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Prevalence and Genomic Structure of Bacteriophage phi3 in Human-Derived Livestock-Associated Methicillin-Resistant *Staphylococcus aureus* Isolates from 2000 to 2015

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ABSTRACT Whereas the emergence of livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) clonal complex 398 (CC398) in animal husbandry and its transmission to humans are well documented, less is known about factors driving the epidemic spread of this zoonotic lineage within the human population. One factor could be the bacteriophage phi3, which is rarely detected in *S. aureus* isolates from animals but commonly found among isolates from humans, including those of the human-adapted methicillin-susceptible *S. aureus* (MSSA) CC398 clade. The proportion of phi3-carrying MRSA *spa*-CC011 isolates, which constitute presumptively LA-MRSA within the multilocus sequence type (MLST) clonal complex 398, was systematically assessed for a period of 16 years to investigate the role of phi3 in the adaptation process of LA-MRSA to the human host. For this purpose, 632 MRSA *spa*-CC011 isolates from patients of a university hospital located in a pig farming-dense area in Germany were analyzed. Livestock-associated acquisition of MRSA *spa*-CC011 was previously reported as having increased from 1.8% in 2000 to 29.4% in 2014 in MRSA-positive patients admitted to this hospital. However, in this study, the proportion of phi3-carrying isolates rose only from 1.1% (2000 to 2006) to 3.9% (2007 to 2015). Characterization of the phi3 genomes revealed 12 different phage types ranging in size from 40,712 kb up to 44,003 kb, with four hitherto unknown integration sites (genes or intergenic regions) and several modified bacterial attachment (*attB*) sites. In contrast to the MSSA CC398 clade, phi3 acquisition seems to be no major driver for the readaptation of MRSA spa-CC011 to the human host.

KEYWORDS *Staphylococcus aureus*, livestock-associated MRSA, clonal complex 398, *spa-CC011*, *spa t034*, *spa t011*, (re)adaptation, bacteriophage, phi3, immune evasion cluster, whole-genome sequencing, zoonosis

During the past decade, attention was drawn to the emergence of livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) of clonal complex 398 (CC398) (1–3). Mainly pigs, but also cattle, poultry, and other livestock act as zoonotic LA-MRSA CC398 reservoirs for humans (4–6). Currently, LA-MRSA CC398 constitutes a significant portion of MRSA detected in human and animal health care centers and substantially contributes to the burden of disease attributable to MRSA, especially in regions with a high density of livestock production (7–9).

Whereas resistance toward antibiotics and heavy metals as well as the general pathogenic potential of MRSA CC398 has been thoroughly investigated (10–13), the factors driving the epidemic of this clonal lineage in humans are only slightly understood. Interestingly, differences in the content of mobile genetic elements (MGEs) between the ancestral methicillin-susceptible CC398 clade that originated in humans, the methicillin-resistant livestock-associated CC398 clade, and an emerging methicillin-susceptible human-specific CC398 clade have been described previously (14–16). In the livestock-associated CC398 clade, the staphylococcal cassette chromosome *mec* (SCC*mec*) types IV and V, the

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tet(M) resistance gene, and the bacteriophages phi2 and phi6 were commonly found, whereas the bacteriophage phi3 and the genes *cadDX* and *rep27* predominated in the human clade (17). Additionally, further studies observed that the host switch of methicillin-susceptible *S. aureus* (MSSA) CC398 from the ancestral human host to livestock was linked to a loss of phage phi3 and an uptake of *tet*(*M*) (15, 18). Therefore, we suggested that the adaptation of the CC398 lineage back to the human host might be linked to an uptake of bacteriophage phi3 into the genome.

MGEs such as prophages are known to serve as drivers in bacterial adaptation, enabling the pathogen to colonize broader host ranges due to the acquisition of new virulence and resistance genes (19, 20). The uptake of the *Siphoviridae* family member phi3, which is recognized as a genetic marker for human-associated, but not for animal-associated, isolates is of particular importance (17, 21, 22). The genomes of *Siphoviridae* are characterized by a mosaic structure, and they are typically organized in six functional modules containing genes for lysogeny, DNA replication, regulation of transcription, packaging, head and tail, and lysis (23). The integration of bacteriophages in the bacterial genome occurs via site-specific recombination by targeting the bacterial attachment site (*attB* site), which contains a 14-bp core sequence (5′-TGTATCCAA ACTGG-3′) (24).

