

First Staphylococcal Cassette Chromosome *mec* Containing a *mecB*-Carrying Gene Complex Independent of Transposon Tn6045 in a *Macrococcus caseolyticus* Isolate from a Canine Infection

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A methicillin-resistant *mecB*-positive *Macrococcus caseolyticus* (strain KM45013) was isolated from the nares of a dog with rhinitis. It contained a novel 39-kb transposon-defective complete *mecB*-carrying staphylococcal cassette chromosome *mec* element (SCC*mec*_{KM45013}). SCC*mec*_{KM45013} contained 49 coding sequences (CDSs), was integrated at the 3' end of the chromosomal *orfX* gene, and was delimited at both ends by imperfect direct repeats functioning as integration site sequences (ISSs). SCC*mec*_{KM45013} presented two discontinuous regions of homology (SCC*mec* coverage of 35%) to the chromosomal and transposon Tn6045-associated SCC*mec*-like element of *M. caseolyticus* JCSC7096: (i) the *mec* gene complex (98.8% identity) and (ii) the *ccr*-carrying segment (91.8% identity). The *mec* gene complex, located at the right junction of the cassette, also carried the β -lactamase gene *blaZm* (*mecRm-mecIm-mecB-blaZm*). SCC*mec*_{KM45013} contained two cassette chromosome recombinase genes, *ccrAm2* and *ccrBm2*, which shared 94.3% and 96.6% DNA identity with those of the SCC*mec*-like element of JCSC7096 but shared less than 52% DNA identity with the staphylococcal *ccrAB* and *ccrC* genes. Three distinct extrachromosomal circularized elements (the entire SCC*mec*_{KM45013}, Ψ SCC*mec*_{KM45013} lacking the *ccr* genes, and SCC_{KM45013} lacking *mecB*) flanked by one ISS copy, as well as the chromosomal regions remaining after excision, were detected. An unconventional circularized structure carrying the *mecB* gene complex was associated with two extensive direct repeat regions, which enclosed two open reading frames (ORFs) (ORF46 and ORF51) flanking the chromosomal *mecB*-carrying gene complex. This study revealed *M. caseolyticus* as a potential disease-associated bacterium in dogs and also unveiled an SCC*mec* element carrying *mecB* not associated with Tn6045 in the genus *Macrococcus*.

The genus *Macrococcus* is composed of seven species of Gram-positive bacteria closely related to staphylococci, including *Macrococcus caseolyticus* (formerly identified as *Staphylococcus caseolyticus*) (1). Unlike staphylococci, macrococci do not usually cause human or animal diseases and are typically isolated from animal skin and food products, such as milk and meat (1, 2). The only association of *M. caseolyticus* with an infection was observed in abscesses from slaughtered lambs in 1992 (3). Even though *M. caseolyticus* is not primarily targeted by antibiotic treatment as an infectious agent, a few strains have acquired antibiotic resistance mechanisms identical or similar to those found in staphylococci, such as *cfr*-mediated multidrug resistance (4) and *mecB*-mediated methicillin resistance (5), respectively.

In staphylococci, methicillin resistance is caused by the synthesis of a modified penicillin binding protein (PBP2a) with low affinity to virtually all β -lactams. This protein is encoded by either the *mecA* or the *mecC* gene (6, 7), whose expression is often regulated by the presence of MecR1 (sensor/signal transducer *mecR1* gene) and MecI (*mec* transcription repressor *mecI* gene). These genes are arrayed in an operon designated the *mec* gene complex, which is located within the staphylococcal cassette chromosome *mec* (SCC*mec*) element. Cassette chromosome recombinases (Ccr), the second essential component of the SCC*mec* element, encoded by different allotypes of the *ccrAB* and *ccrC* genes, are responsible for site-specific integration and excision of the element at the integration site sequence (ISS) of SCC*mec* located at the 3' end of the chromosomal *orfX* gene. The combination of the different allotypes defines the *ccr* gene complex. SCC*mec* elements are flanked by characteristic direct repeats (DRs) containing the ISSs that define the transferable unit (8).

The *mecA* and *mecC* homologue *mecB* genes have been identified in *M. caseolyticus* in two plasmids as well as on a macrococcal chromosomal primordial form of the SCC*mec* element, designated a SCC*mec*-like element due to the location of the *mecB* gene complex (*mecR1m-mecIm-mecB-blaZm*) within a transposon-driven genetic element (5). In this element, transposon Tn6045 was shown to be responsible for the excision of the region peripheral to *mecB* (5). While the SCC*mec*-like element exhibited characteristic 18-bp DRs that potentially also enable spontaneous excisions of Ψ SCC*mec*₇₀₉₆ (SCC*mec* lacking the *ccr* genes) and SCC₇₀₉₆ (SCC lacking the *mecB* gene) as independent units in

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strain JCSC7096, the two *mecB*-containing plasmids lacked *ccr* genes (5).

