activates plasminogen into plasin, which is a serine protease bound to staphylococcal membrane which disrupts opsonization and phagocytosis through degradation of C3b and IgG, and it also blocks the cytolytic effect of human α-defensins (29).

Notably, previous studies showed that these virulence factors were specific for human hosts (30). Among them, SCN inhibits the alternative pathways exclusively found in humans (27), while the human chemotaxis-inhibiting protein had much lower capacity for binding to animal neutrophils, with 30-fold-reduced activation in mouse compared to human neutrophils (28). Similarly, bacterial plasminogen activators (PA) (such as streptokinase or staphylokinase) have a restricted ability to cleave different animal plasminogens (31). In addition, previous studies showed that the β-hemolysin-converting prophages were almost exclusively found in human isolates but absent from animal isolates (32). The results are consistent with previous studies showing that the IEC-harboring φSa3 β-hemolysin prophages were closely associated with S. aureus strains from humans (33, 34). Our results reiterate the loss of IEC is a major molecular
event underlying the host shift from humans to animals during the molecular evolution of ST9 strains.

The second major event in the evolution of ST9 was the acquisition of the SaPlbov4-like element in the backbone of the vSa genomic island (type V). This SaPlbov4-like element harbored an animal-specific virulence factor gene, the vwb gene, which encodes a homolog of the von Willebrand factor binding protein (35). Several vwb alleles showed species-specific coagulation activities in animals through unique N-terminal motifs which activate bovine and equine prothrombin, as an important animal host adaptation mechanism (36). However, the effect of SaPlbov4-encoding vwb on plasma clotting has been evaluated only in bovine and caprine plasma (22, 36). Its function in porcine plasma has not been fully studied, and future work is needed to understand its role in swine pathogenesis. Our analysis showed the acquisition of vwb in isolates from clade IIb and IIc, which contained mainly animal isolates. The SaPlbov4-like element was acquired by several spa types, initially in MSSAspa11430 and later in MRSA spa t899 from clade IIb and clade IIc. This element also carried a second scn variant, which has 52.1% similarity to scn (SCIN-A) encoded in φSa3. A previous study reported the identification of an equine scn (eqSCIN) variant in prophage φSaeq1, detected in different lineages of S. aureus exclusively isolated from horses (37). Remarkably, this variant inhibits C3 convertases from horses but also is a potent inhibitor of human and pig complement (37). However, the role of this scn in interfering complement function from different human and animal hosts remains unclear and deserves future studies.

The third notable event was the acquisition of multiple resistance genes, including SCCmec, QRDR mutations, and the MDRST9 element(s). Interestingly, the acquisition of SCCmec and QRDR mutations correlated with the emergence of spa t899 in ST9 strains. The phylogenomic analysis revealed an ancestral clade composed of MSSA human isolates of diverse spa types and a most recent clade composed of MRSA animal isolates of predominantly spa t899. In addition to our analysis, previous molecular typing reports of MRSA isolates collected from pigs and pig industry-related workers in China have also showed the predominance of ST9 and ST9 single-locus variant (SLV) spa t899 strains (6, 10, 13, 14). In contrast to our findings of spa t899-SCCmec XII predominance among ST9, isolates from previous work were spa t899-SCCmec III and SCCmec IV. These findings also support a human MSSA origin of ST9 MRSA, with independent acquisition of SCCmec elements in the background of different spa types.

Multidrug resistance was also a hallmark of animal strains. Human isolates in clade I and IIa contained only the resistance genes lmrS, mecA, fpaA, tett38, blaZ, and cadD, and the clade IIb isolates (predominantly of animal origin) had obtained multiple antimicrobial resistance genes, including mecA, vgaA, qacG, QRDR mutations (parC_S80F), tettK, and str. The clade IIc isolates, including isolates from human and animal sources, carried the largest number of drug resistance genes, commonly those for resistance to methicillin (mecA), aminoglycosides [aac(6’)-Ie, aadD1, ant(6’)-Ia, aph(2’)-la, spw], arsenite (arsB and arsC), copper (mco), fosfomycin (fosB), lincosamide (lnuB and IsaE), macrolides (ermC), chloramphenicol/orfencinol (fexA), quinolones (gyrA_S84A and parC_S80F), tetracycline (tet38 and tett), and trimethoprim (dfrG) and those for multidrug efflux MFS and MATE transporters (lmrS and mecA). However, resistance markers frequently associated with the LA-MRSA CC398 clone, such as the chromosomal gene tet(M), were nearly absent from the ST9 genomes, suggesting different antimicrobial resistance pressure in CC398 and ST9 strains.

