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Novel antimicrobial genetic elements in methicillin-resistant *Macrococcus armenti*

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Dear Editor:

Macrococcus belongs to the commensal flora of mammals, but some of the species have been occasionally recovered from infection sites in animals and humans.[1] They have the capacity to acquire antimicrobial resistance genes on different types of mobile genetic elements including the methicillin resistance genes *mecB* and *mecD* on staphylococcal cassette chromosome *mec* (SCC*mec*) elements SCC*mecB* and SCC*mecD*, *Macrococcus* resistance islands ($McRI_{mecD}$), and *mecB*-containing plasmids.[1] *M. armenti* was described as a new species colonizing the nasal cavities and skin of calves and pigs in 2022.[2] Following this description, two *Macrococcus* sp. strains sharing the same SmaI pulsotype (CCM 2607= B-P 25 and CCM 2609 =B-P 26) isolated from pig-derived samples (origin not specified, but either skin of pigs or bacon) in 1963 and deposited in the Czech Collection of Microorganisms (CCM) were also classified as *M. armenti*.[3] Here, we determined the antimicrobial susceptibility and compared whole genome sequences of recent strains isolated in 2017, 2019 and 2021 and one of the oldest strains from the 1960s to identify mobile genetic elements containing resistance genes and their genomic locations.

The complete genomes of *M. armenti* were obtained by hybrid assembly with Unicycler v0.4.4 software (https://github.com/rrwick/Unicycler) using Illumina and Oxford Nanopore technologies (ONT) reads for JEK37^{T}, JEK46, JEK29, JEK12, 17Msa1131, 19Msa0295 and 19Msa0966[2] and using IonTorrent and ONT sequence reads for strain CCM 2609. Antimicrobial resistance genes were detected using ResFinder 4.1 (https://cge.food.dtu.dk/services/ResFinder/). Minimal inhibitory concentrations (MICs) of 20 antimicrobials were determined by broth microdilution susceptibility testing in Müller-Hinton broth containing 5% laked horse blood using Sensititre™ EUST plates (Thermo Fischer Scientific) and a 96-well plate containing 2-fold dilutions of oxacillin (Sigma–Aldrich) ranging from 0.5 to 256 mg/L following the standard M07 of the Clinical and Laboratory

Standards Institute (CLSI). Interpretation of the MICs was performed using CLSI and EUCAST clinical resistance breakpoints set for *Staphylococcus* spp. (Table S1).

All strains were susceptible (MIC in parentheses) to chloramphenicol, ciprofloxacin, gentamicin, kanamycin, linezolid, quinupristin/dalfopristin, rifampin, sulfamethoxazole, tetracycline, trimethoprim, and vancomycin (Table S1). The strains also showed low MICs for mupirocin and variable MICs for tiamulin and streptomycin for which no breakpoints are available. All strains, except CCM 2607 and CCM 2609, were resistant to oxacillin and penicillin and contained the methicillin resistance gene *mecD* (Table S1). Three of the *mecD*-containing strains exhibited an MIC lower than the MIC resistance breakpoint of cefoxitin (≥ 8 µg/ml) used for the detection of methicillin resistance in *S. aureus*, indicating that oxacillin should be preferred to predict methicillin resistance in *M. armenti*, as already proposed for other *Macrococcus* species.[4] Nevertheless, further larger studies are still needed to determine whether oxacillin or cefoxitin best predicts methicillin resistance in *Macrococcus* spp. Strains 19Msa0295, JEK29, JEK12 and JEK46 were resistant to erythromycin (MIC >8µg/ml), showed inducible resistance to clindamycin as determined by the D-zone test and contained the macrolide-lincosamide-streptogramin B (MLS_B) resistance gene *erm*(45).[5] Strains 19Msa0966 and 19Msa0295 exhibited high MIC to streptomycin (MIC >32 µg/ml) and harbored the streptomycin O-adenylyl transferase gene *ant(6)-Ia*. All strains except 17Msa1131 were also resistant to fusidic acid (MIC 2–4 µg/ml) but encoded no known resistance genes (Table S1).