In 2013, a 9-year-old male neutered Bernese mountain dog was presented several times to a veterinary practice with coughing and signs of rhinitis, including sneezing, nasal and ocular discharge, and swelling of the tonsils and regional lymph nodes. Bacteriological analysis of a nasal sample revealed massive growth of hemolytic Gram-positive cocci which exhibited resistance to penicillin as well as to oxacillin and ceftiofur, which are used for the prediction of the *mec* genes in staphylococci (9, 10). This prompted us to further identify this bacterium and characterize the genetic background of the β -lactam resistance, revealing a novel *mecB*-containing SCC*mec* element not associated with a transposon in a hemolytic *Macrococcus*.

MATERIALS AND METHODS

Bacterial identification. Strain KM45013, obtained from our diagnostic unit, was identified as *M. caseolyticus* by 16S rRNA gene PCR amplification of cell lysates and sequence analysis (11). *M. caseolyticus* was routinely grown on either Trypticase soy agar containing 5% sheep blood (TSA-S; Becton, Dickinson and Company, Franklin Lakes, NJ) or in LB broth at 37°C with aeration.

Determination of antimicrobial resistance profile. MICs were measured in Mueller-Hinton broth by the microdilution technique using custom-made Sensititre susceptibility plates (NLEUST; Trek Diagnostics Systems, East Grinstead, United Kingdom) and following the Clinical and Laboratory Standards Institute (CLSI) guidelines (9). The production of β -lactamase was tested on nitrocefin dry slides (Becton, Dickinson and Company).

DNA extraction and determination of *mecB* location. Genomic DNA was isolated using a phenol-chloroform method with the following modifications for improved cell lysis (12). Five milliliters of overnight culture in LB broth was centrifuged for 10 min at 15,000 rpm, and cells were resuspended in 100 μ l Tris-EDTA buffer containing 2 mg/ml lysozyme and 0.5 mg/ml lysostaphin and incubated for 20 min at 37°C. Plasmid DNA was obtained by phenol-chloroform extraction as described by Anderson and McKay (13), also including a lysis step with lysozyme and lysostaphin. The integrity and concentration of the extracted DNA were assessed by agarose gel electrophoresis and spectrophotometric measurement (Qubit; Invitrogen), respectively.

Southern blot hybridization was performed on both genomic and plasmid DNA of strain KM45013 using a digoxigenin-labeled *mecB* probe obtained using primers *mecB*-Fw and *mecB*-Rv (see Table S1 in the supplemental material) following the manufacturer's protocol (Roche, Switzerland). Hybridization signals were visualized on the membrane using a LAS-3000 imaging system (Fujifilm), anti-DIG-AP Fab fragments, and the CDP-Star chemiluminescence substrate (Roche, Switzerland). Genomic and plasmid DNA of *M. caseolyticus* strain JCSC5402 (5) served as a positive control for the determination of the location of the *mecB* gene.

Whole-genome sequencing, assembly, and annotation of the novel SCC*mec* element. High-throughput whole-genome sequencing (WGS) of *M. caseolyticus* KM45013 was performed with Roche 454 GS Titanium chemistry according to the manufacturer's standard protocols (GS Junior System; Roche Diagnostics, Switzerland). Resulting contigs were analyzed for the presence of characteristic genes of the SCC*mec*-like element (*mecB*, *mecM*, *mecRm*, *ccrAm*, *ccrBm*) and surrounding chromosomal segments (*orfX*, transposase gene of Tn6045, MCCL_0033, and MCCL_0034) of *M. caseolyticus* JCSC7096 (5) (GenBank accession no. AB498756) using a BLAST search (<http://www.ncbi.nlm.nih.gov>) and specific PCRs (see Table S1 in the supplemental material). Sanger sequencing of PCR products was performed using BigDye Terminator cycle sequencing and an ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA) to fill the gaps between SCC*mec*-containing contigs and to define their specific

orientations. Open reading frames (ORFs) were defined with the help of Prodigal (14), GeneMark (15), and ORF Finder (NCBI) software. Only those with plausible ribosomal binding sites were considered. Annotation of the ORFs was performed by BLAST homology, and motif analysis of the translated reading frames was performed against the ScanProsite database (16), the Pfam database (only significant matches were considered for annotation) (17), and the National Center for Biotechnology Information conserved domain database (CDD) (18).

Detection of ISSs was achieved by searching for the consensus sequence GA[A/G][TCG/ATG]TATCATAAGTGA (positions with possible alternative nucleotides are indicated within square brackets, and the possible nucleotides are separated by slashes) in all contigs. Characteristic inverted repeats (IRs), typically found after element insertion catalyzed by Ccrs, were also examined directly upstream and downstream of the ISSs.

