

The novel macrolide resistance genes *mef(F)* and *msr(G)* are located on a plasmid in *Macrococcus canis* and a transposon in *Macrococcus caseolyticus*

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Objectives: To analyse macrolide resistance in a *Macrococcus canis* strain isolated from a dog with an ear infection, and determine whether the resistance mechanism is also present in other bacteria, and associated with mobile genetic elements.

Methods: The whole genome of *M. canis* Epi0082 was sequenced using PacBio and Illumina technologies. Novel macrolide resistance determinants were identified through bioinformatic analysis, and functionality was demonstrated by expression in *Staphylococcus aureus*. Mobile genetic elements containing the novel genes were analysed *in silico* for strain Epi0082 as well as in other bacterial strains deposited in GenBank.

Results: *M. canis* Epi0082 contained a 3212 bp operon with the novel macrolide resistance genes *mef(F)* and *msr(G)* encoding a efflux protein and an ABC-F ribosomal protection protein, respectively. Cloning in *S. aureus* confirmed that both genes individually confer resistance to the 14- and 15-membered ring macrolides erythromycin and azithromycin, but not the 16-membered ring macrolide tylosin. A reduced susceptibility to the streptogramin B pristinamycin IA was additionally observed when *msr(G)* was expressed in *S. aureus* under erythromycin induction. Epi0082 carried the *mef(F)*–*msr(G)* operon together with the chloramphenicol resistance gene *fexB* in a novel 39 302 bp plasmid pMiCAN82a. The *mef(F)*–*msr(G)* operon was also found in macrolide-resistant *Macrococcus caseolyticus* strains in the GenBank database, but was situated in the chromosome as part of a novel 13 820 bp or 13 894 bp transposon Tn6776.

Conclusions: The identification of *mef(F)* and *msr(G)* on different mobile genetic elements in *Macrococcus* species indicates that these genes hold potential for further dissemination of resistance to the clinically important macrolides in the bacterial population.

Introduction

Macrococcus is a genus of Gram-positive cocci closely related to *Staphylococcus*.^{1,2} Previously they were thought to include non-pathogenic species from the skin of mammals;^{1,3} however, some of them have recently been associated with diseases in animals^{4–6} and humans.⁷ *Macrococcus* has also been considered as a possible reservoir for antimicrobial resistance genes of pathogenic *Staphylococcus* species.^{2,8–10}

Co-localization of *mef(A)* variants and *msr(D)* genes within an operon is well known in *Streptococcus* species that exhibit resistance against 14- and 15-membered ring macrolides but remain susceptible to 16-membered ring macrolides.^{11–15} Recently, a distantly related operon carrying the macrolide resistance genes *mef(D)* and *msr(F)* was identified in both *Macrococcus canis* and *Staphylococcus aureus*.¹⁶ Mef proteins

belong to the major facilitator superfamily (MFS) proteins involved in macrolide efflux, and Msr proteins are ATP-binding cassette subfamily F (ABC-F) proteins that mediate ribosomal protection against macrolides and in some cases also against streptogramin B.¹⁷

Neither the new *mef(D)* and *msr(F)* genes nor any of the macrolide resistance genes commonly found in Gram-positive bacteria could be identified in the macrolide- and chloramphenicol-resistant *M. canis* strain Epi0082 isolated from a dog with otitis externa in Switzerland.⁴ We therefore conducted a next-generation sequencing (NGS)-based analysis to identify the antibiotic resistance genes in *M. canis* Epi0082 and tested possible new macrolide resistance genes for functionality in *S. aureus*. Furthermore, the mobile genetic elements carrying these genes in Epi0082 as well as in other bacterial strains deposited in GenBank were investigated.

Materials and methods

Bacterial strains, vectors and growth conditions

M. canis strain Epi0082 was isolated from a Newfoundland dog with otitis externa in Switzerland and assigned to ST5 in a previous study.⁴ *Escherichia coli* DH5 α was used for cloning and amplification of pTSSCm-based shuttle plasmids.¹⁸ *S. aureus* strain RN4220 was used as the recipient of the pTSSCm-based constructs and for phenotypic gene expression.¹⁹ *M. canis* was routinely cultivated on trypticase soy agar plates containing 5% sheep blood (TSA-SB) (Becton Dickinson Company). *E. coli* and *S. aureus* strains were grown either in LB broth with shaking or on LB agar plates. *E. coli* and *S. aureus* strains containing pTSSCm-based plasmids [*tet*(L)] were selected and grown on LB agar containing tetracycline 10 mg/L. All strains were grown under aerobic conditions at 37°C.

NGS, assembly, annotation and bioinformatic analysis

The genome of *M. canis* Epi0082 was sequenced by both long (Pacific Biosciences) and short reads (HiSeq Illumina, 2×150 bp paired-end) technologies. The initial *de novo* genome assembly was performed with Canu v1.9²⁰ using only long reads. This initial draft genome assembly was subsequently used as a scaffold in Unicycler v0.4.4²¹ together with the Illumina short reads in order to obtain high-quality circular contigs.

