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Identification of a Novel Transposon (Tn*6072*) and a Truncated Staphylococcal Cassette Chromosome *mec* Element in Methicillin-Resistant *Staphylococcus aureus* ST239▽†

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A novel composite transposon (Tn*6072*) resembling staphylococcal cassette chromosome *mercury* (SCC*Hg*) was identified in a collection of sequence type (ST) 239 methicillin (meticillin)-resistant *Staphylococcus aureus* (MRSA) isolates from Romanian hospitals. Tn*6072* is homologous to the 5′ region of SCC*Hg* found in staphylococcal cassette chromosome *mec* (SCC*mec*) type III prototype strain 85/2082 but lacks the characteristic *mer* operon. SCC*Hg* has previously been reported to integrate downstream of *orfX*, at the same chromosomal location as SCC*mec*. Tn*6072*, by contrast, is demarcated by two IS*431* elements, flanked by 8-bp direct repeats, and inserted upstream of the origin of replication, within an open reading frame homologous to SAR2700 of *S. aureus* strain MRSA252. Analysis of a geographically and temporally diverse collection of 111 strains from the ST239 clonal group uncovered 11 additional strains harboring Tn*6072*, demonstrating a lineage-specific insertion pattern. Complete sequence analysis of the SCC*mec* regions of two representative Romanian strains (BK16704, BK16691) revealed two additional novel structures derived from a type III SCC*mec* background. BK16704 possesses an SCC*mec* 3A.1.4 structure, with an IS*256* insertion downstream of the right chromosomal junction. In contrast, the SCC*mec* element of BK16691 is truncated downstream of the *mec* gene complex, with a 24-kb deletion encompassing the right chromosomal junction and an inverted downstream IS*256* element. This structure, tentatively named "ψSCC*mec*$_{16691}$," confers methicillin resistance but lacks most of the J1/J2 region, including the *ccr* gene complex. Taken together, these findings provide evidence for the continuing evolution of SCC elements, as well as the ST239 clonal group.

Methicillin (meticillin) resistance in staphylococci is associated with a heterogeneous mobile element known as staphylococcal cassette chromosome *mec* (SCC*mec*) (19, 22). SCC*mec* elements are characterized by several common features, including (i) integration at a specific chromosomal site; (ii) flanking repeat sequences; and (iii) presence of two essential genetic components, namely, a *mec* gene complex and a cassette chromosome recombinase (*ccr*) gene complex (17). Integration takes place at a unique bacterial chromosomal attachment site (*attB*SCC) located in the *orfX* gene, approximately 34 kb downstream of the origin of replication, and is achieved by recognition of a specific nucleotide sequence designated the integration site sequence of SCC (ISS) (17, 21). SCC*mec* elements are classified by binary combinations of different *ccr* and *mec* gene complex allotypes and can be further differentiated by variation within three "joining" (J) regions: J1, right chromosomal junction to *ccr* gene complex; J2, *ccr* gene complex to *mec* gene complex; and J3, *mec* gene complex to left chromosomal junction. Eight types of SCC*mec* elements (I to VIII) have been described for methicillin-resistant *S. aureus* (MRSA) (17), along with various J-region subtypes, with ongoing diversification driven by both recombination and *de novo* gene acquisition from exogenous sources (39, 40). Other SCC elements which do not harbor *mecA* have also been described for staphylococci (18, 23, 26, 29), integrated in the same chromosomal location as SCC*mec*.

Acquisition of SCC*mec* by methicillin-susceptible *S. aureus* (MSSA) is the defining characteristic of MRSA, and major "epidemic waves" in the evolution of MRSA have been linked to the emergence of SCC*mec* types I to IV (7, 18, 20, 38). SCC*mec* type III, for example, is uniquely associated with a widespread group of nosocomial clones (Brazilian, Hungarian, AUS-2 and -3, CMRSA-3, and EMRSA-1, -4, -7, -9, and -11) assigned by multilocus sequence typing (MLST) (9) to the sequence type (ST) 239 subgroup of clonal complex (CC) 8. In many geographic regions, including Brazil, Eastern Europe, the Middle East, India, East Asia, and Eastern Australia, ST239-III is the predominant nosocomial MRSA genotype, accounting for as many as 70% of hospital-acquired MRSA isolates worldwide (3, 10). The ST239 lineage is believed to have arisen via a large-scale chromosomal recombination event between genotypically disparate strains, with the majority of the chromosome (80%) derived from a CC8 background, whereas a 635-kb region surrounding the origin of replication