Characterization of the chromosomal SCC*mec* structure and detection of spontaneous extrachromosomal excisions. Primers designed for the detection of the *mec* and *ccr* gene complexes of SCC*mec*_{KM45013} and the SCC*mec*-like element of JCSC7096 are shown in Table S1 in the supplemental material. In addition, primers used for identification of the chromosomal *orfX* gene of *M. caseolyticus* strain KM45013, as well as the conserved reading frames MCCL_0033 and MCCL_0034 located at the 3' end of *orfX* in the previously described *mecB*-carrying macrococcal strains (5), are also described (see Table S1). BamHI and BglII restriction analysis of different regions of the SCC*mec* element amplified by long-range PCR (GoTaq long PCR master mix; Promega) was performed for assembling and scaffolding confirmation (see Table S1). The presence of potential circular intermediates (CIs) of SCC*mec* segments delimited by ISS sequences, as well as other possible extrachromosomal circularized structures, was tested by specific PCR and sequenced using primers reading outward from the ISSs or a corresponding region (for an ISS-independent excision event). The chromosomal region, where segment excision was expected to have occurred, was also amplified by PCR using adapted elongation times and was sequenced (see Table S1).

Phylogenetic relationship of *ccr* genes and *blaZ*-containing *mec* gene complexes. The phylogenetic relationships of one representative of each type of *ccr* gene (19) and the *mec* gene complexes containing the *blaZ* gene (5–7, 20) were investigated by the construction of a maximum likelihood phylogenetic tree using the SeaView program, version 4.4.0 (21), with nucleotide sequences deposited in the ENA/GenBank databases. Sequences were aligned using MUSCLE, and the trees were built with PhyML using a general time-reversible (GTR) model.

Nucleotide sequence accession number. The 41,563-bp nucleotide sequence of *M. caseolyticus* strain KM45013 containing the complete 38,941-bp SCC*mec*_{KM45013} and its 602-bp upstream and 2,020-bp downstream chromosomal regions has been deposited in the GenBank/ENA/DBJ databases under the accession number HG970732.

RESULTS AND DISCUSSION

Identification of *M. caseolyticus* KM45013. Strain KM45013 was identified as *M. caseolyticus* based on the 16S rRNA gene sequence, which exhibited 99.7% nucleotide identity with that of *M. caseolyticus* type strain ATCC 13548^T and *M. caseolyticus* JCSC5402, the only macrococcal strain whose genome has been completely sequenced (2). Decreased susceptibility to β -lactams was confirmed by the determination of the MICs for penicillin (MIC, >2 μ g/ml), oxacillin (MIC, >8 μ g/ml), and ceftiofur (MIC, >8 μ g/ml). *M. caseolyticus* KM45013 differed from other members of *Macrococcus* species by the formation of a complete hemolysis on a sheep blood plate. Hemolysins are known virulence factors in staphylococci which have been associated with different types of infections (22, 23). Whether the hemolytic property of strain KM45013, which is so far unique among *Macrococcus caseolyticus*, represents a virulence factor in dogs still remains to be clarified. Nevertheless, the massive presence of *M. caseolyticus* in the nasal sample may be