Acquisition of phi3 in the *S. aureus* CC398 clade might be beneficial for the adaptation process to the human host as it confers a cluster of human-specific immune-modulatory elements, which counteract with the human innate immunity (25). The genes harbored on the immune evasion cluster (IEC) comprise the chemotaxis inhibitory protein (*chp*), the staphylococcal complement inhibitor (*scn*), and the plasminogen activator staphylokinase (*sak*). These proteins are known as human-specific virulence factors due to their high specificity to human immune cells and serum protein (26–28). In some cases, enterotoxin A or P (*sea* or *sep*) is additionally encoded in the IEC (25, 29). Previous studies evaluating the occurrence of phi3 in CC398 isolates derived from animals and humans with livestock contact found a comparatively low prevalence for this bacteriophage (30, 31). Therefore, systematic studies covering the complete time period since the advent of this *S. aureus* lineage in livestock are warranted to evaluate the uptake of bacteriophage phi3 in human LA-MRSA isolates over time.

Consequently, this study aimed to investigate the prevalence of bacteriophage phi3 in human-derived MRSA *spa*-CC011. This *spa*-CC with t011 and t034 as the most frequent *spa* types constitutes presumptively LA-MRSA within the multilocus sequence type (MLST) clonal complex 398. Overall, 632 MRSA *spa*-CC011 isolates covering the entire time period from the beginning of the MRSA CC398 epidemic in 2000 until 2015 and recovered from inpatients at the University Hospital Münster (UKM) were included. Furthermore, a whole-genome sequencing (WGS) approach was applied to detect the genetic location of phi3 within the MRSA *spa*-CC011 genome and to assess the genetic variability within the phi3 genomes.

MATERIALS AND METHODS

Strain collection, molecular typing, and cultivation conditions. Human MRSA isolates were recovered from patients at the University Hospital Münster (UKM) in Germany. The hospital is located in a German region characterized by a very high density of pig production, and epidemiological investigations have demonstrated that carriage of MRSA *spa*-CC011 by patients in this region is predominantly associated with livestock contact (32). At the UKM, all MRSA isolates were identified by matrix-assisted laser desorption–ionization time of flight mass spectrometry (MALDI-TOF MS) (Microflex-LT system, MALDI-Biotyper, version 3.0; Bruker Daltonics, Germany) as previously described (33, 34). Phenotypic and genotypic characterization of methicillin resistance was assessed for every isolate by using a Vitek-2 automated system (bioMérieux, Nürtingen, Germany), applying the antimicrobial susceptibility test card AST-P632, and via *S. aureus*-specific PCR detecting the genes *mecA* and *mecC* (GenoType MRSA; Hain-Lifescience, Germany) as described previously (35, 36).

Since 2006, every first MRSA isolate of each patient had been typed by *S. aureus* protein A (*spa*) sequence-based typing and stored at −80°C (37, 38). For isolates obtained before 2006, *spa* typing was retrospectively performed. The "based upon repeat pattern" (BURP) was used for the identification of MRSA isolates belonging to the *spa* clonal complex CC011 (*spa*-CC011) (39). Therefore, *spa* types clustering to *spa* clonal complex t011 and closely related clades, which are representative for the CC398 clade, were selected (see Fig. S1 in the supplemental materials). Additionally, one isolate of each clade that forms branches in the BURP analysis was selected, and MLST analysis was performed. In early years

of the epidemic (2000 to 2006), all available MRSA *spa*-CC011 isolates were included ($n = 92$). Later (2007 to 2015), the first 15 isolates of each quarter were included in the study, resulting in 60 isolates per year. Only the first isolate of each patient was included in the study. The antibiotic resistance profile of the phi3-positive isolates ($n = 21$) was determined at the genetic level by the use of DNA microarray analysis (Identibac Microarray; Alere Technologies GmbH, Jena, Germany).

For all experiments, *S. aureus* strains were cultivated on Columbia agar (Becton Dickinson, Franklin Lakes, NJ, USA) containing 5% sheep blood (Oxoid, Wessel, Germany).

Genomic DNA isolation and PCR-based detection of IEC genes. Genomic DNA was extracted from *S. aureus* using a QIAamp DNA minikit according to the manufacturer's instructions (Qiagen, Hilden, Germany) with the exception that lysostaphin (20 μg/ml) (Wakchemie, Steinbach, Germany) was used for bacterial cell lysis. To detect the bacteriophage phi3, PCRs specific for genes located on the IEC (*chp*, *scn*, *sak*, *sea*, and *sep*) using the oligonucleotides chp-for, chp-rev, sak-for, sak-rev, scn-for.2, scn-rev.2, sea-for, sea-rev, sep-for, and sep-rev were performed as follows: 95°C for 5 min, followed by 35 cycles of 30 s at 95°C, 30 s at 50°C, 30 s at 72°C, and a final elongation of 7 min at 72°C. To investigate whether phi3 was integrated within the *hlb* gene, PCRs using the oligonucleotides hlb-1-for, hlb-2-rev, hlb-3-for, and hlb-4-rev were performed using the following conditions for all oligonucleotide combinations: 95°C 5 min, followed by 35 cycles of 30 s 95°C, 30 s 59°C, 60 s 72°C, and a final elongation of 7 min at 72°C. A truncated *hlb* gene indicative of phage integration was detected using the oligonucleotide pair hlb-2-rev/hlb-3-for, giving no PCR product after phage integration. The DNA sequences of PCR oligonucleotides are given in Table S1.