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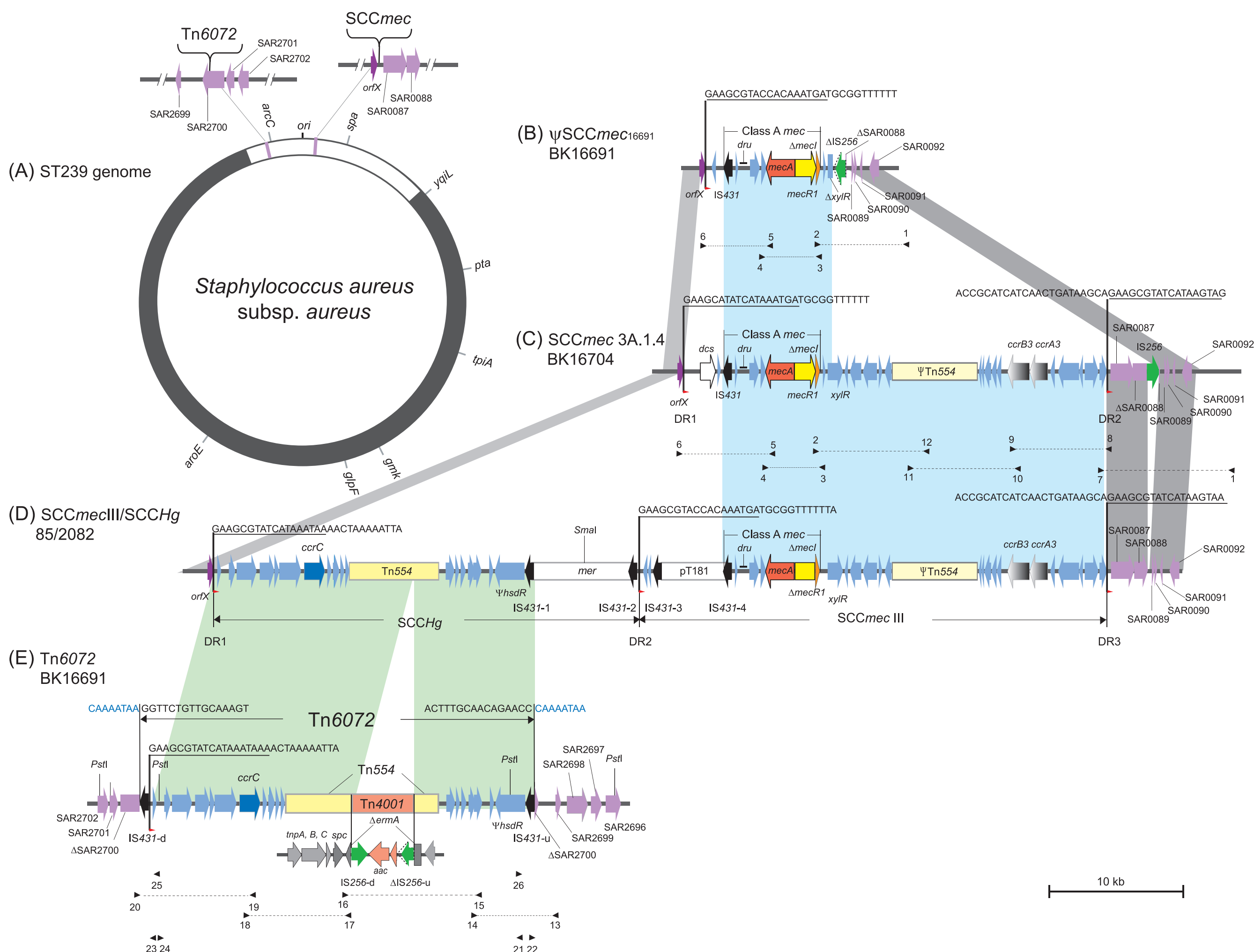

FIG. 1. Structure of Tn*6072* and SCC elements in strains BK16691, BK16704, and 85/2082. (A) Schematic representation of *S. aureus* ST239 chromosome showing the integration sites of Tn*6072* and SCC*mec*. A 635-kb segment corresponding to the ST30-derived chromosomal replacement is depicted in white. The locations of the seven MLST and *spa* loci are also indicated. (B) ψSCC*mec*$_{16691}$ element from strain BK16691; (C) SCC*mec* 3A.1.4 element from strain BK16704; (D) composite SCC*mec* III/SCC*Hg* element from prototype strain 85/2082; (E) Tn*6072* element from strain BK16691, shown in the opposite orientation relative to representation shown in Fig. 1A, with PstI restriction sites used for inverse PCR. Blue shading denotes regions of homology shared by SCC*mec* elements in strains BK16691, BK16704, and 85/2082; green shading denotes homology between SCC*Hg* and Tn*6072*; gray shading indicates homology shared by flanking chromosomal junctions. ORFs are portrayed by colored arrows, with chromosomal ORFs indicated in purple, IS*431* elements in black, and IS*256* elements in green. Dotted lines to the left of IS*256* elements in Fig. 1B and 1E denote the truncated regions of ΔIS*256* and ΔIS*256*-u, respectively, in BK16691. Tn*554*, Tn*4001*, pT181, and *mer* elements are depicted as rectangles. ISS sites are depicted by small red arrowheads, with characteristic ISS sequences underlined. Integration site sequences and direct repeats in Tn*6072* are shown in blue (direct repeats in Tn*6072* are shown in reverse complement, since the orientation of Tn*6072* is inverted relative to SCC*Hg*). Small black arrowheads represent the locations of primers used for long-range, inverse, and outward-directed PCR (see Table S1 in supplemental material).

appears to be derived from CC30 (16, 33) (Fig. 1A). This region includes both SCC*mec* and the *spa* locus, consistent with the presence in ST239 of *spa* repeat motifs (33) characteristic of CC30; SCC*mec* type III in CC30, however, has never been reported (16).

The 67-kb structure originally reported for SCC*mec* III prototype strain 85/2082 (18) was subsequently shown to consist of an SCC*mec* III element (class A *mec* with *ccrAB3*) in tandem arrangement with another SCC element, staphylococcal cassette chromosome *mercury* (SCC*Hg*) (5). The latter element harbors a mercury resistance operon (*mer*), a Tn*554* element, and a site-specific recombinase gene complex (*ccrC*) and integrates into the *S. aureus* genome at the aforementioned insertion sequence site within *orfX* (Fig. 1D). Precise chromosomal excision of SCC*Hg* has been previously demonstrated (5, 18), presumably mediated by *ccrC*, but SCC*Hg* in any other chromosomal location has not been reported.

In this study, a novel transposon (Tn*6072*) closely resembling SCC*Hg* was identified in multiple isolates of two ST239 MRSA clones circulating in Romanian hospitals and in 11 additional strains from 6 other countries. We demonstrate that Tn*6072* is located upstream of the origin of replication, within an open reading frame (ORF) corresponding to SAR2700 in CC30 strain MRSA252 (15). In addition, one of the two Ro-

manian clones is shown to possess a 24-kb deletion encompassing the entire region from the *mec* gene complex to the right chromosomal junction, thereby lacking a *ccr* gene complex and flanking repeat sequences. This structure, while conferring resistance to methicillin, does not satisfy current criteria for SCC*mec* elements (17) and has tentatively been designated "ψSCC*mec*$_{16691}$."