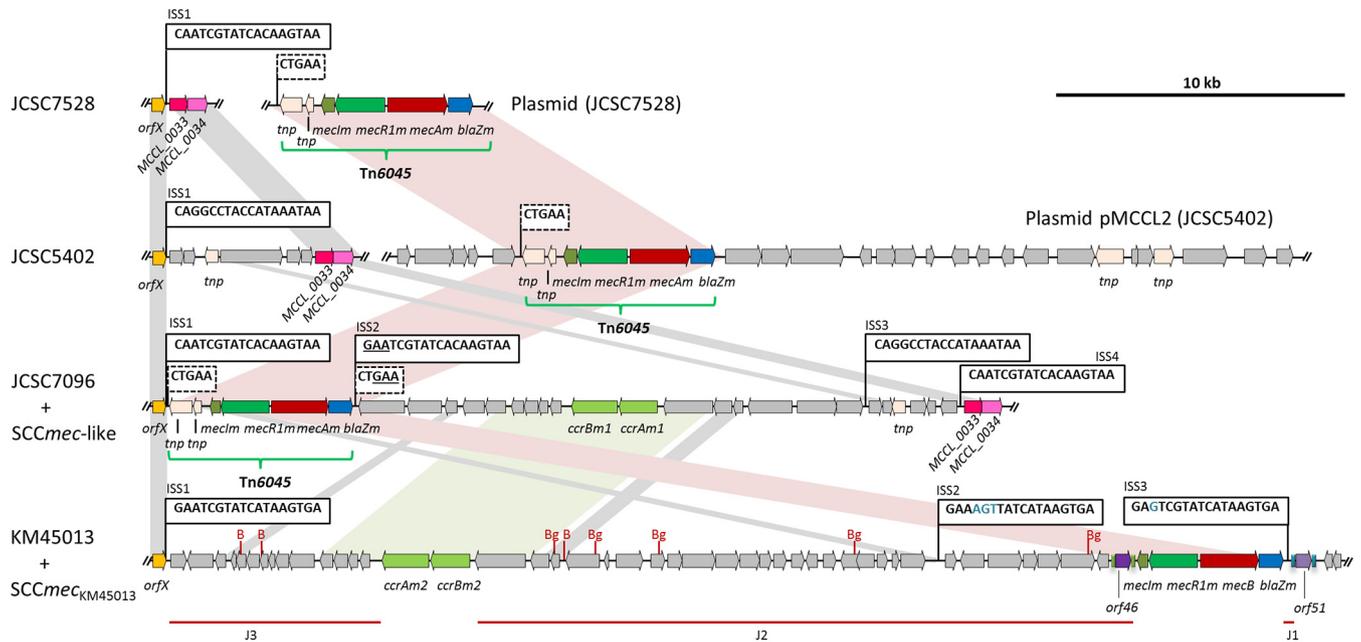


FIG 1 Schematic presentation of the *orfX* downstream region in the *M. caseolyticus* KM45013 chromosome, including the novel SCCmec_{KM45013} (ENA accession no. HG970732), and a comparison with the previous *mecB*-carrying genetic structures detected in macrococci: *M. caseolyticus* strain JCSC7528 (GenBank accession no. AB498758), JCSC5402 (GenBank accession no. AP009486), and JCSC7096 (GenBank accession no. AB498756) and their correspondent chromosomal *orfX* downstream regions (GenBank accession no. AB498757, AP009484, and AB498756, respectively). The arrows indicate the extent and direction of transcription of the open reading frames. All annotated regions of *M. caseolyticus* KM45013 are colored as follows: yellow (*orfX*), pale pink (*tnp*), pale green (*ccrAm*, *ccrBm*), dark green (*mecIm*), green (*mecRm*), red (*mecB*, *mecAm*), blue (*blaZm*), purple (*orf46*), pale purple (*orf51*), magenta (MCCL_0033) and pink (MCCL_0034). The different integration site sequences (ISSs) for SCC (ISS1 to ISS4) are shown within boxes. The direct repeats (CTGAA) of transposon Tn6045 in strains JCSC7528, JCSC5402, and JCSC7096 are shown within dashed boxes. Shaded areas indicate regions with more than 84% nucleotide sequence identity. Tn6045 in strains JCSC7528, JCSC5402, and JCSC7096 (green horizontal curly brackets) and the joining regions J1 to J3 (red horizontal bar) in KM45013 are also shown. B and Bg indicate the BamHI and BglII restriction sites, respectively, within SCCmec_{KM45013}. A size scale in kb is displayed in the upper right-hand corner.

indicative of an association with the disease, even if other causes cannot be excluded. Since this discovery, 2 additional hemolytic *M. caseolyticus* isolates were obtained in our laboratory from 2 dogs diagnosed with otitis and dermatitis, indicating that more attention should be paid to this microorganism.

Characterization of the novel SCCmec_{KM45013} element and comparison with other *mecB*-carrying elements. WGS of strain KM45013 resulted in 92,893 filter reads and coverage equivalent to 14.3 \times . Sequence reads were *de novo* assembled using Newbler 2.6 (Roche) at the Vital-IT Center for High-Performance Computing at the Swiss Institute of Bioinformatics (<http://www.vital-it.ch>), yielding 116 contigs (86 contigs >500 bp) with an N_{50} (length-weighted median) of 46,794 bp, a mean contig size of 19,287 bp, a maximum contig length of 227,824 bp, and a contig sum of 2,275,932 bp. WGS of *M. caseolyticus* KM45013 as well as Southern blot hybridization experiments allowed the identification and characterization of the novel 38,941-bp chromosomally located SCCmec_{KM45013} element. A total of 49 coding sequences (CDSs) were identified in SCCmec_{KM45013}. The genome of strain KM45013 presented a GC content of 37.0%, while that of SCCmec_{KM45013} was 31.5%, suggesting that SCCmec_{KM45013} was integrated as an exogenous element. SCCmec_{KM45013} was located at the 3' end of the chromosomal *orfX* gene and was demarcated at both extremities by DRs with the following ISSs: 5'-GAATCGTATCATAAGTGA-3' (ISS1) and 5'-GAGTCGTATCATAAGTGA-3' (ISS3) (Fig. 1). An additional ISS, ISS2 (5'-GAAAGTTATCATAAGTGA-3'), was

detected 26,883 bp downstream of the *orfX* gene and 6,842 bp upstream of the *mecB* complex. Imperfect inverted repeats (IRs), which have been shown to play a role in the excision but not the integration of SCCmec (8), were detected adjacent to the three ISS elements (data not shown). These IRs had similar sequences to those detected in staphylococcal SCCmec elements and in the SCCmec-like element of *M. caseolyticus* JCSC7096 (5, 24).