WGS and bioinformatics. Whole-genome sequencing (WGS) was performed using the PacBio RS II platform. Staphylococcal DNA was extracted using a Genomic-tip 20/G kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions with lysostaphin incubation (20 μg/ml) (Wakchemie, Steinbach, Germany) for 1 h at 37°C. Five micrograms of extracted, high-quality, double-stranded DNA was sequenced using P6-C4 chemistry and Pacific Biosciences RSII instrumentation using a movie collection time of 4-h and 110 pmol/liter of complexed 20-kb SMRTbell library. Initial *de novo* assembly was performed using the HGAP3, version 2.3.0, pipeline (Icahn Institute for Genomics and Multiscale Biology at the Icahn School of Medicine at Mount Sinai, New York, NY, USA). The sequenced samples presented coverages between 37× and 323× and 9,473 to 84,090 mapped reads, with a mean read length of 4,581 bp to 13,804 bp (N_{50} of 6,271 bp to 19,453 bp). The assembled genomes were annotated using the GenDB pipeline (40). For classification of the functional phage modules, an *in silico* PCR using oligonucleotides characteristic for the specific phage module types was applied using SnapGene (version 4.0.6; GSL Biotech, LLC, Chicago, IL, USA) (41). Structural comparison of the phi3 phages was performed using Easyfig software (42). Detection of genes carried by MGEs and indicative for human or animal origin of the isolates was performed by an *in silico* PCR using oligonucleotides for the genes *int* of phi6, *int* of phi7, *cadDX*, *rep27*, *rep7* (43), and *int* of phi2 (20).

Statistical analysis. A two-tailed Fisher's exact test (GraphPad Prism, version 5; GraphPad Software, Inc.) was used to compare the proportion of phi3-positive isolates among all MRSA *spa*-CC011 isolates detected in early and late years of the epidemic. *P* values of <0.05 were considered significant.

Accession number(s). The assembled genome sequences of the phi3-positive strains were deposited in the European Nucleotide Archive (ENA) under the following accession numbers: LT992456 to LT992458, LT992460 to LT992477, and OVTT01000001 to OVTT01000003.

RESULTS

Prevalence of the bacteriophage phi3 in MRSA *spa*-CC011 over 16 years. In total, 632 MRSA *spa*-CC011 isolates were screened for the presence of bacteriophage phi3. At the study hospital, the MRSA *spa*-CC011 epidemic started in 2000 with first one isolate; in 2013 MRSA *spa*-CC011 accounted for 35% of all MRSA isolates detected among local patients, as published elsewhere (8). Based on BURP analysis, the complete set of isolates comprised 26 different MRSA *spa*-CC011-associated *spa* types, with t011 (50.3%, $n = 318$), t034 (38%, $n = 240$), t108 (2.8%, $n = 18$), t1451 (2.1%, $n = 13$), t2011 (1.1%, $n = 7$), t1255 (0.9%; $n = 6$), t2582 (0.6%, $n = 4$), t2576 (0.5%, $n = 3$), t571 (0.5%, $n = 3$), t1580 (0.3%; $n = 2$), t1793 (0.3%; $n = 2$), and t2346 (0.3%, $n = 2$) being predominant. BURP analysis revealed four closely related clades belonging to *spa*-CC011 that form distinct branches in the clustering (CC034, CC108, CC1451, and CC1580). From each group, one isolate was selected for MLST analysis, which confirmed that these isolates belong to ST398.

Within this group, 21/632 (3.3%) isolates tested positive for IEC genes and were thus classified as phi3 positive (Table 1). Over time, the rate of phi3-positive MRSA *spa*-CC011 isolates increased nonsignificantly from 1.1% ($n = 1/92$ in 2000 to 2006) to 3.9% ($n = 20/540$ in 2007 to 2015) ($P > 0.05$). The majority of phi3-positive isolates were found in 2011 ($n = 7/60$; 11.7%), followed by 2010 ($n = 4/60$, 6.7%), 2012 ($n = 3/60$, 5%), 2008 and 2013 (each, $n = 2/60$; 3.3%), and 2006, 2014, and 2015 (each, $n = 1/60$; 1.7%) (Table 1).