The locations of the antimicrobial resistance genes on integrated mobile genetic elements were analyzed by BLASTN comparative analysis and illustrated using easyFig v2.1 (Figure 1). The *mecD* gene was located in the chromosomal resistance island inserted downstream of the *rpsI* gene, designated $McRI_{mecD}$-1, following a previously established classification system.[1] However, strains JEK37^T, JEK29, JEK46 and JEK12 did not contain the DNA recombination mediator protein gene *dprA*, and strains 19Msa0295 and 19Msa0966 contained

an insertion of 7,980 bp between the *mecD* and *hsmRI-hsrRI* genes (Figure 1A). This insertion contained the *ant(6)-Ia* gene and was delimited by open reading frames (*orfs*) encoding a recombinase and a transposase of an insertion sequence of the IS*1380* family (Figure 1A). This structure was also found in *Enterococcus faecium* plasmids (e.g., GenBank acc. nos. LR134110.1 and CP093943.1) and chromosome (GenBank acc. no. LR135169.1) as well as within an integrative and conjugative element (ICE*Ssu*$_{SC84}$) in *Streptococcus suis* (GenBank acc. no. FM252031.1). The *erm*(45) gene was present on a 10,439-bp chromosomal element flanked by two direct repeats (DRs) downstream of the *guaA* gene, similar to *Mammaliicoccus fleuretti*, where it was first described as a SAPI-like genomic island[5] (Figure 1B). The regions flanking the *erm*(45) gene were identical in both genera, but the 5'-end region containing a similar putative site-specific tyrosine integrase (*int*) differed in both elements. The *M. armenti* strains also contained diverse plasmids and putative prophages as identified by PHASTER, but none of these elements contained antimicrobial resistance genes (Table S2).

This comparative genomic analysis of decades-old and more recent isolates showed that *M. armenti* acquired antimicrobial resistance genes through the integration of mobile genetic elements similar to those found in other *Macrococcus* and *Mammaliicoccus* species, as well as in *Enterococcus* and *Streptococcus*. It also highlighted that additional antimicrobial resistance genes such as *ant(6)-Ia* can insert into McRI$_{mecD}$. The presence of different types of mobile genetic elements in *M. armenti* again underlines the propensity of *Macrococcus* to evolve and adapt its genetic material to survive antimicrobial selective pressure, as is commonly exerted in animal husbandry.

GenBank/EMBL/DDBJ accession numbers: The complete genome sequences of strains JEK37^{T}, JEK46, JEK29, JEK12, 17Msa1131, 19Msa0295, 19Msa0966 and CCM 2609 have been deposited into GenBank under accession numbers CP083608-CP083609, CP083604-

CP083607, CP083602-CP083603, CP083598-CP083601, CP083595-CP083597, CP083594, CP083592-CP08359 and CP094348-CP094350.

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Figure legend

Figure 1. Structure of the *Macrococcus* chromosomal resistance island McRI$_{mecD}$-1 (A) and *erm*(45)-containing elements (B) in *Macrococcus armenti*. Genes are represented by arrows and are color-coded: (A) light yellow, integrase *int0819*; pink, potential virulence factor gene *virE*; orange, *mecD* operon genes; green, genes *hsmRI-hsrRI* encoding a restriction-modification system; light blue, DNA recombination-mediator protein gene *dprA*; light pink, recombinases and transposase of IS*1380*; red, aminoglycoside adenylyl transferase gene *ant(6)-Ia*; (B) orange, GMP synthase gene *guaA* and transcriptional regulator gene *lysR*; blue, integrase *int*; green, 23S rRNA methyltransferase gene *erm*(45) for macrolide-lincosamide-streptogramin B (MLS$_B$) resistance. Integration sites are indicated by black arrows representing direct repeats (DRs) in McRI$_{mecD}$-1 (A) and attachment (attL, attR) and integration (attB) sites in *erm*(45)-containing elements (B). Gray connections indicate regions sharing 74% to 100% nucleotide (nt) sequence identity. 1) The displayed regions of *M. armenti* strains JEK12, JEK29 and JEK46 share the same structure with >99.9% nt sequence identity; 2) The displayed regions of *M. armenti* strains JEK12, JEK29 and JEK46 share >99.7% nt sequence identity. T, type strain. Figures were generated using Easyfig software and Microsoft PowerPoint.

Supplementary Tables

Table S1. Minimal inhibitory concentrations (MICs) of antimicrobials and resistance genes for *Macrococcus armenti*.

Table S2. Acquired genetic elements and their genomic locations in *Macrococcus armenti*.