MATERIALS AND METHODS

Bacterial strains. Thirty-four MRSA strains were selected from a recent study involving approximately 150 *S. aureus* isolates, collected between 2004 and 2005 from three affiliated hospitals of the Clinic County Hospital in Braçsov, Romania (R. Ionescu, J. R. Mediavilla, L. Chen, D. O. Grigorescu, M. Idomir, B. N. Kreiswirth, and R. B. Roberts, unpublished data). All 34 isolates chosen for this study belonged to the ST239 clonal group, as determined by multilocus sequence typing (ST239/ST1312) and *spa* typing (*spa* type 351/WGKAQQ/t030; *spa* type 3/WGKAOMQ/t037; or *spa* type 1020/WFGKAOMQ/t074). SCC*mec* III prototype strain 85/2082 (18) was kindly provided by T. Ito (Juntendo University, Tokyo, Japan), while SCC*mec* IIIB prototype strain HDG2 (32) was a gift from H. de Lencastre (Rockefeller University, New York, NY). A previously described collection of 111 diverse strains from the ST239 clonal group, collected over 34 years from 29 countries (36), was used to assess the distribution of Tn*6072*. Strains MRSA252 (NRS071) and MSSA476 (NRS072) were obtained from the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) (http://www.narsa.net).

MIC testing. MIC values for oxacillin (Sigma-Aldrich, St. Louis, MO) were determined for all 34 Romanian isolates using the broth dilution method, according to CLSI guidelines (6). Bacterial suspensions were incubated for 24 h at 37°C, and optical density at 600 nm (OD_{600}) values were obtained using a VersaMax tunable microplate reader (Molecular Devices, Sunnyvale, CA). *S. aureus* strains MRSA252 and MSSA476 were used as positive and negative controls (15), respectively.

DNA isolation. Single colonies of *S. aureus* were isolated on BBL CHROMagar Staph aureus (Becton-Dickinson, Franklin Lakes, NJ), restreaked onto Luria-Bertani (LB) agar, and grown overnight at 37°C. DNA was isolated using a Wizard genomic DNA purification kit (Promega, Madison, WI), following treatment with 20 μg/ml lysostaphin (Sigma-Aldrich, St. Louis, MO) for 30 min at 37°C. Genomic DNA was stored at 4°C and used as template for all PCRs.

Genotypic analysis. *S. aureus* isolates were *spa* typed using eGenomics software, as described previously (27, 35). Ridom *spa* types were subsequently assigned using the *spa* typing website (http://www.spaserver.ridom.de) developed by Ridom GmbH and curated by SeqNet.org (http://www.SeqNet.org) (13). MLST was also performed as described previously (9), using the database maintained at http://saureus.mlst.net. Haplotype analysis based on sequences at 32 loci (36) was performed on two representative Romanian strains (BK16691 and BK16704) and on reference strain 85/2082 in order to place them within the global population structure of the ST239 clonal group. SCC*mec* typing was performed using a recent multiplex real-time PCR method (4), which targets *mecA* and the two essential gene complexes (*ccr* and *mec*) found in all SCC*mec* elements. Other SCC*mec* or SCC*Hg* targets, such as the *mer* operon, pT181, *dcs*, and the SCC*Hg* J region (CZ055 to CZ066 in strain 85/2082) were detected using conventional PCR methods described elsewhere (24, 31). Lastly, the variable-number tandem repeats (VNTR) adjacent to IS*431mec*, referred to as *dru* (*d*irect *r*epeat *u*nit) or HVR (*h*yper*v*ariable *r*egion), have demonstrated utility in molecular analyses of MRSA (11, 30, 34, 36). *dru* typing was therefore performed as described previously (11) using DruID software, and new *dru* types were submitted to http://www.dru-typing.org. DNA sequencing for *spa* typing, *dru* typing, and MLST was performed commercially.

Long-range PCR. Several long-range PCR assays were employed to amplify the entire SCC*mec* regions (*orfX* to right chromosomal junction) and Tn*6072* elements from two representative Romanian strains (BK16691 and BK16704). Primers used for long-range PCR are listed in Table S1 in the supplemental material and depicted in Fig. 1. Hot-Start *Taq* DNA polymerase Mastermix (Denville Scientific, Metuchen, NJ), 400 nmol of each primer, and 100 ng of template DNA were combined into 50-μl reaction mixtures. Cycling conditions were as follows: initial denaturation at 94°C for 5 min; 10 cycles of 94°C/10 s followed by 65°C/10 min; and an additional 20 cycles of 94°C/10 s and 65°C/10 min, increased by 20 s per cycle at 65°C.

Inverse PCR. Inverse PCR was used in order to sequence the chromosomal junctions surrounding Tn*6072*. Briefly, 5 μg of *S. aureus* genomic DNA was digested overnight using 20 U of PstI (New England BioLabs, Ipswich, MA) and, subsequently, 0.5 μg of PstI-digested fragments was ligated for 3 h at 16°C using T4 DNA ligase (New England BioLabs). Ligation products were purified using equal volumes of phenol-chloroform, and the aqueous phase was precipitated with ethanol and resuspended in double distilled water (ddH_2O). Approximately 100 ng of circularized DNA was used as template to amplify both Tn*6072* flanking regions, using the long-range PCR conditions described above.

Outward-directed PCR. To detect the presence of circularized extrachromosomal Tn*6072* intermediates (minicircles), two outward-directed primers (3F7 and 3R8) (see Table S1 in the supplemental material) matching the terminal sequences of Tn*6072* were designed. Outward-directed PCR was then performed as described elsewhere (37), using genomic DNA as a template. An alternative primer set (3F7 and SCC*Hg*-R) was used to detect the extrachromosomal form of SCC*Hg* in strains 85/2082 and BK16684 (SCC*mec* III with SCC*Hg*).