All MRSA *spa*-CC011 isolates positive for IEC genes were characterized as methicillin and tetracycline resistant by the possession of the *mecA* and *tet*(M) genes,

TABLE 1 phi3-positive isolates belonging to *spa*-CC011-related *spa* types from 2000 to 2015[a]

Year(s) of isolation	Frequency of phi3-positive isolates by *spa* type (no. of positive isolates/no. tested [%])					Total no. (%)
	t011	t034	t1451	t1793	Other	
2000 to 2006	1/50	0/37	0/2	0/0	0/3	1/92 (1.1)
2007	0/34	0/24	0/0	0/0	0/2	0/60 (0)
2008	0/32	2/21	0/0	0/0	0/7	2/60 (3.3)
2009	0/31	0/18	0/1	0/0	0/10	0/60 (0)
2010	1/28	2/21	1/3	0/0	0/8	4/60 (6.7)
2011	2/30	5/20	0/2	0/1	0/7	7/60 (11.7)
2012	3/30	0/29	0/0	0/0	0/1	3/60 (5)
2013	0/27	2/23	0/1	0/0	0/9	2/60 (3.3)
2014	0/30	0/25	0/2	1/1	0/2	1/60 (1.7)
2015	1/26	0/22	0/2	0/0	0/10	1/60 (1.7)
Total	8/318 (2.5)	11/240 (4.6)	1/13 (7.7)	1/2 (50)	0/59 (0)	21/632 (3.3)

[a]As detected by PCR targeting immune evasion cluster (IEC)-associated genes *chp*, *scn*, *sak*, *sea*, and *sep*.

respectively (see Table S3 in the supplemental material). Furthermore, livestock origin was underlined by the presence of the phages phi2 and phi6 in 14.2% (n = 3/21) and 61.9% (n = 13/21) as well as the replication protein 7 in 95.2% (n = 20/21) of the isolates, respectively (Table S4). However, human markers such as the cadmium resistance gene *cadX* were found in 9.5% (n = 2/21). Furthermore, MLST analysis of the phi3-positive isolates revealed that all isolates belonged to ST398.

Most of the IEC-positive isolates (18/21) were associated with colonization and were obtained from classical screening specimens, including nasal, pharyngeal, or superficial skin swabs. The remaining three isolates were derived from clinical specimens (wound swabs and blood culture) potentially associated with infections. Among the phi3-positive isolates, four different *spa* types were observed, with t034 being the most prevalent (n = 11), followed by t011 (n = 8) and t1451 and t1793 (each, n = 1) (Table 1).

The bacteriophages belonged to three different IEC types. The most prevalent IEC type was type B carrying the genes *sak*, *chp*, and *scn* in 66.7% (n = 14/21) of phi3-positive *S. aureus* isolates, followed by IEC E carrying the genes *sak* and *scn* in 23.8% (n = 5/21) and IEC type A (*sea*, *sak*, *chp*, *scn*) in 9.5% (n = 2/21) of these isolates (Table 2).

Comparing the PCR-based typing results of the bacteriophages with the WGS data revealed that the PCR results correspond with the WGS data in 86% of instances. Discrepancies were due to false-positive *sep* PCR results in three isolates. The IEC-carried genes *chp*, *sak*, and *scn* were correctly identified in all isolates by PCR-based typing.

Characterization and classification of the phi3 phage genomes. WGS of the 21 phi3-positive isolates revealed complete assembled genomes for 20/21 isolates whereas for one isolate the genome was available in several contigs. However, the phi3 phage genomes were always located within one contig and ranged between 40,712 kb and 44,003 kb, containing between 55 and 69 genes. Sequence comparison of the phi3 bacteriophages showed homologous regions for the integrase locus, the morphogenesis locus (containing genes for DNA packing), the lysis module (containing genes for

TABLE 2 Diversity of immune evasion cluster types among the phi3-positive isolates

IEC type[a]	Immune-modulatory gene profile				% of phi3-positive isolates[b]
A	*sea*	*sak*	*chp*	*scn*	9.5 (2/21)
B		*sak*	*chp*	*scn*	66.7 (14/21)
C			*chp*	*scn*	
D	*sea*	*sak*		*scn*	
E		*sak*		*scn*	23.8 (5/21)
F	*sep*	*sak*	*chp*	*scn*	
G	*sep*	*sak*		*scn*	

[a]Immune evasion cluster (IEC) type classification according to van Wamel et al. (25).
[b]Values in parentheses are the number of phi3-positive isolates/total number tested.

host cell lysis), and the IEC (Fig. 1). Structural differences were found in modules carrying genes for lysogeny, DNA replication and packaging, and regulation of transcription.