Several of those genes, including aac(6’)-Ie/aph(2’)-Ia, blaR1, blaZ, tet(L), lnu(B), and Isa(E), were located in the MDRST9 chromosomal region(s). Our phylogenetic analysis showed that the acquisition of MDRST9 region(s) was exclusively found in clade IIe from isolates from mainland China and Taiwan. A BLAST search of this element against NCBI database failed to detect similar sequences from other S. aureus clones, suggesting the MDRST9 may originate through the molecular evolution of ST9 strains. Interestingly, the β-lactamase genes (blal, blar, and blaz) and the arsenic resistant genes (arsR, arsB, and...
arsC) were found in plasmids from ancestor clade I strains (Fig. 2). Examination of MDR\textsubscript{ST9} identified multiple genes with insertion elements (IS\textsubscript{256}, IS\textsubscript{6}, and IS\textsubscript{L3}) and a transposon (Tn\textsubscript{552}) (Fig. 2). We therefore hypothesized that MDR\textsubscript{ST9} may have originated from the chromosomal integration of plasmid-borne genes (such as \textit{blaI}, \textit{blaR}, \textit{blaZ}, \textit{arsR}, \textit{arsB}, and \textit{arsC}) along with the acquisition of additional antimicrobial resistance genes encoding tetracycline (\textit{tetL}) and aminoglycoside [\textit{aac(6\')-Ib/aac(6\')-II}] resistance, through IS- or transposon-mediated transposition, as a result of evolution against the increased antibiotic selection pressures. This clone appeared to be widely disseminated in China, and genetically similar isolates were also reported recently in food surveillance for antimicrobial resistance from raw meat products in Hong Kong (38).

Moreover, we also detected evidence of interhost transmission of ST9 strains. Within clade IIC, at least one cluster of isolates from pig and human sources showed very close core SNPs (<20), in support of the likelihood of the transmission of the ST9 strains between humans and animals (Fig. 3). These isolates were recently described in a pork production chain and were found to be carried by pigs and pig workers in several farms (40). Although we could not determine the direction of transmission, both animal-to-human and human-to-animal transmission could be possible (10, 11, 16, 17). Though not as common as animal-to-human transmission, human-to-animal transmission (i.e., reverse zoonosis) has been documented in \textit{S. aureus} infections in livestock or companion animals (39). Interestingly, we detected one isolate carrying the IEC (\textit{scn-chp-sak}) in this clade IIC. This strain was isolated from a patient with a bloodstream infection (BSI) without livestock contact and showed high \textit{in vivo} and \textit{in vitro} virulence, with virulence genetic profiles closely related to those of human-associated ST9 MSSA (12). Nonetheless, this isolate carried the SCC\textit{mec} XII, MDR\textsubscript{ST9}, and the type V genomic island with the SaPI\textit{bov4}-like element. Our results suggest that this isolate may have independently acquired human-specific factors (IEC) and caused severe infection in humans, molecular evidence of an MDR animal-adapted strain (identified by MDR\textsubscript{ST9} and SaPI\textit{bov4}-like elements) that could obtain hypervirulence through horizontal gene transfer. This finding warns of the potential emergence of multidrug-resistant and hypervirulent LA-MRSA strains.

Our results resembled the evolution of CC398, another successful LA-MRSA lineage, which originated in human as MSSA, spread to livestock, and later acquired methicillin resistance. In our study, the evolutionary analysis of ST9 genomes points to a human MSSA origin of ST9, which lost the IEC genes (\textit{sak}, \textit{chp}, and \textit{scn}). The introduction of ST9 strains in animal populations was aligned with the acquisition of the SaPI\textit{bov4}-like element, SCC\textit{mec} (IV and XII), and multidrug resistance. The animal-adapted ST9 LA-MRSA strains showed the ability to infect humans, and the transmission from animal to human hosts could potentially reshape the resistance and virulence genetic pool in these isolates. The rapid clonal expansion of MDR ST9 in China and Taiwan highlights the increasing need for effective surveillance of antibiotic consumption in animal husbandry to control antimicrobial resistance spread.