Excision of SCC*mec*. PCR was also employed to identify the precise excision of SCC*mec*, using methods described elsewhere (5, 19). Briefly, primer pair cR2/cLt2 was used to detect *attB* within the type 3A.1.4 SCC*mec* strains found in this study, while primer pairs LF10/cR2 and LF12/orfR1 were used to detect potential excision of the truncated SCC*mec* element in ψSCC*mec*$_{16691}$-bearing strains.

Other PCR methods. Additional PCR assays were employed to characterize the structures and integration sites of SCC*mec* 3A.1.4, ψSCC*mec*$_{16691}$, and Tn*6072*. Primer pairs LF10/mecI-R and LF10/IS*256*-P3 were used to identify ψSCC*mec*$_{16691}$ and the associated downstream IS*256* insertion, with ψSCC*mec*$_{16691}$-bearing strains yielding 2.5- and 1.1-kb fragments (see Table S1 in the supplemental material). Similarly, primer set LF10/IS*256*-F1 was used to confirm the downstream IS*256* insertion in all type 3A.1.4 SCC*mec*-bearing strains, yielding a single 0.9-kb amplicon. Carriage of Tn*6072* was determined using primers 3F2 and IS431-r-1, while integration within the SAR2700 homolog was confirmed by amplifying the upstream and downstream junctions using primer pairs SAR2699-F3/typeIII-F and 3F2/SAR2700-R4, respectively (see Table S1). Primer set SAR2699-F3/SAR2700-R4 was used as a negative control, yielding a 0.5-kb amplicon for all strains not harboring Tn*6072* (e.g., 85/2082). Lastly, insertion of Tn*4001* within the Tn*554* element of Tn*6072* was identified using primer pairs 3F3/aac2-R1 and ermA-F/aac-R1 (see Table S1).

Sequence analysis. Complete nucleotide sequences of the SCC*mec* and Tn*6072* elements from two representative Romanian strains (BK16691 and BK16704) were obtained using long-range PCR, inverse PCR, and primer walking. Amplicons generated by long-range and inverse PCR were verified by agarose gel electrophoresis and sequenced commercially. ORFs were identified with GLIMMER V3.02 software (8) using default parameters. ClustalW2 (25) was used to align and compare multiple sequences, and annotation was performed using BLASTN and BLASTX (http://blast.ncbi.nlm.nih.gov/).

Nomenclature assignment for Tn*6072*. Transposon number 6072 was assigned using the Tn Number Registry maintained by the Eastman Dental Institute at University College London (http://www.ucl.ac.uk/eastman/tn).

Nucleotide sequence accession number. The complete nucleotide sequences of the following elements have been deposited in GenBank under the following accession numbers indicated: (i) the ψSCC*mec*$_{16691}$ element from strain BK16691, GU235983; (ii) the SCC*mec* 3A.1.4 element from strain BK16704, GU235984; and (iii) the Tn*6072* element from strain BK16691, GU235985.

RESULTS AND DISCUSSION

Characteristics of Romanian strains. Thirty-four clinical MRSA strains used in this study were obtained between March 2004 and June 2005 from the Clinic County Hospital in Braçsov, Romania, as part of another study (Ionescu et al., unpublished data). In that study, approximately 150 *S. aureus* isolates were analyzed by *spa* typing and SCC*mec* typing, among other methods. Of these, 32 MRSA strains possessed the same *spa* repeat pattern (*spa* type 351/WGKAQQ/Ridom t030), while another two strains (BK16684 and BK16672) displayed closely related patterns (3/WGKAOMQ/t037 and 1020/WFGKAOMQ/t074, respectively) (Table 1). Subsequent MLST typing indicated that 32/34 strains belonged to ST239 (2-3-1-1-4-4-3), while 2 *spa* type 351 strains (BK16658 and BK16666) were classified as ST1312 (2-3-176-1-4-4-3), a single-locus variant of ST239. However, SCC*mec* typing suggested the