The functional modules of phage phi3 were classified according to the multiplex scheme of Kahánková et al. (41), and 12 different phage types were identified (Table 3). For the lysogeny control module, all phages were found to belong to the same integrase group (Sa3int), and three different antirepressor types (*ant1a*, *ant4a*, and *ant4b*) were found. Furthermore, most phages contained a DNA replication module harboring the *dnaD* gene ($n = 19/21$) (subtypes *dnaD1a* [$n = 7/21$] and *dnaD1b* [$n = 12/21$]), and two phages were found to carry the *dnaC* gene (subtype *dnaC1*). The phages belonged to three different transcription regulation modules with the dUTPase types *dut1* ($n = 10/21$), *dut2* ($n = 4/21$), and *dut3* ($n = 7/21$). All phages carried the portal protein Fa and the endolysin gene *ami3*.

Alternative integration sites (genes or intergenic regions) of phage phi3 in the MRSA *spa*-CC011 genome. In addition to two already described integration sites (*hlb* gene and alanine racemase), WGS revealed four alternative integration sites for bacteriophage phi3 in the MRSA *spa*-CC011 genome (Table 3). The phi3 phage was integrated within metabolic genes (genes of the pyruvate oxidase [SAPIG2589], the acetolactate synthase large subunit *ilvB* [SAPIG2093], and the alanine racemase [SAPIG2238] [GenBank accession no. AM990992.1]), in between a gene for a nucleoside transporter protein (SAPIG0723) and *yxkD* (SAPIG0724) (GenBank accession no. AM990992.1), or within another phage genome (GenBank accession no. KC595279.1). Moreover, modified bacterial attachment (*attB*) sites were identified (Table 3). Six of the 21 phages integrated within *hlb* or *ilvB* harbored the *attB* site (5′-TGTATCCAAACTGG-3′) published by Coleman et al. (24). The majority of bacteriophages contained an *attB* site with two nucleotide exchanges (5′-TGTATCCGAATTGG-3′; changes are underlined). This *attB* site was found only for phages integrated within the *hlb* gene. Further *attB* sites with one to three nucleotide exchanges (underlined) were found in the remaining bacteriophages integrated within other genes in the *spa*-CC011 genome (5′-TGTATCCAAAATGG-3′; 5′-TGTATCCAAACTGT-3′; 5′-TGTATCCAATCTGG-3′; 5′-TGTATCCAACCAGG-3′; 5′-TGTATCCTTACTGT-3′).

DISCUSSION

Bacterial host switches are often linked to gains and losses of MGEs. Thus, we hypothesized that the livestock phenotype (i.e., methicillin and tetracycline resistance) associated with MRSA *spa*-CC011, which had been transmitted to the human host, would show adaptation by taking up bacteriophage phi3 characterized by its human-specific gene cluster containing the genes *chp*, *sak*, *scn*, *sea*, and *sep*. The livestock origin of the phi3-positive isolates was supported by the presence on the MGEs of the genes *rep7*, *int* of phi2, and *int* of phi6 in the majority of isolates. These genes were shown to be present in livestock-associated clades but were absent in human-specific isolates (17). Furthermore, MGE-carried genes described for human-specific isolates (*cadDX*) were also present sporadically in some isolates. This might indicate an ongoing uptake of human-specific genes contained on MGEs in the livestock-associated MRSA *spa*-CC011 clade and thereby an expanding host range of MRSA *spa*-CC011. However, the percentage of human phi3-carrying MRSA *spa*-CC011 isolates recovered in our study was comparatively low (3.3%). The number of phi3-positive MRSA *spa*-CC011 isolates increased only slightly from 1.1% in 2000 to 2006 to 3.9% in 2007 to 2015. In general, it is known for *S. aureus* that the majority of clinical and laboratory strains from human sources carry the phi3 phage (25). In contrast, the phi3-related gene cluster is only rarely found among the livestock-associated MRSA CC398 (18, 30, 31).

Until now, human-adapted MSSA CC398 isolates harboring the phi3 phage were mainly assigned to *spa* types t571, t034, and t3625 and were associated with human infections (16, 18, 31, 44, 45). Furthermore, bacteriophage phi3 was found in MSSA CC398 isolates belonging to the *spa* types t899, t571, t1451, t5635, t6587, and t9378 obtained from bloodstream infections in France (14). In Spain, an MRSA CC398 isolate