**MATERIALS AND METHODS**

**ST9 genomes.** Seventeen ST9 \textit{S. aureus} isolates, collected in China from three provinces between 2011 and 2016, were included in this study. An additional 174 ST9 \textit{S. aureus} genomes were retrieved from the NCBI whole-genome sequence database or short-read archive comprising publicly available genomes at the time of the study (January 2021). The accession number, host, isolation source, geographical origin, and genotype data are listed in Data Set S1.

**Antimicrobial susceptibility testing.** Antimicrobial susceptibility testing of the 17 ST9 isolates was evaluated using a Vitek-2 microbiology analyzer (bioMérieux, Marcy l’Etoile, France) in accordance with the manufacturer’s instructions. The MICs of 16 antimicrobial agents, including ampicillin, cefoxitin, ciprofloxacin, clindamycin, erythromycin, gentamicin, levofloxacin, linezolid, moxifloxacin, nitrofurantoin, oxacillin, rifampin, tetracycline, tigecycline, trimethoprim-sulfamethoxazole, and vancomycin, were determined, and the results were interpreted using Clinical and Laboratory Standards Institute guidelines (41). \textit{S. aureus} strains ATCC 29213 and ATCC 25923 were used as quality controls.

**Whole-genome sequencing and analysis.** Whole-genome sequencing of the 17 ST9 isolates was carried out using the HiSeq 2500 sequencing platform (Illumina Inc., San Diego, CA), with 2 × 150 bp paired-end reads. The raw data were filtered using Trimmomatic v.0.39 (42), followed by assembly using SPAdes v3.14 (43). Antimicrobial resistance genes were mined using AMRFinderPlus v3.9.8 (44). Virulence genes...
Molecular Evolution of LA-MRSA ST9

were identified by ABRicate v1.01 (https://github.com/tseemann/abricate) using the VFDB database (http://www.mgc.ac.cn/VFs/main.html) with 95% identity and 90% query coverage cutoffs. SCCmec and spa types were determined by SCCmecFinder v1.2 (45) and spaTypeR 1.0 (46), respectively. Comparative genomic analysis of mobile genetic structures was performed using Mauve v2.2.4 (47, 48). In brief, the contigs from the genome assembly were ordered and oriented relative to a closed reference ST9 genome from each clade (Fig. 3) and then concatenated to form a pseudogenome with the Mauve contig mover (48). The resulting pseudogenome was then aligned with the reference genome by progressive Mauve (49) to identify putative mobile genetic structures.

**Dating of ST9 strains.** Filtered reads from each isolate were mapped to the *S. aureus* ST9 reference genome (strain QD-CD9; accession number CP031838) by Snippy 4.4 (https://github.com/tseemann/snippy) using default settings. For genome assemblies downloaded from the NCBI WGS database, 10 million 150-bp paired-end reads were simulated using the wgsim (https://github.com/lh3/wgsim) algorithm from SAMtools (50). The resulting BactDating tree was then annotated using iTOL v5 (55).

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

- DATA SET S1, XLSX file, 0.1 MB.
- DATA SET S2, XLSX file, 0.01 MB.
- FIG S1, EPS file, 1.9 MB.
- FIG S2, EPS file, 0.5 MB.
- FIG S3, EPS file, 0.7 MB.
- FIG S4, TIF file, 0.6 MB.

**ACKNOWLEDGMENTS**

This study was in part supported by grant 81871704 from the National Natural Science Foundation of China.

We have no competing financial or nonfinancial interests.