TABLE 1. Characteristics of Romanian MRSA strains used in this study

Strain ID[a]	Oxacillin MIC (μg/ml)	MLST[b]	*spa* type[c]	*spa* repeat patterns	SCC*mec* type	*dru* type	*dru* repeat patterns	SCC components						Excision of SCC*mec*	Tn*6072*			IS*256* (16691)[e]	IS*256* (16704)[f]
								mecI	*ccrC*	*mer*	*Hg*-J[d]	pT181	*dcs*		SAR2700 insertion	Tn*6072* minicircle	Tn*4001* insertion		
16684	256	ST239	3	WGKAOMQ	III	dt8t	5a-2d-5b-3a-4c-3b-4e-4j	+	+	+	+	+	−	+	−	[g]	−	−	−
16672	8	ST239	1020	WFGKAOMQ	IIIB	dt11ac	5a-2d-4a-0-2d-6f-2a-3o-3b-4e-3e	+	−	−	−	−	−	+	−	−	−	−	−
16645	>512	ST239	351	WGKAQQ	3A.1.4	dt10a	5a-2d-4a-0-2d-5b-3a-2g-3b-4e	+	+	−	+	−	+	+	+	+	+	−	+
16646	512	ST239	351	WGKAQQ	3A.1.4	dt10a	5a-2d-4a-0-2d-5b-3a-2g-3b-4e	+	+	−	+	−	+	+	+	+	+	−	+
16651	512	ST239	351	WGKAQQ	3A.1.4	dt10a	5a-2d-4a-0-2d-5b-3a-2g-3b-4e	+	+	−	+	−	+	+	+	+	+	−	+
16652	512	ST239	351	WGKAQQ	3A.1.4	dt10a	5a-2d-4a-0-2d-5b-3a-2g-3b-4e	+	+	−	+	−	+	+	+	+	+	−	+
16653	512	ST239	351	WGKAQQ	3A.1.4	dt10a	5a-2d-4a-0-2d-5b-3a-2g-3b-4e	+	+	−	+	−	+	+	+	+	+	−	+
16656	512	ST239	351	WGKAQQ	3A.1.4	dt10a	5a-2d-4a-0-2d-5b-3a-2g-3b-4e	+	+	−	+	−	+	+	+	+	+	−	+
16658	256	ST1312	351	WGKAQQ	3A.1.4	dt10g	5a-2d-4a-0-2d-5b-2a-2g-3b-4e	+	+	−	+	−	+	+	+	+	+	−	+
16666	256	ST1312	351	WGKAQQ	3A.1.4	dt10g	5a-2d-4a-0-2d-5b-2a-2g-3b-4e	+	+	−	+	−	+	+	+	+	+	−	+
16670	512	ST239	351	WGKAQQ	3A.1.4	dt10a	5a-2d-4a-0-2d-5b-3a-2g-3b-4e	+	+	−	+	−	+	+	+	+	+	−	+
16671	512	ST239	351	WGKAQQ	3A.1.4	dt10a	5a-2d-4a-0-2d-5b-3a-2g-3b-4e	+	+	−	+	−	+	+	+	+	+	−	+
16676	512	ST239	351	WGKAQQ	3A.1.4	dt10a	5a-2d-4a-0-2d-5b-3a-2g-3b-4e	+	+	−	+	−	+	+	+	+	+	−	+
16677	256	ST239	351	WGKAQQ	3A.1.4	dt7f	5a-2d-5b-3a-2g-3b-4e	+	+	−	+	−	+	+	+	+	+	−	+
16680	512	ST239	351	WGKAQQ	3A.1.4	dt10a	5a-2d-4a-0-2d-5b-3a-2g-3b-4e	+	+	−	+	−	+	+	+	+	+	−	+
16686	512	ST239	351	WGKAQQ	3A.1.4	dt10a	5a-2d-4a-0-2d-5b-3a-2g-3b-4e	+	+	−	+	−	+	+	+	+	+	−	+
16687	512	ST239	351	WGKAQQ	3A.1.4	dt10a	5a-2d-4a-0-2d-5b-3a-2g-3b-4e	+	+	−	+	−	+	+	+	+	+	−	+
16698	512	ST239	351	WGKAQQ	3A.1.4	dt10a	5a-2d-4a-0-2d-5b-3a-2g-3b-4e	+	+	−	+	−	+	+	+	+	+	−	+
16699	256	ST239	351	WGKAQQ	3A.1.4	dt10a	5a-2d-4a-0-2d-5b-3a-2g-3b-4e	+	+	−	+	−	+	+	+	+	+	−	+
16700	512	ST239	351	WGKAQQ	3A.1.4	dt10a	5a-2d-4a-0-2d-5b-3a-2g-3b-4e	+	+	−	+	−	+	+	+	+	+	−	+
16702	512	ST239	351	WGKAQQ	3A.1.4	dt10a	5a-2d-4a-0-2d-5b-3a-2g-3b-4e	+	+	−	+	−	+	+	+	+	+	−	+
16704	512	ST239	351	WGKAQQ	3A.1.4	dt10a	5a-2d-4a-0-2d-5b-3a-2g-3b-4e	+	+	−	+	−	+	+	+	+	+	−	+
16674	256	ST239	351	WGKAQQ	ψSCC*mec*$_{16691}$	dt9x	5a-2d-3p-5b-3a-2g-2c-4e-3e	+	+	−	+	−	−	−	+	+	+	+	−
16678	256	ST239	351	WGKAQQ	ψSCC*mec*$_{16691}$	dt9x	5a-2d-3p-5b-3a-2g-2c-4e-3e	+	+	−	+	−	−	−	+	+	+	+	−
16679	512	ST239	351	WGKAQQ	ψSCC*mec*$_{16691}$	dt9x	5a-2d-3p-5b-3a-2g-2c-4e-3e	+	+	−	+	−	−	−	+	+	+	+	−
16683	256	ST239	351	WGKAQQ	ψSCC*mec*$_{16691}$	dt9x	5a-2d-3p-5b-3a-2g-2c-4e-3e	+	+	−	+	−	−	−	+	+	+	+	−
16688	512	ST239	351	WGKAQQ	ψSCC*mec*$_{16691}$	dt9x	5a-2d-3p-5b-3a-2g-2c-4e-3e	+	+	−	+	−	−	−	+	+	+	+	−
16689	512	ST239	351	WGKAQQ	ψSCC*mec*$_{16691}$	dt9x	5a-2d-3p-5b-3a-2g-2c-4e-3e	+	+	−	+	−	−	−	+	+	+	+	−

16691	256	ST239	351	WGKAQQ	ψSCC*mec*$_{16691}$	dt9x	5a-2d-3p-5b-3a-2g-2c-4e-3e	+	+	−	+	−	−	−	+	+	+	+	−
16692	256	ST239	351	WGKAQQ	ψSCC*mec*$_{16691}$	dt9x	5a-2d-3p-5b-3a-2g-2c-4e-3e	+	+	−	+	−	−	−	+	+	+	+	−
16695	512	ST239	351	WGKAQQ	ψSCC*mec*$_{16691}$	dt9x	5a-2d-3p-5b-3a-2g-2c-4e-3e	+	+	−	+	−	−	−	+	+	+	+	−
16696	512	ST239	351	WGKAQQ	ψSCC*mec*$_{16691}$	dt9x	5a-2d-3p-5b-3a-2g-2c-4e-3e	+	+	−	+	−	−	−	+	+	+	+	−
16703	256	ST239	351	WGKAQQ	ψSCC*mec*$_{16691}$	dt9x	5a-2d-3p-5b-3a-2g-2c-4e-3e	+	+	−	+	−	−	−	+	+	+	+	−
16708	256	ST239	351	WGKAQQ	ψSCC*mec*$_{16691}$	dt9x	5a-2d-3p-5b-3a-2g-2c-4e-3e	+	+	−	+	−	−	−	+	+	+	+	−
HDG2	64	ST239	121	WGKAOM	IIIB	dt11l	5a-2d-4a-2d-2d-5b-3a-2g-3b-4e-3e	+	−	−	−	−	−	+	−	−	−	−	−
85/2082	16	ST239	3	WGKAOMQ	III	dt10c	5a-2d-4a-0-2d-1b-2g-3b-4e-3e	+	+	+	+	+	−	+	−	[e]	−	−	−