SCCmec_{KM45013} also shared the highest identity with the SCCmec-like element of JCSC7096 (BLAST hit of 35% query cover and 92% sequence identity) (Fig. 1). The high nucleotide identity value was mainly due to the presence of two discontinuous regions: the *mec* gene complex and a *ccr*-carrying segment. The *mec* gene complex shared 98.8% DNA identity with the corresponding segments of the three *mecB*-carrying macrococcal strains, and the *ccr*-carrying region shared 91.8% DNA identity with that detected in the SCCmec-like element of JCSC7096 (5). The *ccr* genes were absent in the *mecB*-carrying plasmids of *M. caseolyticus* strains JCSC5402 and JCSC7528, which instead contained transposon-associated transfer mechanisms (5) (Fig. 1).

The *mec* gene complex was located at the right-end junction of the cassette and carried a functionally active β -lactamase resistance gene, *blaZm*, as determined by the nitrocefin test. No other β -lactamase gene was detected in the remaining genomic sequence. In strains JCSC7096, JCSC5402, and JCSC7528, the *mec* gene complex (*mecR1m-mecIm-mecB-blaZm*) formed part of transposon Tn6045, which contains two adjacent transposase genes immediately upstream of the *mec* gene complex and is

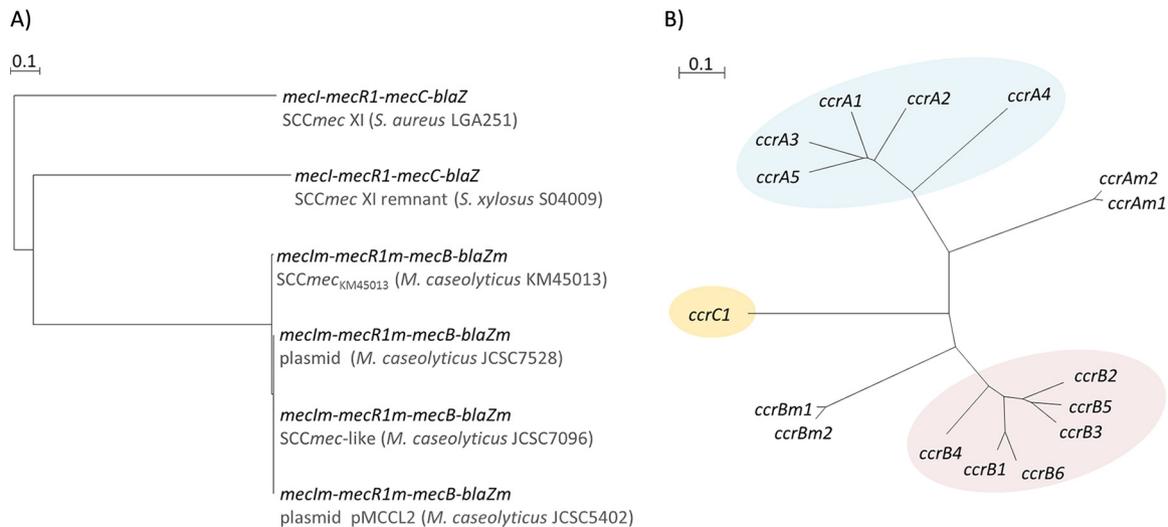


FIG 2 Phylogenetic relationships of the *mec* gene complex and serine recombinase *ccr* genes. Bar length indicates the number of substitutions per site. (A) Phylogenetic relationships of the *mec* gene complex (*mecI-mecR1-mecC-blaZ*) detected in SCCmec XI of *S. aureus* strain LGA251 (GenBank accession no. [FR821779](#)) and SCCmec XI remnant of *S. xylosois* S04009 (GenBank accession no. [HE993884](#)) and those (*mecI-mecR1m-mecB-blaZm* [formerly *mecAm*]-*blaZm*) detected in *M. caseolyticus* strains JCSC7528 (GenBank accession no. [AB498758](#)), JCSC5402 (GenBank accession no. [AP009486](#)), and JCSC7096 (GenBank accession no. [AB498756](#)). (B) Phylogenetic relationship of the *ccr* genes currently described in macrococci (*ccrAm1* and *ccrBm1* [SCCmec-like element of *M. caseolyticus* strain JCSC7096, GenBank accession no. [AB498756](#)] and *ccrBm2* [SCCmec_{KM45013} of *M. caseolyticus* strain KM45013, GenBank accession no. [HG970732](#)]) and one representative staphylococcal *ccr* per type (*ccrA1* and *ccrB1* [SCCmec I of *S. aureus* strain NCTC10442, GenBank accession no. [AB033763](#)], *ccrA2* and *ccrB2* [SCCmec II of *S. aureus* strain N315, GenBank accession no. [BA000018](#)], *ccrA3* and *ccrB3* [SCCmec III of *S. aureus* strain 85/2082, GenBank accession no. [AB037671](#)], *ccrA4* and *ccrB4* [SCCmec VI of *S. aureus* strain HDE288, GenBank accession no. [AF411935](#)], *ccrA5* and *ccrB5* [SCCmec VII-241 of *S. pseudintermedius* strain KM241, GenBank accession no. [AM904731](#)], *ccrB6* [SCCmec X of *S. aureus* strain JCSC6945, GenBank accession no. [AB505630](#)], and *ccrC1* [SCCmec VII of *S. aureus* strain JCSC6082, GenBank accession no. [AB373032](#)]).

