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MECHANISMS OF RESISTANCE

The Novel Macrolide Resistance Genes *mef*(D), *msr*(F), and *msr*(H) Are Present on Resistance Islands in *Macrococcus canis*, *Macrococcus caseolyticus*, and *Staphylococcus aureus*

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ABSTRACT Chromosomal resistance islands containing the methicillin resistance gene *mecD* (McRI$_{mecD}$) have been reported in *Macrococcus caseolyticus*. Here, we identified novel macrolide resistance genes in *Macrococcus canis* on similar elements, called McRI$_{msr}$. These elements were also integrated into the 3′ end of the 30S ribosomal protein S9 gene (*rpsI*), delimited by characteristic attachment (*att*) sites, and carried a related site-specific integrase gene (*int*) at the 5′ end. They carried novel macrolide resistance genes belonging to the *msr* family of ABC subfamily F (ABC-F)-type ribosomal protection protein [*msr*(F) and *msr*(H)] and the macrolide efflux *mef* family [*mef*(D)]. Highly related *mef*(D)-*msr*(F) fragments were found on diverse McRI$_{msr}$ elements in *M. canis*, *M. caseolyticus,* and *Staphylococcus aureus*. Another McRI$_{msr}$-like element identified in an *M. canis* strain lacked the classical *att* site at the 3′ end and carried the *msr*(H) gene but no neighboring *mef* gene. The expression of the novel resistance genes in *S. aureus* resulted in a low-to-moderate increase in the MIC of erythromycin but not streptogramin B. In the *mef*(D)-*msr*(F) operon, the *msr*(F) gene was shown to be the crucial determinant for macrolide resistance. The detection of circular forms of McRI$_{msr}$ and the *mef*(D)-*msr*(F) fragment suggested mobility of both the island and the resistance gene subunit. The discovery of McRI$_{msr}$ in different *Macrococcus* species and *S. aureus* indicates that these islands have a potential for dissemination of antibiotic resistance within the *Staphylococcaceae* family.

KEYWORDS McRI$_{msr}$, antibiotic resistance, chromosomal resistance island, erythromycin esterase, macrolides, *mef*(D)-*msr*(F), *msr*(H)

The genus *Macrococcus* was first described in 1998 as a group of bacteria closely related to *Staphylococcus*, which currently includes 8 species already recognized by the nomenclature and 3 novel species not yet recognized (1–4). *Macrococcus* species are mainly animal commensals with low pathogenic potential, even though isolates from human clinical samples have been reported (4). Of concern is rather the fact that *Macrococcus* species can carry antibiotic resistance genes, including the alternative methicillin resistance genes *mecB* and *mecD*, on mobile genetic elements (5–7). The recent detection of highly similar *mecB*-carrying plasmids in both *S. aureus* and *M. canis* suggests that horizontal gene transfer may occur between *Macrococcus* and *Staphylococcus* species (8, 9). The chromosomal *M. caseolyticus* resistance island containing *mecD* (McRI$_{mecD}$) also has the potential to be site-specifically integrated into *Staphylococcus* chromosomes (10). Other clinically relevant resistance genes often present in *Staphylococcus* species were also detected in *Macrococcus* species, including the macrolide resistance genes *erm*(B) and *erm*(C) (11–13).

Three main mechanisms leading to macrolide resistance in Gram-positive bacteria are currently known, as follows: (i) inhibition of drug binding to the ribosome by target modification or protection, (ii) active efflux, and (iii) enzymatic inactivation (14). The

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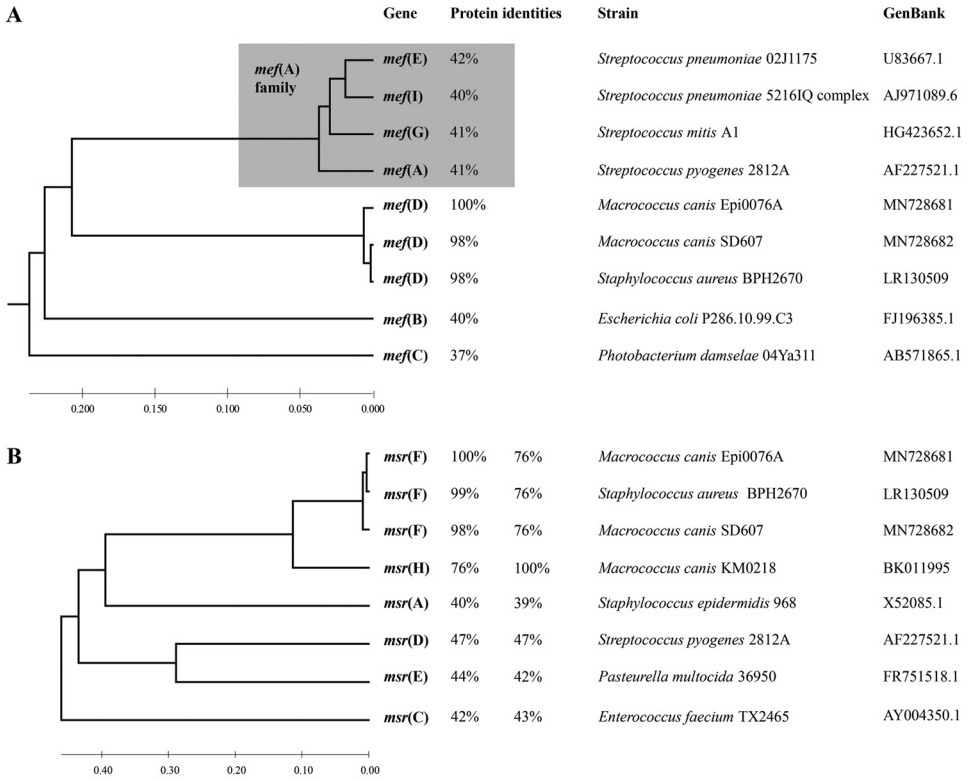

FIG 1 Phylogenetic trees of macrolide resistance genes belonging to the *mef* and *msr* families. Evolutionary analysis was performed for nucleotide sequences using the unweighted pair group method using average linkages (UPGMA) method in MEGA7 (56). The percentages of the deduced amino acid identities between the novel gene and the genes recognized by the MLS nomenclature (https://faculty.washington.edu/marilynr/) were determined by alignment with Clustal OMEGA (https://www.ebi.ac.uk/Tools/msa/clustalo/). (A) Comparison of *mef* macrolide efflux genes encoding major facilitator superfamily (MFS) membrane transporters. Note that *mef*(E), *mef*(I), *mef*(G), and *mef*(A) (gray shading) shared more than 80% aa identities with each other (87 to 94% for the selected representatives), and are all assigned to the *mef*(A) class by the MLS nomenclature. (B) Comparison of *msr* genes encoding ABC-F-type ribosomal protection proteins. The amino acid identities between the recognized genes by the MLS nomenclature and the two novel genes *msr*(F) and *msr*(H) are indicated.