[a] Prototype strains used in this study are shown in boldface. ID, identification number.
[b] Multilocus sequence types correspond to the following allelic profiles: ST239 (2-3-1-1-4-4-3) and ST1312 (2-3-176-1-4-4-3).
[c] Ridom equivalents for eGenomics *spa* types are as follows: *spa* type 3, Ridom t037; *spa* type 121, Ridom t421; *spa* type 351, Ridom t030; and *spa* type 1020, Ridom t074.
[d] J region of SCC*Hg* element (corresponding to CZ055 to CZ066 in strain 85/2082), detected using primers MN21/MN22 (see Table S1 in supplemental material) (24).
[e] Downstream ΔIS*256* within ΔSAR0088 in strain BK16691, detected with primer set LF10/IS256-P3 (see Table S1 in the supplemental material) (12).
[f] Downstream IS*256* within ΔSAR0088 in strain BK16704, detected with primer set LF10/IS256-F1 (see Table S1 in the supplemental material).
[g] Tn*6072* minicircle is not present, but an SCC*Hg* minicircle was detected using primer set 3F7/SCC*Hg*-R (see Table S1 in the supplemental material).

presence of two different variants among the 32 *spa* type 351 strains. The majority (20/32) exhibited a signature (class A *mec*, *ccrAB3*, *ccrC*) consistent with SCC*mec* III prototype strain 85/2082, which harbors the SCC*Hg* element (Fig. 1). The remaining 12/32 *spa* type 351 strains, however, were positive only for class A *mec* and *ccrC* and were therefore classified as nontypeable. Sequencing of the entire SCC*mec* region from one of the latter strains (BK16691) revealed the presence of a truncated SCC*mec* element, subsequently dubbed ψSCC*mec*$_{16691}$ (see below). Of the remaining two MRSA strains, one possessed an SCC*mec* III signature (BK16684), while the other strain (BK16672) possessed a signature consistent with SCC*mec* IIIB (class A *mec* and *ccrAB3*, without *ccrC*). Conventional PCR analysis of other SCC targets (Table 1) showed that all of the *spa* type 351 strains possessed the SCC*Hg* J region (CZ055 to CZ056 in strain 85/2082) but did not harbor the *mer* operon or pT181. Interestingly, the 20 *spa* type 351 strains with the SCC*mec* III signature also possessed the *dcs* locus, previously reported only for the J3 regions of SCC*mec* types I, II, and IV (31) but recently described in an ST239 strain from Saudi Arabia (JCSC1716, SCC*mec* type 3A.1.4) (5). These 20 strains were accordingly classified SCC*mec* type 3A.1.4. In contrast, the *dcs* locus was not present with the 12 *spa* type 351 strains with the nontypeable ψSCC*mec*$_{16691}$ element. The latter group of strains all possessed the same *dru* type (dt9x), whereas three different *dru* types (dt10a, dt10g, and dt7f) were found among the 20 SCC*mec* 3A.1.4 strains (Table 1). Lastly, MIC testing indicated that all 34 strains were highly resistant to oxacillin ($\geq$256 μg/ml), with the exception of BK16672 (8 μg/ml) (Table 1).

SCC*mec* structure in BK16691 and BK16704. Complete SCC*mec* sequencing of BK16704 confirmed it as a type III SCC*mec* element, possessing *ccrAB3* in combination with the class A *mec* gene complex (IS*431*-*mecA*-*mecR1*-*mecI*) (Fig. 1C). Two SCC*mec* integration site sequences (ISS) containing direct repeats (DR) were likewise identified. A *dcs* locus was identified in the J3 region of this strain, in agreement with PCR results described above. The right chromosomal junction of the SCC*mec* element in BK16704 was also sequenced, revealing an IS*256* insertion within an open reading frame corresponding to SAR0088 of MRSA252, which is located downstream of SCC*mec* in the genome of MRSA252 (Fig. 1C) (15).

In contrast to BK16704, complete nucleotide sequencing of the SCC*mec* region of BK16691 (Fig. 1B) confirmed the presence of a class A *mec* gene complex with no downstream *ccr* loci, in agreement with real-time PCR SCC*mec* typing results (Table 1) (4). Surprisingly, BK16691 possesses a 24-kb deletion spanning most of the region between the *mec* gene complex and the right chromosomal junction. The deleted region originates in the *xylR* gene, just downstream of *mecI*, and terminates within a truncated homolog of MRSA252 SAR0088 (Fig. 1B). Moreover, the J3 region of BK16691 lacks the aforementioned *dcs* locus, while the IS*256* insertion in BK16691 is inverted relative to the one in BK16704. Only one ISS sequence was found within the *attB*SCC attachment site in *orfX*, and no DR were identified at either chromosomal junction. Since the SCC region of BK16691 does not possess *ccr* loci or flanking direct repeats, it cannot be designated an SCC*mec* element according to current nomenclature guidelines (20), despite possessing a *mec* gene complex and chromosomal integration