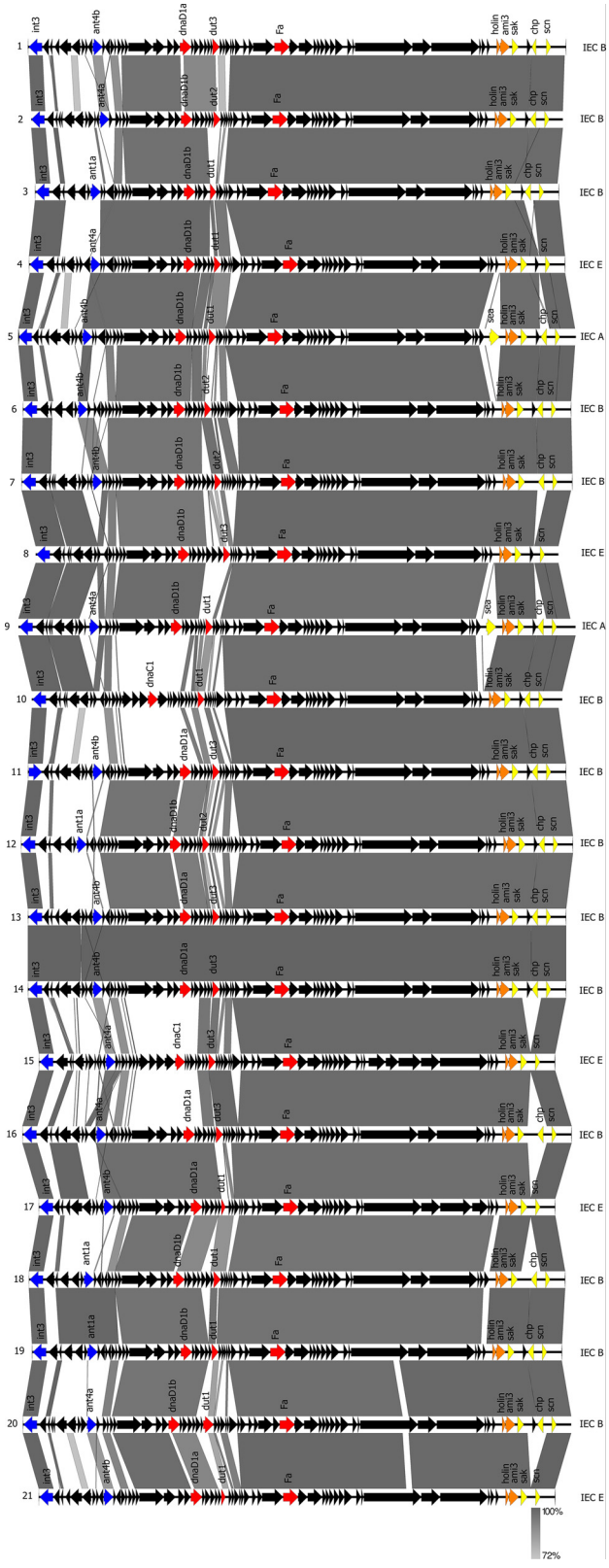

FIG 1 Structural comparison of the phi3 genomes of the phi3-positive LA-MRSA (strain numbers on the left) performed by Easyfig software. Gray areas represent regions with nucleotide sequence similarities ranging between 72% and 100%. The integrase and antirepressor genes (lysogeny module) are shown in blue, the replication protein (*dnaD* or *dnaC*), UTPase genes, and the portal protein (DNA metabolism and DNA packaging modules) are shown in red, holin and amidase (lysis module) are shown in orange, and IEC genes (IEC type on the right) are shown in yellow.