erythromycin ribosome methylation (*erm*) genes are responsible for adenine methylation at position 2058 (A2058) in the 23S rRNA, which prevents drug binding and confers cross-resistance to macrolides, lincosamides, and streptogramin B (MLS_B). Antibiotic resistance ATP-binding cassette subfamily F (ARE ABC-F) proteins protect the ribosomal function by actively displacing antibiotics from their target sites (15, 16). ARE ABC-F genes specifically responsible for macrolide resistance include the plasmid-carried staphylococcal *msr*(A) gene (17), the intrinsic chromosomal *msr*(C) gene in *Enterococcus faecium* (18), as well as *msr*(D) (formerly called *mel*) in streptococci (19–21) and *msr*(E) in Gram-negative bacteria (22). In streptococci, the *msr*(D) gene is found together with the macrolide efflux (*mef*) gene on an operon located on accessory chromosomal elements (23–25). In contrast to *msr*(A) and *msr*(C), *msr*(D) confers lower levels of resistance to 14- and 15-membered macrolides but no resistance to streptogramin B (19, 26). The cotranscribed *mef*(A) gene [or highly related variants also called *mef*(E), *mef*(I), or *mef*(G); (Fig. 1A)] encodes a major facilitator superfamily (MFS) membrane transporter involved in macrolide efflux (20, 21, 27). Other *mef* genes, *mef*(B) and *mef*(C), were not associated with an *msr* gene and were only found in Gram-negative bacteria. Macrolide antibiotics can also be inactivated enzymatically by macrolide phosphotransferases (*mph*) and macrolide esterases (*ere*). Several different *mph* genes have been reported, with *mph*(C) commonly found in *Staphylococcus* species often on plasmids carrying *msr*(A) (28). Of the four *ere* genes mainly found in Gram-negative bacteria, *ere*(A) and *ere*(B) genes were detected in some *Staphylococcus* species in rare cases (29, 30).

TABLE 1 Antimicrobial susceptibilities of *Staphylococcus aureus* and *Macrococcus canis* to erythromycin and pristinamycin IA, as determined by broth microdilution

Strain/vector	Reference(s) or source	Characteristics[a]	MIC (μg/ml)[b]		
			ERY	PIA	iPIA[c]
S. aureus					
RN4220	44	NCTC8325-4 derivative, antibiotic susceptible	0.25	8	NA
RN4220/pTSSCm	36	Plasmid with *tet*(L)	0.25	4	NA
RN4220/pTSSCm-P_{cap}	36	Plasmid with *tet*(L)	0.25	ND	ND
RN4220/pTSSCm::*mef*(D)-*msr*(F)	This study	Plasmid with *mef*(D)-*msr*(F) genes, including upstream sequence (493 bp), *tet*(L)	16	8	8
RN4220/pTSSCm::*mef*(D)	This study	Plasmid with *mef*(D), including upstream sequence (493 bp), *tet*(L)	1	8	NA
RN4220/pTSSCm::*msr*(F)	This study	Plasmid with *msr*(F), including 217 bp upstream sequence [3′ end of *mef*(D) and intergenic region], *tet*(L)	0.5	4	NA
RN4220/pTSSCm::Δ*mef*(D)-*msr*(F)	This study	Plasmid with *msr*(F) fused to upstream sequence of *mef*(D) (493 bp), *tet*(L)	32	8	8
RN4220/pTSSCm::*msr*(H)	This study	Plasmid with *msr*(H), including upstream sequence (698 bp), *tet*(L)	2	8	4
RN4220/pTSSCm::*ere*-like	This study	Plasmid with *ere*-like, including upstream sequence containing a transcriptional regulator (*tr*) (710 bp), tet(L)	0.25	ND	ND
RN4220/pTSSCm::Δ*tr-ere*-like	This study	Plasmid with *ere*-like, including *tr*-deleted upstream sequence (345 bp), *tet*(L)	0.25	ND	ND
RN4220/pTSSCm-P_{cap}::*ere*-like	This study	Plasmid with *ere*-like gene controlled by promoter P_{cap}, *tet*(L)	0.25	ND	ND
M. canis					
KM45013^T	3, 43	*mecB*, $blaZ_m$	0.25	2	NA
Epi0076A	9, 12	*mef*(D), *msr*(F), *mecB*	4	2	2
SD0607	12	*mef*(D), *msr*(F), *ere*-like	16	2	2
KM0218	9, 12	*msr*(H), *mecB*, $blaZ_m$, *aac(6′)-aph(2″)*, *sat4*, *aph(3′)-IIIa*, *erm*(B), *ant(6)-Ia*, *tet*(S)	>64	>64	ND

[a]Antibiotic resistance genes and their functions are as follows: *mef*(D), macrolide efflux protein; *msr*(F) and *msr*(H), macrolide ABC-F ribosomal protection protein; *ere*-like, putative erythromycin esterase; *tet*(L), tetracycline efflux protein; *tet*(S), ribosome protection tetracycline resistance gene; *mecB*, penicillin-binding protein 2a (PBP2a) with low affinity for β-lactams; $blaZ_m$, β-lactamase; *aac(6′)-aph(2″)*, gentamicin and kanamycin acetyltransferase and phosphotransferase; *sat4*, streptothricin acetyltransferase; *erm*(B), macrolide, lincosamide, and streptogramin B 23S rRNA methylase gene; *ant(6)-Ia*, streptomycin nucleotidyltransferase gene; *aph(3′)-III*, kanamycin phosphotransferase gene.

[b]ERY, erythromycin; PIA, pristinamycin IA; iPIA, inducible PIA; ND, not determined; NA, not applicable.

[c]iPIA, inducible resistance to pristinamycin IA was measured in the presence of 1 μg/ml erythromycin, except for RN4220/pTSSCm::*msr*(H), for which the erythromycin concentration was 0.25 μg/ml.

In a previous study, two *M. canis* strains, Epi0076A and SD607, isolated from dogs were found to be resistant to macrolides but contained none of the known resistance genes (12), prompting us to search for the underlying resistance mechanism. We used whole-genome sequencing for the identification of a new *mef-msr* operon as well as recombinant gene expression in *S. aureus* for proof of functionality. In addition, comparative sequence analysis by BLAST search allowed the identification of the same *mef-msr* operon in *M. caseolyticus* and *S. aureus* strains deposited in the NCBI GenBank database, as well as another novel *msr* gene integrated similarly into the chromosome of the *erm*(B)-carrying *M. canis* strain KM0218 obtained from a dog (9, 12).

RESULTS

New *mef*(D)-*msr*(F) genes in *Macrococcus canis*. Both *M. canis* strains, Epi0076A and SD607 (12), showed increased MICs of erythromycin (4 μg/ml and 16 μg/ml, respectively) compared to the *M. canis* type strain KM45013 (0.25 μg/ml) (Table 1). The MICs of clindamycin were identical in all three strains (0.25 μg/ml).