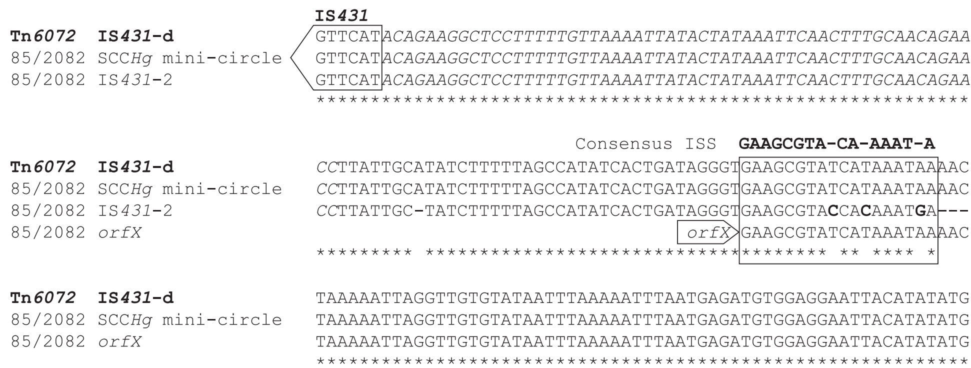

FIG. 2. Sequence comparison of the region upstream of IS*431*-d in the Tn*6072* element of BK16691 (shown in Fig. 1E) and the extrachromosomal circular form of SCC*Hg* from 85/2082, with the regions downstream of *orfX* and IS*431*-2 in the SCC*Hg* element of 85/2082 (shown in Fig. 1D). Asterisks denote nucleotide identity in two or more strains, while the consensus ISS region common to all three sequences is surrounded by a box. The ISS sequences for *orfX*, IS*431*-2, and IS*431*-d correspond to the underlined sequences in Fig. 1D and 1E, respectively. Flanking inverted repeat (IR) sequences for the IS*256* elements are displayed in italics, and the transcriptional directions of *orfX* and the IS*431* elements are indicated by boxed arrows.

site (*attB*SCC) identical to those of other SCC*mec* strains. Consequently, the entire SCC region of strain BK16691, from the ISS in *orfX* to the inverted copy of IS*256*, has tentatively been named ψSCCmec_{16691}.

Structure of Tn*6072*. As described above, SCC*mec* typing demonstrated that BK16704 and BK16691 both possess a *ccrC* locus, and an additional PCR confirmed the presence of an element closely resembling the J region of SCC*Hg*, minus the characteristic *mer* operon. However, complete sequencing from *orfX* to the right chromosomal junctions of both strains did not reveal any *ccrC* loci, suggesting that an SCC*Hg*-like fragment might be located elsewhere on the chromosome or within an extrachromosomal element. Inverse PCR was therefore used to determine the insertion junctions for the SCC*Hg* homolog in both BK16691 and BK16704. A single PCR product was obtained using primer pairs typeIII-R/IS431-r-1 (8.7 kb) and 3F2/3R2 (5.3 kb), corresponding to the 5′ and 3′ junctions, respectively (data not shown). The results of inverse PCR in both BK16691 and BK16704 also indicated that the SCC*Hg* homology is located in an open reading frame corresponding to MRSA252 SAR2700, within the 635-kb chromosomal replacement region previously described for ST239 (Fig. 1A) (33). The SAR2700 homolog is located 108 kb upstream from the origin of replication (*ori*), thereby confirming the insertion of an SCC*Hg*-like element outside the SCC*mec* region in BK16691 and BK16704. Surprisingly, complete nucleotide sequencing of the putative SCC*Hg* homolog from strain BK16691 revealed it to be a novel composite transposon, and it was accordingly named Tn*6072*.

The Tn*6072* element from strain BK16691 is illustrated in Fig. 1E, with its chromosomal location depicted in Fig. 1A. Tn*6072* is 29,422 bp in length, with an overall structure highly similar to the 5′ region of the SCC*Hg* element from 85/2082 (18). However, the chromosomal orientation of Tn*6072* is inverted relative to the full-length SCC*Hg* element in 85/2082 (Fig. 1A, D, and E). In strain BK16691, Tn*6072* is flanked by two IS*431* elements, one of which displaces the *orfX* locus found in previously described SCC*Hg* elements. Inspection of the junction sequences at both termini determined that each IS*431* element is also flanked by an 8-bp direct repeat (TTAT TTTG, shown as a reverse complement in Fig. 1E, CAAAA TAA) as a result of target site duplication following insertion. Moreover, a characteristic ISS sequence (GAAGCGTATCA TAAATAA) was identified near the downstream IS*431* element (IS*431*-d), as depicted in Fig. 1E. The 36-bp sequence between the ISS and IS*431*-d is nearly identical to the one between IS*431*-2 and DR-2 in the SCC*Hg* element of strain 85/2082, while the right contiguous sequence of ISS is identical to the one immediately downstream of *orfX* in 85/2082 (Fig. 2).

SCC*Hg* can be excised from the chromosome of 85/2082, as reported previously (5); in this study, we found that SCC*Hg* can also form extrachromosomal circles in 85/2082 (Table 1), using outward-oriented PCR with primer set 3F7/SCC*Hg*-R (see Table S1 in the supplemental material). Sequence analysis revealed that the outward PCR product of SCC*Hg* comprises both termini, with only one intervening copy of IS*431*. The sequence upstream of IS*431* in the SCC*Hg* minicircle is identical to that of IS*431*-d in Tn*6072* and includes the characteristic ISS sequence (Fig. 2). These results suggest that Tn*6072* may have originated from an IS*431*-flanked SCC*Hg* homolog containing the region from DR1 to DR2 in 85/2082, but without the *mer* operon and IS*431*-1 (Fig. 1D). Such an element may have been excised from the chromosomal ISS sites, forming a transpositionally active extrachromosomal circle in the same manner as the SCC*Hg* minicircle, and then integrated into the aforementioned SAR2700 homolog.