TABLE 3 Integration sites of phi3 phages and bacteriophage classification

Isolate no.	Integration site	GenBank accession no.[a]	*attB* site (5′– 3′)[b]	Bacteriophage classification[c]							
				Phage genome size (bp)	Integrase	Genetic control	Replication module	Transcription module	Morphogenesis subtype	Amidase	Phage type
1439	*hlb*		TGTATCCGAATTGG	42,460	Sa3	*ant4b*	*dnaD1a*	*dut3*	Fa	*ami3*	Sa3int-*ant4b-dnaD1a-dut3*-Fa-*ami3*
LA86	Pyruvate oxidase (SAPIG2589)	AM990992.1[f]	TGTATCCAAAATGG	41,927	Sa3	*ant4a*	*dnaD1b*	*dut2*	Fa	*ami3*	Sa3int-*ant4a-dnaD1b-dut2*-Fa-*ami3*
LA115	*hlb*		TGTATCCAAACTGG	41,335	Sa3	*ant1a*	*dnaD1b*	*dut1*	Fa	*ami3*	Sa3int-*ant1a-dnaD1b-dut1*-Fa-*ami3*
LA208	*hlb*		TGTATCCAAACTGG	42,292	Sa3	*ant4a*	*dnaD1b*	*dut1*	Fa	*ami3*	Sa3int-*ant4a-dnaD1b-dut1*-Fa-*ami3*
3949	Phage StauST398-5	KC595279.1[f]	TGTATCCAACCAGG	44,003	Sa3	*ant4b*	*dnaD1b*	*dut1*	Fa	*ami3*	Sa3int-*ant4b-dnaD1b-dut1*-Fa-*ami3*
LA232	*hlb*		TGTATCCAAACTGG	43,254	Sa3	*ant4b*	*dnaD1b*	*dut2*	Fa	*ami3*	Sa3int-*ant4b-dnaD1b-dut2*-Fa-*ami3*
4623	*hlb*		TGTATCCAAACTGG	43,456	Sa3	*ant4b*	*dnaD1b*	*dut2*	Fa	*ami3*	Sa3int-*ant4b-dnaD1b-dut2*-Fa-*ami3*
LA272	*ilvB* (SAPIG2093)	AM990992.1[e]	TGTATCCAAACTGG	41,228	Sa3	NT	*dnaD1b*	*dut3*	Fa	*ami3*	Sa3int-*dnaD1b-dut3*-Fa-*ami3*
LA281	Between SAPIG0723 and SAPIG0724[d]	AM990992.1[e]	TGTATCCAAACTGT	43,946	Sa3	*ant4a*	*dnaD1b*	*dut1*	Fa	*ami3*	Sa3int-*ant4a-dnaD1b-dut1*-Fa-*ami3*
5235	Between SAPIG0723 and SAPIG0724[d]	AM990992.1[e]	TGTATCCTTACTGT	41,849	Sa3	NT	*dnaC1*	*dut1*	Fa	*ami3*	Sa3int-*dnaC1-dut1*-Fa-*ami3*
LA290	*hlb*		TGTATCCGAATTGG	43,453	Sa3	*ant4b*	*dnaD1a*	*dut3*	Fa	*ami3*	Sa3int-*ant4b-dnaD1a-dut3*-Fa-*ami3*
LA293	*hlb*		TGTATCCGAATTGG	43,543	Sa3	*ant1a*	*dnaD1b*	*dut2*	Fa	*ami3*	Sa3int-*ant1a-dnaD1b-dut2*-Fa-*ami3*
LA301	*hlb*		TGTATCCGAATTGG	42,458	Sa3	*ant4b*	*dnaD1a*	*dut3*	Fa	*ami3*	Sa3int-*ant4b-dnaD1a-dut3*-Fa-*ami3*
5418	*hlb*		TGTATCCGAATTGG	42,458	Sa3	*ant4b*	*dnaD1a*	*dut3*	Fa	*ami3*	Sa3int-*ant4b-dnaD1a-dut3*-Fa-*ami3*
LA305	*hlb*		TGTATCCAAACTGG	40,712	Sa3	*ant4a*	*dnaC1*	*dut3*	Fa	*ami3*	Sa3int-*ant4a-dnaC1-dut3*-Fa-*ami3*
LA309	*hlb*		TGTATCCGAATTGG	43,331	Sa3	*ant4a*	*dnaD1a*	*dut3*	Fa	*ami3*	Sa3int-*ant4a-dnaD1a-dut3*-Fa-*ami3*
LA343	*hlb*		TGTATCCGAATTGG	40,746	Sa3	*ant4b*	*dnaD1a*	*dut1*	Fa	*ami3*	Sa3int-*ant4b-dnaD1a-dut1*-Fa-*ami3*
LA388	*hlb*		TGTATCCGAATTGG	42,310	Sa3	*ant1a*	*dnaD1b*	*dut1*	Fa	*ami3*	Sa3int-*ant1a-dnaD1b-dut1*-Fa-*ami3*
LA415	*hlb*		TGTATCCGAATTGG	41,810	Sa3	*ant1a*	*dnaD1b*	*dut1*	Fa	*ami3*	Sa3int-*ant1a-dnaD1b-dut1*-Fa-*ami3*
LA436	Alanine racemase (SAPIG2238)	AM990992.1[e]	TGTATCCAATCTGG	43,263	Sa3	*ant4a*	*dnaD1b*	*dut1*	Fa	*ami3*	Sa3int-*ant4a-dnaD1b*-dut1-Fa-*ami3*
LA562	*hlb*		TGTATCCGAATTGG	40,747	Sa3	*ant4b*	*dnaD1a*	*dut1*	Fa	*ami3*	Sa3int-*ant4b-dnaD1a-dut1*-Fa-*ami3*

[a]GenBank accession numbers correspond to genes used for alignment in WGS data.
[b]Nucleotides differing from the original *attB* site of phage phi3 are underlined (24).
[c]Classification of bacteriophage modules according to Kahánková et al. (41). NT, nontypeable.
[d]SAPIG0723 encodes a nucleoside transporter protein; SAPIG0724 is the *yxkD* gene.
[e]*S. aureus* ST398 (GenBank accession no. NC_017333.1) (20).
[f]*S. aureus* phage StauST398-5pro (GenBank accession no. KC595279.1) (16).

containing bacteriophage phi3 belonging to *spa* type t1451 was identified in a poultry farmer with abscesses and septic arthritis (46).