The genome sequences of Epi0076A and SD607 analyzed using TBLASTN with protein query of known macrolide resistance determinants listed in the MLS nomenclature (https://faculty.washington.edu/marilynr/) revealed the presence of putative new *mef* and *msr* genes. In both strains, the putative *mef* gene was situated directly upstream of the *msr* gene on 3.6-kb DNA fragments, which shared 98% nucleotide (nt) identity. The new putative *mef* gene was only distantly related to other *mef* family members, with the deduced amino acid (aa) sequence sharing less than 43% identity to all other Mef proteins (Fig. 1A). It represented a new *mef* gene, named *mef*(D), according to the MLS nomenclature that defines a new gene if the aa sequence shares less than 80% identity to any previously characterized MLS genes (31). The Mef(D) protein possessed the major facilitator superfamily (MFS) profile (PROSITE entry PS50850) that covers the 12 putative transmembrane helices typically found in MFS efflux pumps. A MefA-like domain (Conserved Domain Database [CDD] entry cd06173)

suggested that this new Mef protein could be involved in macrolide efflux. The *mef*(D) genes of Epi0076A and SD607 differed from each other by a 6-bp deletion in the *mef* gene of SD607, generating 399-aa and 397-aa proteins with 98% identities. Downstream of *mef*(D), Epi0076A and SD607 encoded a similar new 486-aa Msr protein that contained the features of ARE ABC-F family proteins with two nucleotide-binding domains separated by a 100-aa linker (PROSITE entry PS50893). This new putative *msr* gene shared 47% aa identity to the closest related *msr*(D) of streptococci and was named *msr*(F), following the MLS nomenclature (Fig. 1B).

The *mef*(D)-*msr*(F) genes were organized in tandem similar to *mef*(A)-*msr*(D) genes in streptococci with a short intergenic region of 111 bp in Epi0076A and 112 bp in SD607. Both *mef*(D) genes contained identical upstream sequences possibly involved in controlling *mef*(D)-*msr*(F) expression. A promoter sequence (−35 box [5′-TTGACT] and −10 box [5′-GTTTATAAT]) was identified 35 bp and 15 bp upstream of the putative transcription start site represented by a guanine located 414 bp upstream of *mef*(D). The 414-bp region upstream of *mef*(D)-*msr*(F) contained imperfect inverted repeats capable of folding into stem-loops, two ribosomal binding sites (RBS), a coding sequence for a small leader peptide [Mef(D)L, MTHAMKLRF], and a rho-independent terminator sequence (see Fig. S1 in the supplemental material). Similar features were observed in the regulatory 5′ region upstream of *mef*(A)-*msr*(D) of streptococci and *erm*(K) of *Bacillus licheniformis* and were shown to be involved in transcriptional attenuation of the antibiotic resistance genes (32–34). Even though the overall sequence identity of the putative cotranscribed 5′ region upstream of *mef*(D)-*msr*(F) (414 bp) to those of *mef*(A)-*msr*(D) (327 bp; 49% nt identity) and *erm*(K) (357 bp; 56% nt identity) was low, the structural similarity suggests possible transcriptional attenuation control of *mef*(D)-*msr*(F).

The association of *mef*(D) and *msr*(F) from *M. canis* Epi0076A with antimicrobial resistance was analyzed by cloning the genes with their putative regulatory DNA region into the promoterless plasmid pTSSCm and subsequent expression in *S. aureus* RN4220. Thus, *S. aureus* RN4220 was transformed with pTSSCm carrying both *mef*(D) and *msr*(F) [plasmid pTSCCm::*mef*(D)-*msr*(F)], as well as with pTSSCm containing either *mef*(D) [pTSSCm::*mef*(D)] or *msr*(F) [pTSSCm::*msr*(F)] alone (see Fig. 2 for plasmid constructs). Susceptibility testing showed that RN4220 containing pTSCCm::*mef*(D)-*msr*(F) exhibited a 64-fold increased erythromycin MIC (16 μg/ml) compared to those of the parental strain and RN4220 containing only the empty vector (0.25 μg/ml) (Table 1). RN4220 containing pTSSCm::*mef*(D) exhibited only a modestly increased (4-fold) erythromycin MIC value of 1 μg/ml. *S. aureus* RN4220 containing pTSSCm::*msr*(F) did not exhibit a clear increase in phenotype, with an MIC for erythromycin of 0.5 μg/ml. Of note, the cloned region preceding *msr*(F) consisted of the 111-bp intergenic region between *mef*(D) and *msr*(F) and the 106-bp 3′ end of *mef*(D). A promoter search in the proximate upstream region of *msr*(F) predicted a possible −10 box (5′-TTTTATTAT) and a −35 box (5′-TAGGAG) with a low probability score, suggesting that *msr*(F) is also under the control of the native regulator of the *mef*(D), with the two genes forming a *mef*(D)-*msr*(F) operon. The *mef*(D) gene was therefore deleted from plasmid pTSCCm::*mef*(D)-*msr*(F) (Fig. 2). The resulting plasmid, pTSCCm::Δ*mef*(D)-*msr*(F), conferred a high MIC value of 32 μg/ml when inserted into RN4220 (Table 1), indicating that DNA upstream of *mef*(D) is necessary for *msr*(F) expression and that *msr*(F) is the predominant gene of the *mef*(D)-*msr*(F) operon causing macrolide resistance. Resistance to the streptogramin B pristinamycin IA was not observed in *S. aureus* strains containing any of the *mef*(D)-*msr*(F)-expressing plasmids nor in *M. canis* strains carrying *mef*(D)-*msr*(F) (Table 1). The MIC for pristinamycin IA also remained unchanged in the presence of 1 μg/ml erythromycin used for the induction of gene expression.

Diverse structures of McRI$_{msr}$ macrolide resistance islands. In *M. canis* strains Epi0076A and SD607, the *mef*(D)-*msr*(F) operon was located in the chromosome downstream of the 30S ribosomal protein S9 gene (*rpsI*) (Fig. 3). This locus has already been reported to contain the integration site for resistance islands carrying *mecD*