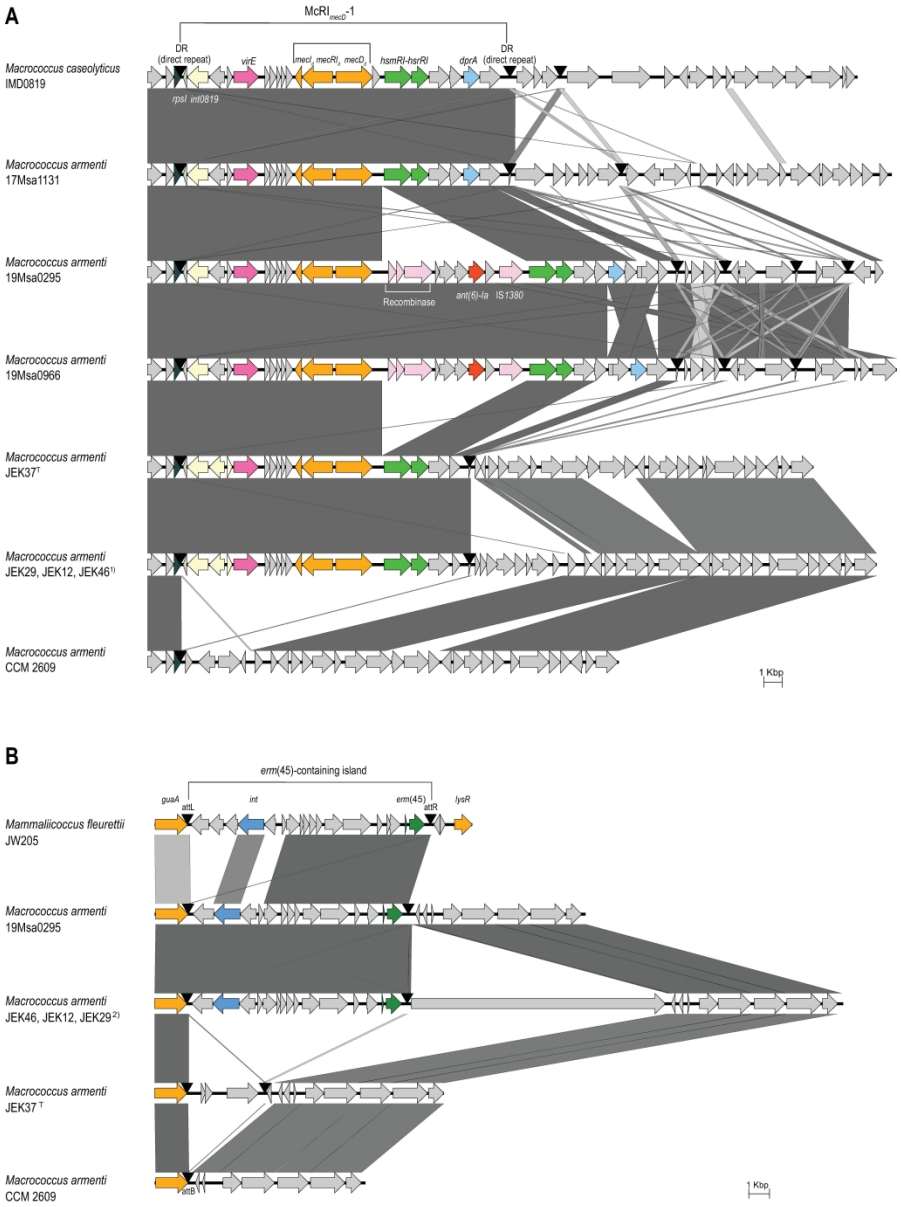

Figure 1. Structure of the *Macrococcus* chromosomal resistance island McRI$_{mecD}$-1 (A) and *erm*(45)-containing elements (B) in *Macrococcus armenti*.

242x326mm (600 x 600 DPI)

Supplementary Table S1. Minimal inhibitory concentrations (MICs) of antibiotics and antibiotics resistance genes for *Macrococcus armenti*

Antibiotics and antibiotic resistance genes[a)]	Resistance breakpoints[b)]	MIC [in µg/ml] of antibiotics and presence of resistance genes for *M. armenti* strains								
		JEK29	JEK37	JEK12	JEK46	19Msa0966	19Msa0295	17Msa1131	CCM2607	CCM2609
Oxacillin	≥1	64	256	64	128	16	16	>256	≤0.5	≤0.5
mecD		+	+	+	+	+	+	+	-	-
Cefoxitin	≥8	16	>16	≤0.5	16	2	2	>16	≤0.5	≤0.5
mecD		+	+	+	+	+	+	+	-	-
Penicillin	≥0.25	>2	>2	>2	>2	2	2	>2	≤0.12	≤0.12
mecD		+	+	+	+	+	+	+	-	-
Erythromycin	≥8	>8	≤0.25	>8	>8	≤0.25	>8	≤0.25	≤0.25	≤0.25
erm(45)		+	-	+	+	-	+	-	-	-
Clindamycin	≥4	1[c)]	0.25	0.5[c)]	1[c)]	0.25	0.25[c)]	0.25	0.25	0.5
erm(45)		+	-	+	+	-	+	-	-	-
Streptomycin	NA	8	8	≤4	8	>32	>32	8	≤4	≤4
ant(6)-Ia		-	-	-	-	+	+	-	-	-
Gentamicin	≥16	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1
Kanamycin	>8	≤4	≤4	≤4	≤4	≤4	≤4	≤4	≤4	≤4
Tetracycline	≥16	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5
Rifampicin	≥4	≤0.016	≤0.016	≤0.016	≤0.016	≤0.016	≤0.016	≤0.016	≤0.016	≤0.016
Fusidic acid	>1	4	2	4	4	4	4	≤0.5	2	2
Chloramphenicol	≥32	≤4	≤4	≤4	≤4	≤4	≤4	≤4	≤4	≤4
Tiamulin	NA	4	>4	4	>4	>4	>4	>4	>4	>4
Quinupristin/Dalfopristin	≥4	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	1	≤0.5	1	1
Vancomycin	≥32	≤1	≤1	≤1	2	≤1	≤1	≤1	≤1	≤1
Ciprofloxacin	≥4	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25
Linezolid	≥8	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1
Mupirocin	NA	1	1	2	1	≤0.5	1	1	1	1
Trimethoprim	≥16	≤2	≤2	≤2	≤2	≤2	≤2	≤2	8	8
Sulfamethoxazole	≥512	≤64	≤64	≤64	≤64	≤64	≤64	≤64	≤64	≤64