In our study, we found MRSA *spa*-CC011 from colonization and clinical specimens carrying the IEC genes in a human health care setting located in a German pig-dense area. Interestingly, the MRSA *spa*-CC011 isolates carrying the phage phi3 belonged to the *spa* types t011, t034, t1451, and t1793. This is of particular importance since isolates with *spa* types t011 and t034 represent the majority of MRSA *spa*-CC011 isolates recovered from the human population in general as well as from the hospital environment (8, 9, 47).

In a previous study by Cuny et al. (31), a slightly higher prevalence for MRSA *spa*-CC011 carrying the phi3 phage (10%) was found in horses, veterinary personnel from horse clinics, and humans with infections, including wound infections, furuncles, and septicemia (31). However, bacteriophage phi3 was absent in MRSA *spa*-CC011 isolated from nasal specimens of pig farmers and pigs. The higher percentage of phi3-positive isolates found by Cuny et al. might be due to diverse strain origins, whereas our isolates were exclusively obtained from human specimens in a clinical setting.

Recently, Kraushaar et al. identified nine alternative integration sites for a bacteriophage belonging to integrase group 3 in the CC398 genome (48). These alternative integration sites were all within genes (mostly metabolic genes), and the phage integration was found after experimental infection of CC398 strains with an *int3* bacteriophage. Furthermore, phage transfer from a human-associated *S. aureus* donor strain to an *S. aureus* CC398 strain was found after treatment with sublethal concentrations of biocides, and nine alternative integration sites for prophage Φ13 were described in an LA-MRSA CC398 isolate (49). The authors speculated that mutations in *attB* might be the reason for the missing phage integration into *hlb* in their study (49). However, we found that phage phi3 was able to integrate within the *hlb* gene containing both the original *attB* site and *attB* sites containing two mutations. Additionally, atypical integration sites for bacteriophages encoding the *sak* gene were previously described for disease-related *S. aureus* isolates within genes or intergenically (50). In contrast, we found six naturally occurring alternative integration sites, four of which have not been previously described. The integration sites were within metabolic genes or in between metabolic and transporter protein genes. Interestingly, for one phi3 phage, we identified another bacteriophage genome as an integration site.

This study provides evidence that the PCR-based detection of the IEC genes described here is a reliable method to detect phage phi3 in MRSA *spa*-CC011. For the IEC-carried genes *scn*, *sak*, and *chp*, no discrepancies were found between WGS and PCR-based typing, and the IEC genes were found only in isolates harboring phage phi3. However, for the precise typing of isolates, especially for the genes *sea* and *sep* and the integration site of the phi3 phage, WGS is an essential tool.

Previous studies showed the presence of three separate clades of Sa3 prophages, as detected by the prophage integrase gene Sa3int (18). The PCR-based detection of IEC genes located on the phi3 phage could therefore lead to missing the identification of other Sa3 prophages without IEC genes and has to be seen as a limitation of the study.

Due to our study design, which was based on *spa* typing and BURP clustering, only MRSA isolates clustering to *spa*-CC011 and closely related clades such as CC034, CC108, and CC1451 were included. A previous study by Strommenger et al. revealed that *spa* typing together with BURP clustering is a useful tool for typing of *S. aureus* isolates (51). However, limitations in using *spa* typing for the assignment of MRSA isolates to clonal complexes have been reported (18). This was observed for *spa* type t899, which was shown to be associated with ST398 or ST9 (52). Previous MLST of isolates in our study with *spa* type t899 showed that these isolates belonged to ST9 (8).

In conclusion, the acquisition of phage phi3 does not seem to be a major factor leading to the readaptation of LA-MRSA *spa*-CC011 back to the human host over a period of 16 years, as shown before for human-adapted methicillin-susceptible *S. aureus* (MSSA) CC398 (15, 16). Moreover, the absence of bacteriophage phi3 in the

MRSA *spa*-CC011 genome does not impair the ability of MRSA *spa*-CC011 to colonize and infect the human host. Therefore, further surveillance of *S. aureus* CC398 dissemination is necessary, in particular, in regions with a high density of livestock production since the high prevalence of this lineage in animal husbandry correlates with its prevalence in persons with occupational livestock exposure and its occurrence in health care facilities (7, 53–55). These findings highlight the importance of monitoring programs for emerging pathogens, such as the *S. aureus* lineage CC398. Furthermore, investigations on the adaptation of livestock-associated MRSA *spa*-CC011 to the human host are of major importance because MRSA *spa*-CC011 presents a zoonotic threat for the human environment and for health care settings (56).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JCM.00140-18.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.
SUPPLEMENTAL FILE 2, PDF file, 0.1 MB.

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We do not have any conflicts of interest to declare.

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