flanked by a set of short DRs (5'-CTGAA-3'), presumably generated by transposon integration (5). Neither transposons nor transposase genes were detected in the entire SCCmec_{KM45013} element. In contrast, the *mec* gene complex was flanked by two 775-bp to 777-bp duplicated sequence fragments that shared 93.2% identity. This duplicated DNA fragment comprised two CDSs (ORF46, ORF51), with 94.6% amino acid identity, and their flanking regions (121 bp upstream of both genes in addition to 81 and 83 bp downstream of *orf46* and *orf51*, respectively). *orf51* was located downstream of ISS3 and was therefore outside SCCmec_{KM45013}. Analysis of the putative functional domains of the ORF46 and ORF51 proteins revealed a helix-turn-helix (HTH) domain of the XRE family (CDD accession no. cd00093) and a HipB domain profile (CDD accession no. COG1396), both associated with transcriptional regulators. The transposase of Tn6045 (GenPept accession no. [BAI83381](#)) also exhibits an HTH-like domain of the family HTH_21 (CDD accession no. pfam13276) but has two additional integrase domains of the rve superfamily (rve CDD accession no. pfam00665 and rve_3 CDD accession no. pfam13683). These domains, which are necessary for transposition, were not detected in ORF46 or ORF51. The presence of a *mecB* gene complex independent of Tn6045 and the detection of an entire SCCmec are novel characteristics for this bacterial genus. The high sequence and structure similarity levels between the *mecB* complex of JCSC7096 and KM45013, both driven by a completely different circularization machinery (see below), illustrate different possible mechanisms for the appearance of SCCmec in this species.

The *ccr*-carrying region was located 5.3 kb downstream of *orfX* and was comprised of five additional CDSs, three of which

encoded proteins with domains (pfam07799 [DUF1643], pfam06124 [DUG960], and pfam04002 [PF04002]) that were also detected in the *mecC*-containing staphylococcal SCCmec XI (6).

In addition to the *mec* gene complex and the *ccr*-carrying region, SCCmec_{KM45013} contained three joining (J) regions (Fig. 1). Two of them (J3 and J2) carried additional CDSs, encoding hypothetical proteins for the vast majority but also proteins with putative metabolic functions (GenBank accession no. [HG970732](#)). Neither additional antimicrobial nor heavy-metal resistance genes were detected within SCCmec_{KM45013}.

Variable regions downstream of the integration site of SCCmec have been previously observed in staphylococci (25, 26). In *M. caseolyticus* strains JCSC7096, JCSC5402, and JCSC7528, the *orfX* downstream region contains two adjacent conserved ORFs, named MCCL_0033 and MCCL_0034, coding for proteins of unknown function. These ORFs were not detected downstream of SCCmec_{KM45013} nor in the entire KM45013 genome. Instead, *orf51* was present, sharing 72% identity with a sequence downstream of *orfX* of the methicillin-resistant *Staphylococcus pseudintermedius* strain 57395 (comprising a CDS named *mrsP-29*) (24) and of methicillin-susceptible *S. pseudintermedius* ED99 and HKU10-03 (27, 28), all encoding putative transcriptional regulators.

Phylogenetic analysis of *ccr* and *mec* gene complexes. The *mecB* complex of SCCmec_{KM45013} presented structural similarities to the *mecC* complexes detected in SCCmec elements of *Staphylococcus aureus* LGA251 (*mecI-mecR1-mecC-blaZ*) (6) and of *Staphylococcus xylosois* S04009 (*mecI-mecR1-mecC1-blaZ*) (20), both belonging to the class E *mec* complex. Phylogenetic comparison of the *mec* gene complex from macrococci with the class E *mec* gene complexes revealed 57.4% nucleotide identity to that of *S. xylosois*