Recently, an extrachromosomal SCC*Hg* element was described in plasmid pTW20_1 from ST239 strain TW20, with IS*431* elements flanking both the plasmid and chromosomal SCC*Hg* homologs (16). Similar findings have been described elsewhere (1, 14), suggesting that IS*431*-mediated recombination may provide a hypothesis to explain the homoplasies inferred from the distribution of SCC*Hg* and other SCC components within the ST239 clonal group (36). Outward-directed PCR experiments indicate that Tn*6072* also forms extrachromosomal circles (Table 1), almost identical in structure to the SCC*Hg* minicircles described above, with both termini connected by a single copy of IS*431*. Tn*6072* is therefore a potentially active transposon, and integration of Tn*6072* into other genomic locations, including other *S. aureus* clonal back-

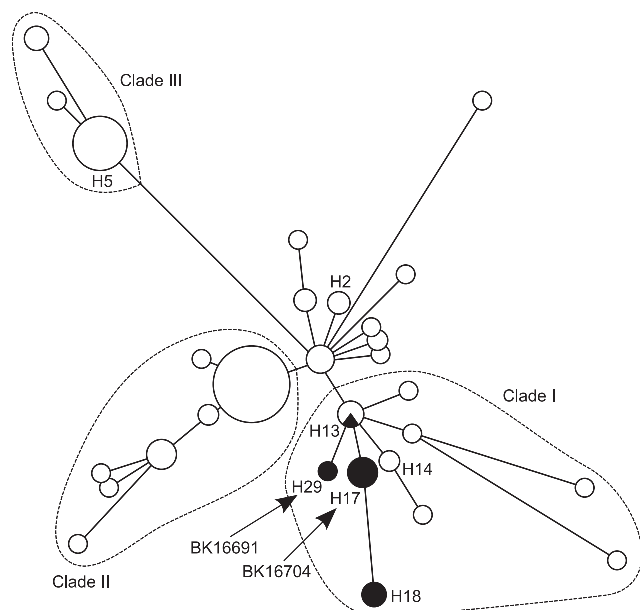

FIG. 3. Distribution of Tn*6072* within the ST239 clonal group. Maximum parsimony tree depicts the global population structure of this clonal group, as described previously (36). Each circle represents a distinct haplotype, defined by sequences at 32 chromosomal loci. Circle sizes represent the relative frequencies of individual haplotypes within a sample of previously characterized strains (36). Dark shading within circles denotes the presence of Tn*6072*, while the locations of Tn*6072*-bearing strains BK16691 and BK16704 are indicated by arrows. Haplotype H18 represents the location of Czech clone prototype strain 2HK, while haplotypes H2, H5, and H14 denote SCC*mec* prototype strains HDG2 (Portuguese clone, SCC*mec* IIIB), HU25 (Brazilian clone, SCC*mec* IIIA), and 85/2082 (SCC*mec* III), respectively. (Adapted from reference 36 with permission of the publisher.)

grounds and staphylococcal species, is possible. Consequently, screening for the presence of Tn*6072*-like elements in staphylococcal strains bearing SCC*Hg*-associated loci is advisable, since current SCC*mec* typing methods cannot differentiate between Tn*6072* and SCC*Hg* (4, 24, 31, 41).

Distribution of Tn*6072* and ψSCC*mec*$_{16691}$. An additional 19 Romanian strains were found with an SCC*mec* 3A.1.4 structure like that of BK16704, whereas another 11 Romanian strains possessed a truncated ψSCC*mec*$_{16691}$ element (Table 1). Subsequent screening of a geographically and temporally diverse collection of 111 strains from the ST239 clonal group (36) identified 11 additional strains carrying Tn*6072* elements, all integrated within the same chromosomal site as the Romanian strains (see Table S2 in the supplemental material). Comparative analysis suggested that the distribution of Tn*6072* is limited to a predominantly European clade of the ST239 clonal group (clade I, Fig. 3) which includes the previously described "Czech clone" (28). BK16691 comprises a novel haplotype (H29) within this lineage, whereas BK16704 bears the same haplotype (H17) consistent with Tn*6072*-positive strains from the Netherlands, Germany, Hungary, Russia, and Turkey (see Table S2). All but one of the non-Romanian strains possessed an SCC*mec* 3A.1.4 structure, whereas no additional strains with truncated ψSCC*mec*$_{16691}$-like elements were observed. These results demonstrate that Tn*6072* insertions have been present in European ST239 strains for at least 2 decades (as early as 1994), whereas ψSCC*mec*$_{16691}$ has been observed only with the Romanian collection analyzed in this study.

To our knowledge, the only other description of a *mec* gene complex with no accompanying *ccr* loci involves the putative "primordial" *mecIRA*$_m$ element described recently for plasmid pMCCL2 of *Macrococcus caseolyticus* strain JCSC5402 (2). ψSCC*mec*$_{16691}$ is therefore a novel example of a chromosomal *mecA*-bearing SCC element without *ccr* loci, which appears to have lost the capability for chromosomal excision (Table 1). All ψSCC*mec*$_{16691}$-bearing isolates used in this study were highly resistant to oxacillin (Table 1), consistent with carriage of *mecA* and the associated *mec* gene complex. Consequently, ψSCC*mec*$_{16691}$ may represent the smallest example to date (10.5 kb) of a genetic element conferring methicillin resistance in staphylococci. In addition, ψSCC*mec*$_{16691}$-bearing strains were isolated from more than one hospital on multiple occasions, suggesting that they remain viable in nosocomial environments despite losing most of the SCC*mec* III element. Further studies are required to compare the relative fitness levels of these strains with those of other ST239-MRSA-III clones. It remains to be seen if ψSCC*mec*$_{16691}$-bearing strains are still circulating in Romanian hospitals and whether they will spread beyond this setting. In conclusion, our findings provide additional evidence of the ongoing diversification of type III SCC*mec* elements within the ST239 clonal group, while further elucidating the evolutionary mechanisms of SCC elements.

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