a) Antibiotic resistance genes and function: *ant(6)-Ia*, streptomycin nucleotidyltransferase gene; *erm*(45), macrolide, lincosamide and streptogramin 23S-rRNA methylase gene; *mecD*, transpeptidase coding gene [PBP2a (penicillin binding protein)] for resistance to all β-lactam antibiotics. +, presence of the gene; -, absence of the gene.

b) The resistance breakpoints presented here are those for *Staphylococcus* spp. from The Clinical and Laboratory Standards Institute (CLSI) for human isolates (CLSI supplement M100, 31st ed., 2022), except for those of fusidic acid and kanamycin which come from EUCAST (www.eucast.org). NA, not available.

c) The inducible resistance to clindamycin was determined by D-test as recommended by the Clinical and Laboratory Standards Institute (CLSI) for staphylococci spp. (CLSI supplement M100, 31st ed., 2022).

Supplementary Table S2. Acquired genetic elements and their genomic locations in *Macrococcus armenti*

Strains	Chromosome GenBank acc. no.	Chromosomal elements									Plasmids		
		McRI$_{mecD}$-1			*erm*(45)-containing island			Candidate prophage					
		Position	Size (bp)	Antibiotic resistance gene	Position	Size (bp)	Antibiotic resistance gene	Position	Size (bp)	Closest related phage	Name	Size (bp)	Plasmid GenBank acc. no
JEK29	CP083602	222687-238666	15980	*mecD*	2206741-2196303	10439	*erm*(45)	1019793-1069893 1652445-1691675	50101 39231	*Staphylococcus* phage StB12 *Staphylococcus* phage 2638A	pJEK29	2566	CP083603
JEK37	CP083608	225422-241401	15980	*mecD*	-	-	-	1362983-1399705 1675672-1729924	36723 54253	*Staphylococcus* phage phiRS7 *Bacillus* phage Mgbh1	pJEK37	1451	CP083609
JEK12	CP083598	222687-238666	15980	*mecD*	2206843-2196405	10439	*erm*(45)	1019730-1069830 1652382-1691612	50101 39692	*Staphylococcus* phage StB12 *Staphylococcus* phage 2638A	pJEK12-1 pJEK12-2 pJEK12-3	62574 28153 2566	CP083599 CP083600 CP083601
JEK46	CP083604	222778-238757	15980	*mecD*	2216171-2205733	10439	*erm*(45)	1019821-1069921 1652473-1691703	50101 39231	*Staphylococcus* phage StB12 *Staphylococcus* phage 2638A	pJEK46-1 pJEK46-2 pJEK46-3	62574 28227 2566	CP083605 CP083606 CP083607
19Msa0966	CP083592	223643-251043	27401	*mecD* *ant(6)-Ia*	-	-	-	1393515-1436408	42893	*Staphylococcus* phage StB20	p19Msa0966	25171	CP083593
19Msa0295	CP083594	220880-248280	27401	*mecD* *ant(6)-Ia*	2207271-2196833	10439	*erm*(45)	1370838-1410899 1880388-1925657	40062 45269	*Staphylococcus* phage CNPx *Listeria* phage 2389	-	-	-
17Msa1131	CP083595	221566-239760	18195	*mecD*	-	-	-	1023582-1071604 1415633-1456733	48023 41101	*Bacillus* phage PM1 *Staphylococcus* phage SpT99F3	p17Msa1131-1 p17Msa1131-2	1447 1446	CP083596 CP083597
CCM2609	CP094348	-	-	-	-	-	-	1003539-1049107	45569	*Geobacillus* phage TP 84	-	-	-