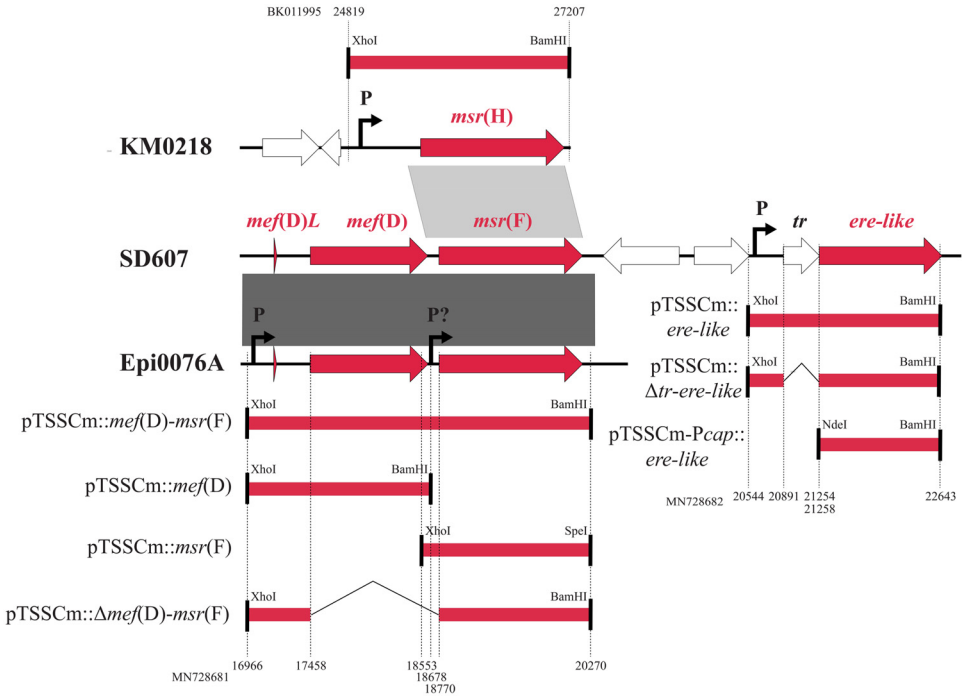

FIG 2 Cloning of *msr*(H), *mef*(D)-*msr*(F), and *ere*-like DNA fragments of *Macrococcus canis* strains KM0218, Epi0076A, and SD607 into the *E. coli-S. aureus* shuttle vector pTSSCm or pTSSCm-P$_{cap}$. The fragments are represented by thick lines below/above the gene structure of the strain used for PCR amplification (see Table S1 for PCR conditions). Flanking restriction sites are indicated, and nucleotide positions are given for the corresponding GenBank accession numbers, BK011995 for KM0218, MN728682 for SD607, and MN728681 for Epi0076A. Gray areas between the gene structures indicate regions with nucleotide sequence identities of 81% (light gray) and 98% (dark gray). The positions of promoter (P) or possible promoter (P?) are indicated.

(McRI$_{mecD}$) in methicillin-resistant *M. caseolyticus* strains (7, 13). Similar to McRI$_{mecD}$, the *mef*(D)-*msr*(F)-containing element in Epi0076A and SD607 was delimited at both sides by characteristic attachment (*att*) sites of 61 bp and carried a related site-specific integrase gene (*int*) of the tyrosine recombinase family preceded by its regulator's *intR* and *xis* (Fig. 3). The integrase gene of McRI$_{mecD}$-1 has been shown to be responsible for the mobilization of *att*-delimited elements (10). Based on these similar features to McRI$_{mecD}$, the *mef*(D)-*msr*(F)-containing elements were called *Macrococcus* resistance island *msr* (McRI$_{msr}$) and represent inserts of 18,834 bp in Epi0076A and 20,836 bp in SD607. The *int* of McRI$_{msr}$ of Epi0076A showed 96% nt identity to the *int0473* of McRI$_{mecD}$-2/3 but only 75% to the *int* of McRI$_{msr}$ of SD607, which, on the other hand, was more similar to the *int0819* of McRI$_{mecD}$-1 (90% nt identity). The McRI$_{msr}$ elements of Epi0076A and SD607 were different from the integrase gene, the *mef*(D)-*msr*(F) fragment, and unrelated types of genes coding for DNA methylase (*mtase*) and a putative AAA-type ATPase (*aase*) (Fig. 3).

While the McRI$_{msr}$ of SD607 seems to be unique, two elements similar to McRI$_{msr}$ of Epi0076A were found by a nucleotide similarity search in the GenBank database. The first strain, *M. caseolyticus* DaniaSudan, isolated from a wound from a donkey in Sudan (GenBank accession no. NZ_RBVL01000008), carried an McRI$_{msr}$ that lacks only the IS*3* element of Epi0076A. Second, *M. caseolyticus* strain CCM7927, isolated from human clinical material in the Czech Republic (accession no. NZ_MJBJ02000004), differed additionally by an alternative first open reading frame (ORF) of an island previously named McRI$_{CCM7927}$ (4) (Fig. S2). The McRI$_{msr}$ elements of the two *M. caseolyticus* strains were also delimited by the characteristic *att* sites. A second direct repeat (DR), DR2, flanking the 3,886-bp *mef*(D)-*msr*(F) segment was identified in all Epi0076A-like McRI$_{msr}$ sequences. These imperfect 93-bp DR2 elements contained at the 3′-end 7 bases (5′-GAACGTA) overlap the 5′ end of the *att* site (Fig. S2). Interestingly, such a DR2-