**REFERENCES**

1. Voss A, Loeffen F, Bakker J, Klaassen C, Wulf M. 2005. Methicillin-resistant *Staphylococcus aureus* in pig farming. Emerg Infect Dis 11:1965–1969. [https://doi.org/10.3201/eid1112.050428](https://doi.org/10.3201/eid1112.050428).
2. van de Sande-Bruinsma N, Leverstein van Hall MA, Janssen M, Nagtzaam N, Leenders S, de Greeff SC, Schneeberger PM. 2015. Impact of livestock-associated MRSA in a hospital setting. Antimicrob Resist Infect Control 4:2. [https://doi.org/10.1186/s13756-015-0053-8](https://doi.org/10.1186/s13756-015-0053-8).
3. de Greeff SC, Hoving AF, Verdun CM. 2020. NethMap 2020: consumption of antimicrobial agents and antimicrobial resistance among medically important bacteria in the Netherlands in 2019/MARAN 2020: monitoring of antimicrobial resistance and antibiotic usage in animals in the Netherlands in 2019. National Institute for Public Health and the Environment, Bilthoven, the Netherlands.
4. Kinross P, Petersen A, Skov R, Van Hauwermeiren E, Pantosti A, Laurent F, Voss A, Klyutmans J, Struelens MJ, Heuer O, Monnet DL, The European Human-LA-Mrsa Study Group. 2017. Livestock-associated methicillin-resistant *Staphylococcus aureus* (MRSA) among human MRSA isolates, European Union/European Economic Area countries, 2013. Euro Surveill 22:160039. [https://doi.org/10.2807/1560-7917.ES.2017.22.16.160039](https://doi.org/10.2807/1560-7917.ES.2017.22.16.160039).
5. He W, Liu Y, Li J, Chen H, Zhao C, Zhang F, Li LH, Wang H. 2013. Food-animal related *Staphylococcus aureus* multidrug-resistant ST9 strains with toxin genes. Foodborne Pathog Dis 10:782–788. [https://doi.org/10.1089/fpd.2012.1452](https://doi.org/10.1089/fpd.2012.1452).
6. Ho PL, Chow KH, Lai EL, Law PYT, Chan PY, Ho AYM, Ng TK, Yam WC. 2012. Clonality and antimicrobial susceptibility of *Staphylococcus aureus* and methicillin-resistant *S. aureus* isolates from food animals and other animals. J Clin Microbiol 50:3735–3737. [https://doi.org/10.1128/JCM.02053-12](https://doi.org/10.1128/JCM.02053-12).
7. Fang H-W, Chiang P-H, Huang Y-C, HsinWei F, PoHong C, YhuChering H. 2014. Livestock-associated methicillin-resistant *Staphylococcus aureus* ST9 in pigs and related personnel in Taiwan. PLoS One 9:e88826. [https://doi.org/10.1371/journal.pone.0088826](https://doi.org/10.1371/journal.pone.0088826).
8. Neela V, Zafural AM, Mariana NS, Van Belkum A, Liew YK, Rad EG. 2009. Prevalence of ST9 methicillin-resistant *Staphylococcus aureus* among pigs and pig handlers in Malaysia. J Clin Microbiol 47:4138–4140. [https://doi.org/10.1128/JCM.01363-09](https://doi.org/10.1128/JCM.01363-09).
9. Wang W, Liu F, Baloich Z, Zhang CS, Ma K, Peng ZX, Yan SF, Hu YJ, Gan X, Dong YP, Bai Y, Li FQ, Yan XM, Ma AG, Xu J. 2017. Genotypic characterization of methicillin-resistant *Staphylococcus aureus* isolated from pigs and retail foods in China. Biomed Environ Sci 30:570–580. [https://doi.org/10.3967/bes2017.076](https://doi.org/10.3967/bes2017.076).
10. Bi Z, Sun C, Börjesson S, Chen B, Ji X, Berglund B, Wang M, Nilsson M, Yin H, Sun Q, Hulth A, Wang Y, Wu C, Bi Z, Nilsson LE. 2018. Identical genotypes of community-associated MRSA (ST59) and livestock-associated MRSA (ST9) in humans and pigs in rural China. Zoonoses Public Health 65:367–371. [https://doi.org/10.1111/zph.12443](https://doi.org/10.1111/zph.12443).
27. Rooijakkers SHM, Ruyken M, Roos A, Daha MR, Presanis JS, Sim RB, van Xiao Y. 2020. Characterization of highly virulent community-associated methicillin-resistant \textit{Staphylococcus aureus} ST9-SCC mec XII causing bloodstream infection in China. Emerg Microbes Infect 9:2526–2535. https://doi.org/10.1093/iumi/mju120.

30. Sung M, Liu M, Hall D, Lindsay JA. 2008. \textit{Staphylococcus aureus} host specificity: comparative genomics of humans versus animal isolates by multistage microarray. Microbiology (Reading) 154:1949–1959. https://doi.org/10.1099/micro.0.015289-0.

37. de Jong NWM, Vrieling M, Garcia BL, Koop G, Brettmann M, Aerts PC, de Vries EFM, van Belkum A, van Wamel WJ. 2011. Minor immune evasion cluster in \textit{Staphylococcus aureus} CC8 is associated with the loss of a \(\beta\)-hemolysin converting prophage and the acquisition of a new staphylococcal cassette chromosome. PLoS One 6:e23562. https://doi.org/10.1371/journal.pone.0023562.