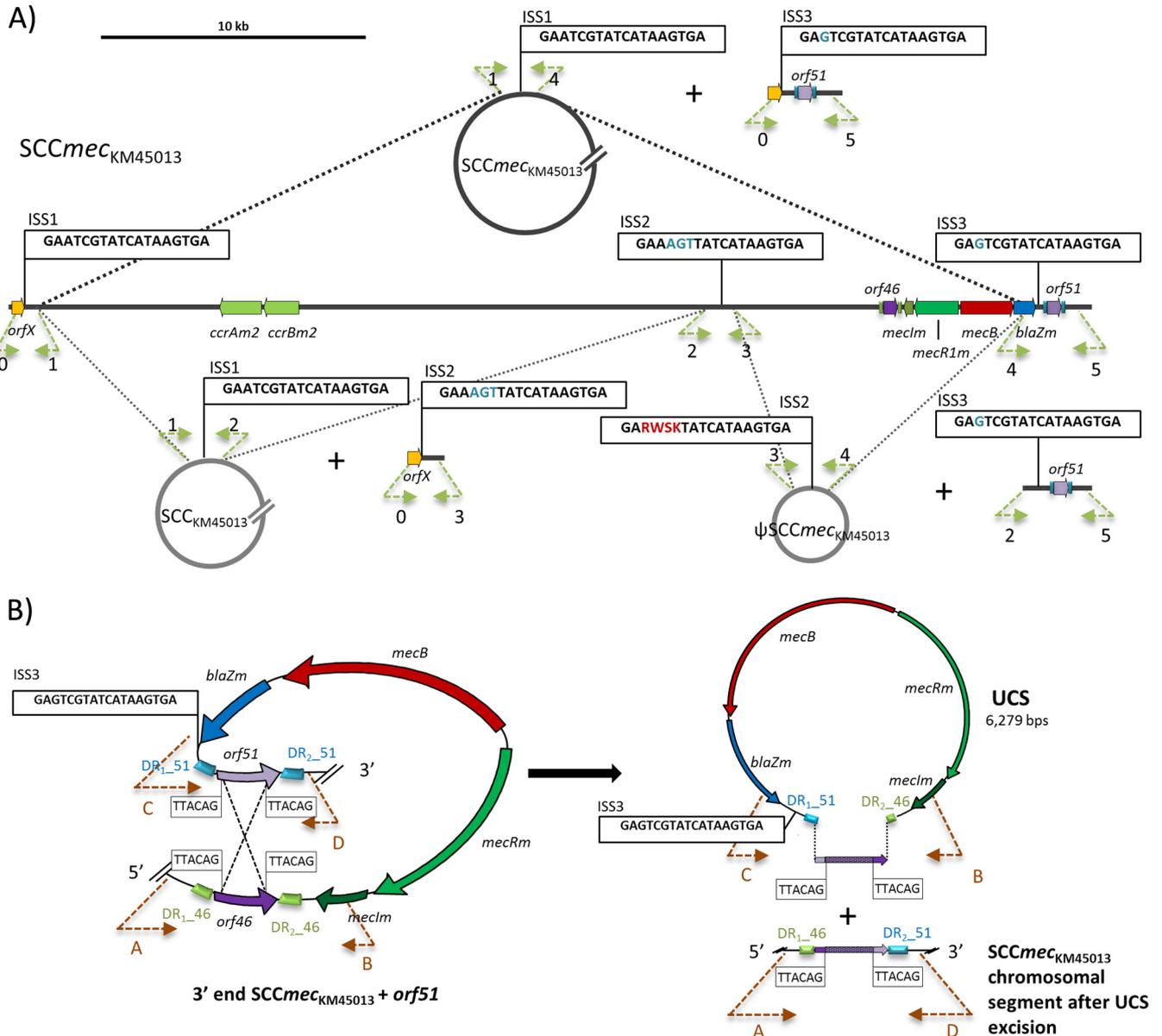


FIG 3 Graphical representation of the spontaneous circular chromosomal excisions detected in SCCmec_{KM45013}. (A) Display of the circular intermediates (CIs) designated to those with integration site sequences (ISSs) as delimiting region (SCCmec_{KM45013}, SCC_{KM45013}, and ΨSCCmec_{KM45013}), and the resulting chromosomal regions after spontaneous loss. The arrows indicate the extent and direction of transcription of *orfX*, *orf46*, and *orf51*, the site-specific recombinase genes (*ccrAm2*, *ccrBm2*), and the genes comprising the *mecB* operon (*meclm*, *mecRm*, *mecB* and *blaZm*). ISS1 to ISS3 are boxed. Bases in blue indicate divergences from ISS1, while letters in red indicate the presence of double peaks (Sanger sequencing) in the sequence chromatograms: R (G or A), W (A or T), S (G or C), and K (T or G). A size scale in kb is displayed in the upper left-hand corner. (B) Spontaneous chromosomal excision of an unconventional circularizable structure (UCS) carrying the *mecB* complex. The left panel shows the potential homologous recombination event between *orf46* and *orf51* in *M. caseolyticus* strain KM45013. The right panel shows the resulting UCS and the chromosomal region after excision. The recombined area is colored in purple with white dots. The imperfect direct repeats (DR₁, DR₂) flanking *orf46* and *orf51* are indicated as blue (DR_{1_51}) or green (DR_{2_46}) blocks. The suggested recombination sites (5'-TTACAG-3') and the ISS3 are indicated within black boxes. Primers used to detect the different circularized elements and the excision sites are indicated as dashed arrows, with the arrowhead indicating the direction of amplification. They are named 0 to 5 for the named CIs and A to D for the designated UCS. See Table S1 in the supplemental material for nomenclature.

and 56.8% to that of *S. aureus* (Fig. 2A). However, since the current nomenclature of the International Working Group on Staphylococcal Cassette Chromosome elements (IWG-SCC) is set for staphylococcal species, in particular for *S. aureus*, this *mecB* complex was not assigned to a specific class (19).