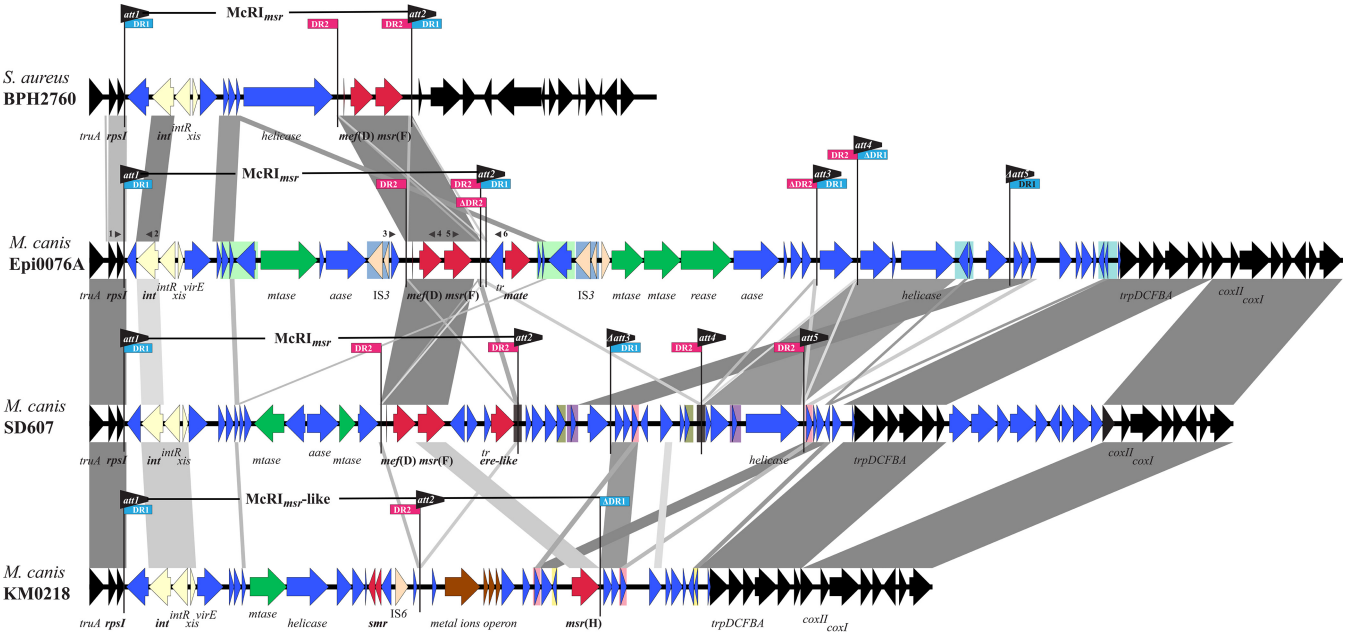

FIG 3 Structures of *Macrococcus* resistance islands (McRI) chromosomally integrated into the 3′ end of the 30S ribosomal protein S9 gene (*rpsI*) of *Staphylococcus aureus* and *Macrococcus canis*. Three different McRI$_{msr}$ containing the macrolide resistance genes *mef*(D)-*msr*(F) are shown, all containing a related integrase (*int*) at the left end and are flanked by characteristic attachment (*att*) sites. The macrolide resistance gene *msr*(H) is found downstream of an *att*-flanked island in a McRI$_{msr}$-like structure. Imperfect direct repeats (DR1 and DR2) often associated with *att* sites are indicated. More extended direct repeats are shaded equally. Genes are represented by arrows and colored by putative function, as follows: resistance genes are shown in red, genes involved in the excision and integration of McRI are in light yellow, recombinase genes are in darker yellow, genes of DNA restriction-modification systems are in green, genes involved in heavy metal metabolism are in brown, other possible accessory genes are shown in blue, and those assumed to be usually present in the chromosome are in black. Arrowheads labeled with numbers indicate primers used to detect circular excision of *mef*(D)-*msr*(F) subunits. Primer names are as follows: 1, rpsI-MC-F; 2, int-0473-F; 3, Epi0076A_18-2-F; 4, mefD-R; 5, msrF_F; and 6, Epi0076A_22-2-F. Gray areas indicate regions with between 75% and 100% nucleotide sequence identity. The figure was generated using the Easyfig software (57) and the sequences of the *S. aureus* strains BPH2760 (GenBank accession no. NZ_LR130509.1, positions 2178720 to 2208720), *M. canis* Epi0076A (accession no. MN728681), *M. canis* SD607 (accession no. MN728682), and *M. canis* KM0218 (accession no. BK011995).

flanked *mef*(D)-*msr*(F) segment (99% nt identity) was also detected in *S. aureus* strain BPH2760, isolated from human blood in Australia (accession no. NZ_LR130509). In this *S. aureus* strain, the *mef*(D)-*msr*(F) operon was also part of an McRI$_{msr}$. The 15,178-bp element of BPH2760 was also integrated into the chromosome at the 3′ end of the *rpsI* gene, carried *int-intR-xis* genes at the 5′ end, and was flanked by *att* sites (Fig. 3). It formed a third type of McRI$_{msr}$ that contained an *int* gene that shared 97% nt identity with that of Epi0076A and unique genes (e.g., ORF1 and a helicase) not present in the McRI$_{msr}$ elements of *M. canis* strains Epi0076A and SD607. A DR2-flanked segment containing *mef*(D)-*msr*(F) was also observed in *M. canis* SD607, but this segment was larger (7,173 bp) and contained a macrolide esterase-like gene (*ere*-like) (see below) (Fig. 3).

Mobility of McRI$_{msr}$. The presence of an integrase gene (*int*) related to that of McRI$_{mecD}$ and intact flanking *att* sites suggests that McRI$_{msr}$ could also be mobilized by an Int-catalyzed site-specific recombination reaction as observed for *mecD* elements (7, 10, 13). To determine whether McRI$_{msr}$ was able to excise itself, the genomic DNA of Epi0076A was analyzed for the presence of circular forms. Circular McRI$_{msr}$ was detected by PCR using divergent primers specific for *int* and *msr*(F) (primers 2 and 5 in Fig. 3; see Fig. S3 for PCR products). A PCR product corresponding to the chromosomal segment after McRI$_{msr}$ deletion was also obtained using short extension time and convergent primers specific for *rpsI* and a transcriptional regulator (*tr*) downstream of *msr*(F) (Fig. 3, primers 1 and 6, and Fig. S3). Sequencing confirmed that circular excision of McRI$_{msr}$ results from recombining the *att1* and *att2* sequences in Epi0076A.

The highly similar DR2-flanked *mef*(D)-*msr*(F) segments seen in a different genetic context in McRI$_{msr}$ of *M. canis* Epi0076A and McRI$_{msr}$ of *S. aureus* BPH2670 indicate

potential mobility of this subunit. Excision and circularization of the *mef*(D)-*msr*(F) subunit were tested using pairs of divergent and convergent primers to detect the circular form and chromosomal deletion of the *mef*(D)-*msr*(F) subunit, respectively (Fig. 3 and S3). The sequence of the amplicon obtained with the *mef*(D) reverse and *msr*(F) forward primers (primers 4 and 5 in Fig. 3) showed the circular *mef*(D)-*msr*(F) subunit joined by a DR2 identical to DR2-2 (Fig. S3). The sequence chromatograms showed no double peaks at the DR2-1/DR2-2 mismatch position, indicating that a site-specific strand exchange occurred at the end of the DR2 sequence. However, the chromosomal segment that remained after excision of the *mef*(D)-*msr*(F) subunit amplified with primers Epi0076A_18-2-F and Epi0076A_22-2-F (primers 3 and 6 in Fig. 3) showed ambiguous bases at the mismatch position within the joining DR2 (Fig. S3). This result suggests that deletion of the *mef*(D)-*msr*(F) subunit can also occur by random strand exchange within the DR2 sequences. The McRI$_{msr}$ and downstream region in Epi0076A contain 5 DR2 that could also become involved in homologous recombination. The *att* sequences were often found embedded in more extended repeats with the DR2 sequence upstream and another partially overlapping repeat (49-bp overlap with *att*) called DR1 downstream (see Fig. S2 for sequences). Additional repetitive sequences formed by an insertion sequence (IS) of the IS*3* family or other long imperfect duplicated sequences were also detected (indicated by shading in Fig. 3). Such extended repeats may also play a role in recombination leading to loss or gain of fragments within McRI$_{msr}$.

Putative resistance genes associated with McRI$_{msr}$. The region downstream of McRI$_{msr}$ in Epi0076A and SD607 carried unique sequences organized in segments flanked by the *att* sites (Fig. 3). In Epi0076A, the *rpsI* downstream region was divided into the 18,834-bp McRI$_{msr}$, followed by a 17,789-bp segment (*att2*-*att3*), a 2,137-bp segment (*att3*-*att4*), and an 8,044-bp segment (*att4*-Δ*att5*) flanked at the right side by a 5′-truncated *att* site. On these segments, putative genes for enzymes of restriction-modification systems (*mtase* and restriction endonuclease [*rease*]), a helicase, and a multidrug and toxic compound extrusion (MATE)-like proteins were identified (Fig. 3). The putative 446-aa MATE protein of Epi0076A contains the MepA (cd13143) and the MATE-like superfamily domain (cl09326) found in integral membrane proteins involved in low-level resistance against a broad spectrum of compounds, including several monovalent and divalent biocides (35).