43. Bankovic-Borbulo N, Surk I, Antipov D, Gurovic AA, Dvorkin M, Kulkov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD, Pyshkin AV, Sirotkin AV, Sakwinska O, Moreillon P. 2013. Human-to-bovine jump of \textit{Staphylococcus aureus} CC8 is associated with the loss of a \(\beta\)-hemolysin converting prophage and the acquisition of a new staphylococcal cassette chromosome. PLoS One 8:e58187. https://doi.org/10.1371/journal.pone.0058187.

44. Feldgarden M, Brover V, Haft DH, Lipsky E, Prasad AB, Slotta DJ, Tolstoy I, Tyson GW. 2012. Comparative genomics of von Willebrand factor-binding protein. Mol Microbiol 77:1583–1594. https://doi.org/10.1111/j.1365-2958.2012.07312.x.

48. de Jong NWM, Vrielin M, Garcia BL, Koop G, Brettmann M, Aerts PC, Buyken M, van Stipria JAS, Holmes M, Harrison EM, Geisbrecht VB, Rooijakkers SMH. 2018. Identification of a staphylococcal component inhibitor with broad host specificity in equids. J Biol Chem 293:4466–4477. https://doi.org/10.1074/jbc.RA117.005599.

52. Bankovic-Lasocka J, Surk I, Antipov D, Gurovic AA, Dvorkin M, Kulkov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD, Pyshkin AV, Sirotkin AV, Vayhni N, Tesler G, Alexseyev MA, Pezvner PA. 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol 19:455–477. https://doi.org/10.1089/cmb.2012.0021.

55. Feldgarden M, Brover V, Haft DH, Prasad AB, Slotta DJ, Tolstoy I, Tyson GH, Zhao S, Hsu CH, McDermott PF, Tadesse DA, Morales C, Simmons M, Tillman G, Waslenko J, Feloter JP, Klimke W. 2019. Validating the AMBIGlider tool and resistance gene database by using antimicrobial resistance genotype-phenotype correlations in a collection of isolates. Antimicrob Agents Chemother 63:e00483-19. https://doi.org/10.1128/AAC.00483-19.
45. Kaya H, Hasman H, Larsen J, Stegger M, Johannesen B, Allesøe L. 2018. SCCmecFinder, a web-based tool for typing of staphylococcal cassette chromosome mec in Staphylococcus aureus using whole-genome sequence data. mSphere 3:e00612-17. https://doi.org/10.1128/mSphere.00612-17.

46. Bartels MD, Petersen A, Worning P, Nielsen JB, Larner-Svensson H, Johansen HK, Andersen LP, Jarløv JO, Boye K, Larsen AR, Westh H. 2014. Comparing whole-genome sequencing with sanger sequencing for spa typing of methicillin-resistant Staphylococcus aureus. J Clin Microbiol 52:4305–4308. https://doi.org/10.1128/JCM.01979-14.

47. Darling ACE, Mau B, Blattner FR, Perna NT. 2004. Mauve: multiple alignment of conserved genomic sequence with rearrangements. Genome Res 14:1394–1403. https://doi.org/10.1101/gr.2289704.

48. Rissman AI, Mau B, Biehl BS, Darling AE, Glasner JD, Perna NT. 2009. Reordering contigs of draft genomes using the Mauve aligner. Bioinformatics 25:2071–2073. https://doi.org/10.1093/bioinformatics/btp356.

49. Darling AE, Mau B, Perna NT. 2010. progressiveMauve: multiple genome alignment with gene gain, loss and rearrangement. PLoS One 5:e11147. https://doi.org/10.1371/journal.pone.0011147.

50. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R. 1000 Genome Project Data Processing Subgroup. The Sequence Alignment/Map format and SAMtools. Bioinformatics 25:2078–2079. https://doi.org/10.1093/bioinformatics/btp352.

51. Arndt D, Grant JR, Marcu A, Sajed T, Pon A, Li X, Wysoker A, Marth G, Abecasis G, Handsaker B. 2009. The Sequence Alignment/Map format and SAMtools. Bioinformatics 25:2078–2079. https://doi.org/10.1093/bioinformatics/btp352.

52. Didelot X, Croucher NJ, Bentley SD. 2018. Bayesian inference of ancestral dates on bacterial phylogenetic trees. Nucleic Acids Res 46:e134–e134. https://doi.org/10.1093/nar/gky783.

53. Letunic I, Bork P. 2019. Interactive Tree Of Life (iTOL) v4: Recent updates and new developments. Nucleic Acids Res 47:W256–W259. https://doi.org/10.1093/nar/gkz239.