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 (SCCmecKM45013). SCCmecKM45013 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). SCCmecKM45013 presented two discontinuous regions of homology (SCCmec coverage of 35%) to the chromosomal and transposon Tn6045-associated SCCmec-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-ccrAB-mecB-blaZm). SCCmecKM45013 contained two cassette chromosome recombinase genes, ccrAm2 and ccrBm2, which shared 94.3% and 96.6% DNA identity with those of the SCCmec-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 SCCmecKM45013; SCCmecKM45013 lacking the ccr genes, and SCCKM45013 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 SCCmec 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, macroccii 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 (SCCmec) element. Cassette chromosome recombinases (Ccr), the second essential component of the SCCmec 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 SCCmec located at the 3′ end of the chromosomal orfX gene. The combination of the different allotypes defines the ccr gene complex. SCCmec elements are flanked by characteristic direct repeats (DRs) containing the ISSs that define the transferable unit (8).
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. Bacteriologic analysis of a nasal sample revealed massive growth of hemolytic Gram-positive cocci which exhibited resistance to penicillin as well as to oxacillin and cefoxitin, 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 SCCmec 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 hemolysins was tested on nitrocefin dry slides (Becton, Dickinson and Company). The presence of potential circular intermediates (CIs) of SCCmec segments delimited by ISS sequences, as well as other possible extrachromosomal circularized structures, was tested by specific PCR and sequencing using primers reading outward from the ISS 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 using 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 SCCmec\textsubscript{M40513} and its 602-bp upstream and 2,020-bp downstream chromosomal regions has been deposited in the GenBank/ENA/DDJB 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\textsuperscript{T} and M. caseolyticus JCSC5402, the only macrococcus 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 μg/ml), oxacillin (MIC, >8 μg/ml), and cefoxitin (MIC, >8 μg/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
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 element and comparison with other mecB-carrying elements.** WGS of strain KM45013 resulted in 92,893 filter reads and coverage equivalent to 14.3×. 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 N50 (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 strain KM45013 was 31.5%, suggesting that SCCmecKM45013 was integrated as an exogenous element. SCCmecKM45013 was located at the 3′ end of the chromosomal orfX gene and was demarcated at both extremities by IRs with the following ISSs: 5′-GAACTGATATCATAGTGA-3′ (ISS1) and 5′-GAGTCTGATATCATAGTGA-3′ (ISS5) (Fig. 1). An additional ISS, ISS2 (5′-GAAAGTTATCATAGTGA-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).

SCCmecKM45013 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-mecI-mecB-blaZm) formed part of transposon Tn6045, which contains two adjacent transposase genes immediately upstream of the mec gene complex and is

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**FIG 1** Schematic presentation of the orfX downstream region in the *M. caseolyticus* KM45013 chromosome, including the novel SCCmecKM45013 (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 (trp), pale green (ccrAm, ccrBm), dark green (mecAm), green (mecBm), red (mecB, mecam), blue (blaZm), purple (orf651), 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. Shadowed 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 SCCmecKM45013. A size scale in kb is displayed in the upper right-hand corner.
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 SCCmecKM45013 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 hypothetical proteins for the vast majority but also proteins with putative metabolic functions (GenBank accession no. HG970732).

Neither antimicrobial nor heavy-metal resistance genes were detected within SCCmecKM45013.

Variable regions downstream of the integration site of SCCmec have been previously observed in staphylococci (25, 26). In M. caseolyticus strains JCS7096, JCS5402, and JCS7528, 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 SCCmecKM45013 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 mrs-29) (24) and of methicillin-susceptible S. pseudintermedius ED99 and HKU10-03 (27, 28), all encoding putative transcriptional regulators.

Phylogenetic analysis of orfX and mec gene complexes. The mecB complex of SCCmecKM45013 presented structural similarities to the mecC complexes detected in SCCmec elements of Staphylococcus aureus LGA251 (mec-mecR1-mecC-blaZ) (6) and of Staphylococcus xylosus S04009 (mec-mecR1-mecC-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. xylosus.

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 (mec-mecR1-mecC-blaZ) detected in SCCmec XI of S. aureus strain LGA251 (GenBank accession no. FR821779) and SCCmec XI remnant of S. xylosus S04009 (GenBank accession no. HE993884) and those (mec-mecR1-mecB [formerly mecAm]-blaZm) detected in M. caseolyticus strains JCS7528 (GenBank accession no. AB498758), JCS5402 (GenBank accession no. AP009486), and JCS7096 (GenBank accession no. AB498756). (B) Phylogenetic relationship of the ccr genes currently described in macrococci (ccrA1 and ccrBm1 [SCCmec-like element of M. caseolyticus strain JCS7096, GenBank accession no. AB498756]) and ccrAm2 and ccrBm2 [SCCmecKM45013 of M. caseolyticus strain KM45013, GenBank accession no. HG970732]) and one representative staphylococcal ccr per type (ccrA1 and ccrB1 [SCCmec of S. aureus strain NCTC10442, GenBank accession no. AB033763], ccrA2 and ccrB2 [SCCmec II of S. aureus strain N315, GenBank accession no. BA0000018], ccrA3 and ccrB3 [SCCmec III of S. aureus strain 852082, 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 JCS6945, GenBank accession no. AB505630], and ccrCI [SCCmec VII of S. aureus strain JCS6682, GenBank accession no. AB373032]).
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 SCCmecKM45013 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-
bakushita 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 macrococcoci SCCmec elements with the other ccr types revealed that the macrococal ccr genes formed two separate branches outside the staphylococcal ccrA, ccrB, and ccrC clades (Fig. 2B).

Analysis of spontaneous chromosomal excision of different SCCmecKm45013 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 SCCmec-like element of JCSC7096 (5): (i) SCCmecKm45013 (entire cassette), (ii) SCCKm45013 (SCC lacking the mec gene) (26.9 kbp, 37 CDSs, GC content of 32.7%), and (iii) cSCCmecKm45013 (SCCmec lacking the ccr genes) (12 kbp, 12 CDSs, GC content of 29%) (Fig. 3A). This excision ability was also observed for the SCCmec-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 recombinated copy of the putative transcriptional regulator genes orf66 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 recombined region located in the UCS element, the recombinated copy that remains 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 SCCmec 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 SCCmec element in Macrooccus. 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 SCCmec in Macrooccus. The detection of several excised circularized elements may also contribute to the further diversification of SCCmec 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 SCCmec with high potential genomic plasticity.

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