M. canis SD607 contained, downstream of McRI$_{msr}$, further *att*-flanked segments of 4,896 bp (*att2*-Δ*att3*), 4,782 bp (Δ*att3*-*att4*), and 5,418 bp (*att4*-*att5*) (Fig. 3). An *ere*-like gene was found downstream of *mef*(D)-*msr*(F) within the DR2-flanked subunit and was analyzed further for macrolide resistance by recombinant gene expression. The *ere*-like gene of SD607, including 710 bp upstream of its translational start site, was cloned into pTSSCm, and the resulting pTSSCm::*ere*-like plasmid was introduced into *S. aureus* RN4220 (see Fig. 2 for plasmid constructs). The measured MIC of erythromycin remained unchanged for RN4220/pTSSCm::*ere*-like compared to that of RN4220 without plasmid and with pTSSCm (Table 1). The cloned fragment contained a putative transcriptional regulator (*tr*) that overlapped by 4 bp with the 5′ end of the *ere*-like gene. To exclude the possibility that *tr* represses *ere*-like expression, the *tr* sequence was deleted in pTSSCm::*ere*-like. However, the RN4220 strain containing pTSSCm::Δ*tr*-*ere*-like also did not exhibit erythromycin resistance (Table 1). It included an upstream region containing possible native promoter sequences with a −35 box (5′-TGGATA) and a −10 box (5′-TACTATCAT) located 327 bp and 303 bp upstream of the start codon, respectively. Additionally, the *ere*-like gene alone was placed under the control of the strong constitutive *S. aureus* type 1 capsule gene 1A promoter (P$_{cap}$) to ensure expression in *S. aureus* (36). The lack of a phenotype observed in RN4220/pTSSCm-P$_{cap}$::*ere*-like indicated that this *ere*-like gene is probably not involved in erythromycin inactivation (Table 1). The 415-aa Ere-like protein of SD607 contains the Ere-like domain (cd14728), including strictly conserved residues in the active-site cleft (E63, H66, E94, H276, and H279), suggesting that the enzyme functions as a hydrolase (37). The

similarity of the Ere-like protein of SD607 to the erythromycin esterases recognized by the MLS nomenclature [Ere(A) and Ere(B) enzymes of *Escherichia coli* and Ere(C) and Ere(D) proteins of *Riemerella anatipestifer*] was low and less than 21% overall aa identity.

A comparison of the McRI$_{msr}$ elements of Epi0076A and SD607 with other *Macrococcus* species sequences from the GenBank database revealed another McRI$_{msr}$-like structure with a putative novel *msr* gene in *M. canis* KM0218 (Fig. 3). The novel *msr*(H) gene of KM0218 encoded a 486-aa ARE ABC-F protein (PROSITE entry PS50893) that shared 76% aa identity to the closest related *msr*(F) gene, hence representing a new *msr* gene named *msr*(H), according to MLS nomenclature (Fig. 1B). KM0218 showed high-level resistance to erythromycin and pristinamycin IA due to an *erm*(B) gene carried on a multidrug resistance plasmid (9) (Table 1). Whether the *msr*(H) gene detected in the *rpsI* region also contributed to the macrolide and streptogramin B phenotype was therefore not clear. A complete *mef* gene was not present in KM0218 but the upstream region of *msr*(H) contained an 81-bp remnant resembling the 3′ end of *mef*(D) as well as a 109-bp region similar to the intergenic region of *mef*(D)-*msr*(F). Features found in the 5′ region of inducibly expressed *mef*, *msr*, and *erm* genes were not identified in the *msr*(H) upstream region. The expression of *msr*(H) from native promoter [*msr*(H), including 698 bp upstream of its translational start site of KM0218 cloned into pTSSCm::*msr*(H) (Fig. 2)] in *S. aureus* mediated low-level resistance to erythromycin, with an MIC value of 2 μg/ml, but not resistance to pristinamycin IA (Table 1).

M. canis KM0218 contained only one *att*-delimited segment of 15,707 bp (*att1-att2*) downstream of the *rpsI* gene that also carried the *int-intR-xis* genes at the 5′ end (Fig. 3). The *msr*(H) gene was found outside this segment and therefore reported to be part of an McRI$_{msr}$-like region (*att1-*Δ*DR1*). A unique helicase and a DNA methylase gene were also identified in this accessory DNA of strain KM0218. This region also contains genes involved in heavy metal metabolism and two genes belonging to the small multidrug resistance (SMR) family (Fig. 3).

DISCUSSION

Three new macrolide resistance genes were characterized in this study, *msr*(F) and *msr*(H), encoding ARE ABC-F family proteins, and *mef*(D), encoding an MFS protein. While *msr*(H) was found alone, *mef*(D) and *msr*(F) were present in tandem within the same operon. The expression of these genes in *S. aureus* resulted in a low-to-moderate increase in erythromycin resistance but did not affect streptogramin B resistance, even under inducing conditions. Such an M-type resistance phenotype has been described for streptococci (38) associated with the *mef*(A)-*msr*(D) operon. The *mef*(D)-*msr*(F) operon identified in *M. canis* also showed structural similarity to the *mef*(A)-*msr*(D) operon of streptococci, even though the overall nucleotide sequence identity was low (53%), and elements carrying the genes in *Macrococcus* and *Streptococcus* species were unrelated. The 414-bp sequence upstream of *mef*(D)-*msr*(F), suggested to be cotranscribed with the two genes, also encodes a leader peptide [Mef(D)L, MTHAMKLRF] that contains the possible pause sequence M(R/K)L(R/K) (32, 39). The *mef*(A)-*msr*(D) genes might therefore be regulated by a macrolide-dependent ribosome stalling mechanism which could lead to reorganization of the mRNA structure between the leader ORF and the *mef*(D) gene. The presence of a rho-independent terminator sequence in this region suggests a transcriptional attenuation control as found for *mef*(A)-*msr*(D) and *erm*(K) (32, 33). Cloning experiments in *S. aureus* showed that the promoter sequence upstream of *mef*(D) is also needed for the expression of *msr*(F). Moreover, the *msr*(F) gene is the principal determinant of the *mef*(D)-*msr*(F) operon responsible for macrolide resistance and also functions without the presence of *mef*(D). This finding is in accordance with the current model of ARE ABC-F proteins that protect ribosomes independently of a partner protein (15, 16). The *mef*(D) gene alone only mediated a slight decrease in susceptibility to erythromycin, and its presence upstream of *msr*(F) seems even to diminish the effect of *msr*(F), as a 2-fold-lower MIC value was repeatedly obtained with a plasmid containing the complete *mef*(D)-*msr*(F) operon. In streptococci,

msr(D) also has a predominant role over *mef*(A) in macrolide resistance, but an additive effect was observed for the two genes (20, 21).

The *msr*(H) gene was most closely related to the *msr*(F) gene and was not preceded by a *mef* gene. However, the presence of an upstream region exhibiting similarity to the end of *mef*(D) and the intergenic region of *mef*(D)-*msr*(F) suggests that the 5′ region of a former *mef-msr* operon could have been truncated. This would also explain the lack of structural similarity of the upstream region of *msr*(H) to the 5′ regulatory region of other *msr*, *mef*, and *erm* genes. The expression of *msr*(H) under the control of its own promoter in *S. aureus* mediated only a moderate increase in the erythromycin MIC, which could also reflect suboptimal gene expression rather than a *per se* less-effective ARE ABC-F protein.