Integration and excision of SCCmec at the *orfX* gene is mediated by CcrAB or CcrC, which are responsible for catalyzing DNA

cleavage, strand exchange, and recombination between the two attachment sites, one within the SCC element (*attSCC*) and the other on the bacterial chromosome (*attB*) (8). The *ccr* genes detected in SCCmec_{KM45013}, showed 94.3% and 95.6% identity with the *ccrAm1* and *ccrBm1* genes, respectively, from the SCCmec-like element of *M. caseolyticus* JCSC7096 and were designated *ccrAm2* and *ccrBm2* according to the nomenclature first described by Tsu-

bakishita et al. (5) with the agreement of the members of the IWG-SCC (see reference 19 for a list of the members). The *ccrAm2* and *ccrBm2* genes showed the closest identity to the staphylococcal *ccr* genes from methicillin-resistant *S. aureus* strain HDE288 (GenBank accession no. AF411935), with an overall nucleotide identity of 51.6% to *ccrA4* and 47.3% to *ccrB4*, respectively (Fig. 2B). Phylogenetic comparative analysis of the *ccr* genes from the macrococcal SCC*mec* elements with the other *ccr* types revealed that the macrococcal *ccr* genes formed two separate branches outside the staphylococcal *ccrA*, *ccrB*, and *ccrC* clades (Fig. 2B).

Analysis of spontaneous chromosomal excision of different SCC*mec*_{KM45013} element units. PCR and sequence analysis detected four distinct extrachromosomal CIs, three of them carrying one ISS copy as joining regions, which is characteristic of Ccr-mediated excision. These three ISS-associated CIs have been named with the same nomenclature as that described for the SCC*mec*-like element of JCSC7096 (5): (i) SCC*mec*_{KM45013} (entire cassette), (ii) SCC_{KM45013} (SCC lacking the *mec* gene) (26.9 kbp, 37 CDSs, GC content of 32.7%), and (iii) Ψ SCC*mec*_{KM45013} (SCC*mec* lacking the *ccr* genes) (12 kbp, 12 CDSs, GC content of 29%) (Fig. 3A). This excision ability was also observed for the SCC*mec*-like element of JCSC7096 (5), indicating a high functional activity of the *ccrABm* gene complex. In addition, all corresponding chromosomal segments remaining after excision were detected (Fig. 3).

The fourth CI (6,279 bp, GC content of 28.1%) consisted of the *mec* gene complex (*mecR1m-mecIm-mecB-blaZm*) joined by one recombinant copy of the putative transcriptional regulator genes *orf46* and *orf51* (Fig. 3B). The GC content of this CI was remarkably lower than that in the genome of *M. caseolyticus* KM45013 (37.0%), indicating that it probably originated from another bacterial species with a lower GC content. Additionally, the presence of long DRs as a joining region instead of ISSs suggested a *ccrABm2*-independent mechanism for excision, categorizing this CI as an unconventional circularized structure (UCS). UCSs have been recently described as particular genetic structures, mostly carrying antimicrobial resistance determinants (29) which, despite the lack of their own recombinase genes, are able to be excised in circular forms thanks to extensive flanking DRs (29). Moreover, UCSs are frequently carried by conventional mobile genetic elements. Mobilization via site-specific recombination and usage of host *trans*-acting functions has been suggested for UCSs; however, an active role of the *ccrABm2* genes in the excision of this UCS cannot be excluded.

Nucleotide sequence alignment of the recombinant region located in the UCS element, the recombinant copy that remains in the chromosome after excision, and the individual *orf46* and *orf51* genes revealed that the CDS located in the UCS and the one that remained in the chromosome after excision resulted from a recombination event between *orf46* and *orf51*, with a 6-bp sequence DR (5'-TTACAG-3') present at the 5' and 3' ends of both CDSs as a presumptive homologous recombination site (see Fig. S1 in the supplemental material). Both extensive repeated regions on each side of *mecB* may still play a role in the UCS integration/excision. Additionally, this UCS contained ISS3 (Fig. 3B) and thus retained the potential to be integrated by Ccrs.

In conclusion, a *mecB*-carrying SCC*mec* element was discovered in a clinical hemolytic *M. caseolyticus* strain of canine origin. The *mecB* gene complex was not associated with transposases of Tn6045, revealing for the first time a true SCC*mec* element in

Macrocococcus. The high sequence and structure similarity between Tn6045 and the *mecB* complex of KM45013 within two structurally different elements gives new insight into the acquisition of *mecB* and the birth of SCC*mec* in *Macrocococcus*. The detection of several excised circularized elements may also contribute to the further diversification of SCC*mec* elements in *M. caseolyticus*. This study underlines the role of commensal bacteria both as potential opportunistic animal pathogens and as reservoirs for novel and primordial forms of SCC*mec* with high potential genomic plasticity.

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