The putative *ere* gene of strain SD607 cloned under the control of P_{cap} did not affect the erythromycin susceptibility of *S. aureus*. The capability of *ere*(A) and *ere*(B) of *E. coli* to hydrolyze the macrolactone rings has been proven experimentally (37, 40, 41), but their functionality in a staphylococcal host has not yet been definitively clarified. Li and colleagues found *ere*(A) in both macrolide-resistant and -susceptible strains (29). Another protein related to Ere(A) and Ere(B), Bcr136 of *Bacillus cereus*, has been shown to possess esterase activity but was unable to inactivate macrolides (37). Current annotation tools are assigning many proteins, including the Bcr136 and Ere-like proteins of SD607, to the erythromycin esterase family. These proteins might possess esterase activity, but it seems that their specificity for erythromycin is not always guaranteed, as seen for Bcr136 and the putative macrolide esterase of SD607.

The McRI$_{msr}$ genetic elements carrying *mef*(D)-*msr*(F) detected in *M. canis* and *S. aureus* showed, despite an overall high diversity, some common important features, as follows: they share the same DR2-flanked *mef*(D)-*msr*(F) fragment, possess highly related integrases and flanking *att* sites, and are found to be site-specifically integrated into the chromosome at the *rpsI* locus. Similarly, the *msr*(H) gene of *M. canis* KM0218 was also associated with an McRI$_{msr}$-like structure integrated at the *rpsI* locus. The 3′ end of the *rpsI* gene, a highly conserved locus in *Staphylococcaceae*, has already been shown to hold integrated elements carrying an *int* gene or small accessory islands without an obvious mobilization function (4, 7, 10, 13). The *att*-flanked segments found in *M. canis* Epi0076A and SD607 suggests that the site-specific *int* gene is active in accumulating accessory DNA and diversifying the *rpsI* locus. Circular excision of McRI$_{msr}$ was demonstrated for Epi0076A. In addition, a circular intermediate of the DR2-flanked *mef*(D)-*msr*(F) subunit was detected, emphasizing the mobility potential of this subunit also present in *S. aureus*. Analysis of the joining DR2 sequence in the circular *mef*(D)-*msr*(F) subunit indicated that strand cleavage occurred at the 3′ end of the homologous DR2 sequences. Since the 3′ end of the second DR2 overlaps the 5′ end of the *att2* site, the *int* of McRI$_{msr}$ could mediate this excisive recombination reaction. Analysis of recombination products of McRI$_{mecD}$-1-McCI$_{IMD0819}$ subunits indicated that strand cleavage occurred within the first 8 bases of the core *att* site (7). The *int* of *rpsI*-associated islands seems to be flexible in *att* site selection and be influenced by regulators like *xis* (10). Furthermore, *M. canis* has previously been shown to have potential to use imperfect direct repeats for the formation of unconventional circularizable structures (9, 42, 43).

Novel erythromycin resistance genes, *mef*(D), *msr*(F), and *msr*(H), were found on the McRI$_{msr}$ and McRI$_{msr}$-like chromosomal resistance islands, site-specifically integrated into the *rpsI* locus. The same type of resistance islands, McRI$_{mecD}$ carrying the methicillin resistance gene *mecD*, were described before. While McRI$_{mecD}$ was so far only found in *M. caseolyticus*, diverse McRI$_{msr}$ islands containing the same *mef*(D)-*msr*(F) fragment were detected in both *Macrococcus* species and *S. aureus* in this study. This finding showed that McRI$_{msr}$ elements play an important role in the distribution of the M-type phenotype in both commensal and opportunistic pathogens of the *Staphylococcaceae* family.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Three erythromycin-resistant *M. canis* strains from dogs, KM0218 [sequence type 12 (ST12); resistance genes *mecB*, $blaZ_m$, *aac(6′)-aph(2″)*, *sat4*, *aph(3′)-IIIa*, *erm*(B), *ant(6)-Ia*, and *tet*(S)], Epi0076A (ST2; *mecB* and *grlA* [Ser80Leu]), and SD607 (ST21), were obtained from a study by Cotting and colleagues (12). The *E. coli* strains TOP10 (Thermo Fisher Scientific [Invitrogen], Waltham, MA) and DH5α were used for cloning and plasmid amplifications. *S. aureus* strain RN4220 (44) was used for plasmid transformation and phenotypic gene expression. The strains were routinely cultivated under aerobic conditions at 37°C on either Trypticase soy agar plates containing 5% sheep blood (TSA-SB; Becton, Dickinson Company, Franklin Lakes, NJ, USA) in Luria-Bertani (LB) broth with shaking or on LB agar plates. DH5α and RN4220 strains containing pTSSCm-derived plasmids [*tet*(L)] were selected and grown in LB medium containing 10 μg/ml tetracycline (36). TOP10 cells carrying pCRII-TOPO-derived plasmids [with *aph(3′)-II* and bla_{TEM}] were cultivated in LB medium containing 50 μg/ml kanamycin.

DNA preparation and PCR. High-quality genomic DNA was extracted from pure cultures using the either peqGOLD bacterial DNA kit (Peqlab Biotechnologie GmbH, Jena, Germany) or the DNeasy blood and tissue kit (Qiagen GmbH, Hilden, Germany). Lysis of *M. canis* cells was improved through a 30-min initial incubation at 37°C in the presence of 50 μg/ml lysostaphin and 2 mg/ml lysozyme. Chelex-based genomic DNA extractions were prepared without enzymatic predigestion of cell walls. To do so, *M. canis* or *S. aureus* cells taken from the plate with a 1-μl loop were incubated in 200 μl of 10% (wt/vol) Chelex 100 molecular biology-grade resin (Bio-Rad, Hercules, CA, USA) solution (100 mM Tris-HCl [pH 8], 0.5 mM EDTA) at 56°C for 30 min, followed by a 15-min boiling step. The DNA-containing supernatant was separated from Chelex resins by centrifugation (5 min at 10,000 $\times$ *g*). Plasmid DNA from *E. coli* was isolated using the peqGOLD plasmid miniprep kit I (Peqlab Biotechnologie GmbH).

PCRs were performed according to the manufacturer's instructions using FIREPol DNA polymerase (Solis BioDyne, Tartu, Estonia) for analytical PCRs, Phusion Hot Start II high-fidelity DNA polymerase (Thermo Fisher Scientific) for cloning, and GoTaq long PCR master mix (Promega, Madison, WI, USA) for the detection of circular excision fragments. All relevant primers and PCR conditions are listed in Table S1. PCR fragments were purified using the High Pure PCR product purification kit (Roche Diagnostics, Rotkreuz, Switzerland) before incubation with restriction endonucleases, T4 DNA ligase, and Sanger sequencing.

Construction of recombinant plasmids. Target sequences for cloning were amplified from high-quality genomic DNA using Phusion Hot Start II high-fidelity DNA polymerase. The resulting blunt-end PCR products were introduced into the pCR-Blunt II-TOPO vector using the topoisomerase-based Zero Blunt TOPO PCR cloning kits (Thermo Fisher Scientific [Invitrogen]) and transformed into One Shot TOP10 chemically competent *E. coli* cells (Thermo Fisher Scientific [Invitrogen]), according to the manufacturer's protocol. TOP10 colonies obtained on selective agar were screened by colony-PCR for insert orientation using combinations of vector and insert primers. Inserts were subcloned into the pTSSCm or pTSSCm-P_{cap} vector (36) (see Fig. 2 for plasmid constructs) using endonuclease restriction and T4 DNA ligation (Promega). Restriction sites in the flanking pCRII-TOPO sequence or incorporated in the 5′ overhangs of cloning primers were used for that purpose (Table S1). The generated pTSSCm/-P_{cap} constructs were transformed through heat shock into chemically competent DH5α cells (45). To construct the plasmids pTSSCm::*mef*(D)-*msr*(F), pTSSCm::*mef*(D), and pTSSCm::*msr*(F), inserts were amplified from Epi0076A DNA using the PCR conditions listed in Table S1. Inserts for pTSSCm::*ere*-like and pTSSCm-P_{cap}::*ere*-like were amplified from SD607 DNA, and that for pTSSCm::*msr*(H) was obtained using KM0218 DNA (Table S1). pCRII-TOPO constructs containing the inserted genes in antisense orientation respective to backbone genes were used for restriction endonuclease-based subcloning into the shuttle vectors pTSSCm and pTSSCm-P_{cap}. The enzymes XhoI and BamHI were used to generate pTSSCm::*mef*(D)-*msr*(F), pTSSCm::*mef*(D), pTSSCm::*ere*-like, and pTSSCm::*msr*(H) (Fig. 2). The enzymes XhoI and SpeI were used to obtain pTSSCm::*msr*(F). The enzymes NdeI and BamHI were used to construct pTSSCm-P_{cap}::*ere*-like. Plasmids pTSSCm::Δ*mef*(D)-*msr*(F) and pTSSCm::Δ*tr*-*ere*-like were generated through PCR-based mutagenesis of pTSSCm::*mef*(D)-*msr*(F) and pTSSCm::*ere*-like, respectively (Table S1 and Fig. 2). Deletion of the *mef*(D) gene in the template plasmid pTSSCm::*mef*(D)-*msr*(F) and of the *tr* gene in pTSSCm::*ere*-like was obtained using primers that excluded amplification of *mec*(D) or *tr* and that contained homologous 5′ ends. The PCR products were treated with the restriction enzyme DpnI and directly transformed into *E. coli* DH5α cells, which allowed recombining their homologous ends *in vivo*. All plasmid structures were verified by restriction digestion, and the sequences of the DNA inserts were confirmed by Sanger sequencing. All of the generated plasmids were transformed into RN4220 strain bacteria through electroporation using the protocol of Schenk and Laddaga (46).

Antimicrobial susceptibility testing. Antimicrobial susceptibility was determined for *M. canis* strains and *S. aureus* RN4220 strains containing pTSSCm/-P_{cap}-derived plasmids. The MICs were measured by the microdilution method in Mueller-Hinton broth, according to CLSI guidelines (47), using both Sensititre EUST plates (Thermo Fisher Scientific) and 96-well plates containing serial 2-fold dilutions ranging from 0.125 μg/ml to 64 μg/ml erythromycin or pristinamycin IA. Inducible resistance to pristinamycin IA was measured in the presence of 1 or 0.25 μg/ml erythromycin. MICs were determined in duplicate.

Assembly, annotation, and analysis of genome sequences. The genome of *M. canis* strain KM0218 was obtained from GenBank (accession no. CP035309.1). Strain Epi0076A was sequenced using Illumina MiSeq technology within a previous study (9). During this study, strain SD607 was sequenced using Illumina HiSeq technology (2 $\times$ 150-bp paired-end; Eurofins, Constance, Germany), and both strains, SD607 and Epi0076A, were sequenced with MinION Oxford Nanopore Technology (ONT). The ONT library

was prepared from mechanically fragmented DNA (g-TUBE; Covaris) using ONT 1D ligation sequencing kits (SQK-LSK108 for Epi0076A and SQK-LSK109 for SD607) with the native barcoding expansion kit (EXP-NBD103; Oxford Nanopore). MinION sequencing was done on an R9.4 SpotON flow cell with a MinION MK1b device. The generated fast5 ONT reads were base called and demultiplexed using the ONT Albacore software (v2.0.1) for Epi0076A and the Guppy software (v3.2.4) for SD607. The software Cutadapt (v2.5) was used for end trimming and size sorting (48). The complete genomes were *de novo* assembled using the Unicycler assembly pipeline (v0.4.4) run with default parameters, paired-end Illumina reads, and ONT reads with a minimum length of 12 kb for Epi0076A and 10 kb for SD607 (49). The Illumina HiSeq reads of SD607 were used without processing, while the MiSeq reads of Epi0076A were prior quality filtered using Trimmomatic v0.36 to ensure removal of Illumina adaptor sequences (parameter, ILLUMINACLIP: NexteraPE-PE.fa:2:30:10), an average quality per base of at least 15 (parameters, LEADING:3, TRAILING:3, and SLIDINGWINDOW:4:15), and a minimum length of 36 bases (parameter, MINLEN:36) (50). Illumina reads of SD607 were additionally assembled using SPAdes (v3.12.0), with the mismatch careful option (51). Calculations were performed on UBELIX (https://www.unibe.ch/universitaet/campus__und__infrastruktur/rund_um_computer/soft_und_hardware/hardware/hochleistungsrechner_hpc_grid/index_ger.html), the High-Performance Computing (HPC) cluster at the University of Bern. The complete genomes of Epi0076A and SD607 were annotated using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) service. Additionally, features present in the McRI$_{msr}$ islands of strains Epi0076A, SD607, and KM0218 were annotated with Prokka (v1.13) (52) and edited manually. Repeat sequences were identified by BLASTN, promoters and transcription start sites by PBROM (53), and transcription terminators by ARNOLD (54). The secondary structure of the nucleotide sequence upstream of *mef*(D)-*msr*(F) was predicted using the RNAfold Web server (55). Putative protein function was analyzed by searching against PROSITE entries and Conserved Domains Databases (Conserved Domain Search Service [CD] search with NCBI-curated position-specific scoring matrices [PSSMs]) (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

Detection of recombination products. Circular excision of *mef*(D)-*msr*(F) fragments in strain Epi0076A was analyzed by PCR (Table S1). A high-quality genomic DNA template (around 200 ng per reaction) was used for the detection of chromosomal deletion products and Chelex extracts (2 μl per reaction) for the detection of circular forms. The obtained PCR products were analyzed on agarose gels and confirmed by Sanger sequencing (Fig. S3).

Data availability. The nucleotide sequences of *rpsI*-downstream regions with manually curated features were deposited in GenBank under the accession numbers MN728681, MN728682, and BK011995 for *M. canis* strains Epi0076A, SD607, and KM0218, respectively. The complete genome sequences of Epi0076A and SD607 are available under GenBank accession numbers CP047363 to CP047365 and CP047361 and CP047362, respectively (NCBI BioProject no. PRJNA596733).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.5 MB.

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