ORFs were predicted and annotated using Prokka²² and the NCBI Prokaryotic Genome Annotation Pipeline.²³ Antibiotic resistance genes were identified using ResFinder v3.2²⁴ and by manual BLAST analysis. The hypothetical functions of ORFs of plasmid pMiCAN82a were predicted with BLASTP,²⁵ UniProt²⁶ and PROSITE^{27,28} databases. For the *mef*(F)–*msr*(G) operon, promoter and transcription start sites (TSSs) were identified using BPROM,²⁹ and the mRNA secondary structure of the operon's leader region was analysed using RNAfold³⁰ and ARNold³¹ tools.

A search for *mef*(F)–*msr*(G)-like genes was performed against the nucleotide collection (nr/nt) and the whole-genome shotgun contigs (wgs) databases of the NCBI. Evolutionary analysis of the amino acid sequences of Mef and Msr protein was performed using the UPGMA method (Phylogeny test by Bootstrap method, 2000 replications) in MEGA v10.1.5³² based on a multisequence alignment executed using MUSCLE³³ (Gap open = –2.90, Gap extend = 0.0). The percentage amino acid identity was determined by multiple sequence alignment using ClustalO.³⁴

Gene structures were visualized using DNAPlotter Release 18.1.0³⁵ for plasmid pMiCAN82a and Easyfig v2.1³⁶ for transposon Tn6776.

Plasmid construction

Plasmids used for gene expression were generated using the promoterless *E. coli*–*S. aureus* shuttle vector pTSSCm¹⁸ and DNA inserts amplified from *M. canis* Epi0082. Template DNA was extracted from strain Epi0082 with the DNeasy Blood & Tissue Kits (Qiagen GmbH) using the protocol for Gram-positive bacteria and enzymatic lysis buffer supplemented with 50 mg/L lysostaphin. Plasmids pJEFBG, pJEFMEF and pJEFMSR were constructed by restriction enzyme-based cloning (Figure S1, available as [Supplementary data](#) at JAC Online). DNA inserts were amplified with the Phusion Hot Start II High-Fidelity DNA polymerase (Thermo Scientific) using the primers and conditions listed in Table S1. PCR amplicons and vector pTSSCm were digested with restriction endonucleases KpnI and PstI to facilitate T4 DNA ligation (Table S1). Plasmid pJEFMSR2 was generated through PCR-based mutagenesis of pJEFBG that resulted in deletion of *mef*(F) and an amplicon with homologous ends for *in vivo* recombination (Table S1). The pJEFBG template DNA was digested with DpnI. Constructs were obtained in *E. coli* DH5 α by heat shock transformation and selection on LB agar containing 10 mg/L tetracycline. Plasmid DNA from *E. coli* was isolated using the peqGOLD Plasmid Miniprep Kit I (Peqlab Biotechnologie GmbH). Generated plasmids were confirmed by restriction digestion and Sanger sequencing prior to electroporation into *S. aureus* RN4220.³⁷

Antimicrobial resistance profiles

The MICs of one representative of each of the 14-, 15- and 16-membered ring macrolides, namely erythromycin, azithromycin and tylosin, as well as the streptogramin B pristinamycin IA, were determined for the *S. aureus* RN4220 and *M. canis* strains in Müller–Hinton broth by the microdilution method according to the CLSI recommendations (Table 1).³⁸ Serial fold dilutions ranging from 0.125 to 64 mg/L were prepared in 96-well plates for erythromycin, azithromycin and pristinamycin IA. A Sensititre™ NLVET9 plate (Thermo Fisher Scientific) was used to determine the MIC for tylosin (range 0.06 to 128 mg/L). Inducible resistance to pristinamycin IA and tylosin was measured in the presence of 1 and 2 mg/L erythromycin.

Reverse transcription–PCR (RT–PCR)

To analyse the transcription of *mef*(F) and *msr*(G) genes, total RNA was extracted from strains *M. canis* Epi0082 and *S. aureus* RN4220 containing pJEFBG using the Quick-RNA Fungal/Bacterial Miniprep Kit (Zymo Research) and following the standard manufacturer's protocol. RNA was extracted from a culture grown in the presence or absence of 2 mg/L erythromycin, followed by a DNA digestion treatment using DNase I (provided in the kit). cDNA was generated by reverse transcription using the murine leukaemia virus reverse transcriptase (Roche Diagnostics) and the leading region-targeting LR-F primer (Table S1). DNA fragments targeting a 170 bp leading region of the operon and a 404 bp region spanning the end of the *mef*(F) gene, the intergenic region and the beginning of the *msr*(G) gene were amplified by PCR using the Taq polymerase (FIREPol® DNA Polymerase, Solis BioDyne) with specific primers and conditions listed in Table S1. Samples not incubated with the reverse transcriptase during cDNA generation were included as negative controls to ensure the absence of genomic DNA.

Nucleotide sequence accession numbers

The complete genome of *M. canis* Epi0082 is available under GenBank accession numbers CP046363–CP046367 (NCBI Bioproject PRJNA590936). Plasmid pMiCAN82a was assigned accession number CP046364, and Tn6776 of *M. caseolyticus* 5459_5_49 was assigned accession number BK012114.

Results and discussion

Identification and structural organization of the novel macrolide resistance genes *mef*(F) and *msr*(G)

The genome of *M. canis* strain Epi0082 consisted of a 2 324 457 bp circular chromosome and four plasmids of 39 302 bp (pMiCAN82a), 19 741 bp (pMiCAN82b), 10 540 bp (pMiCAN82c) and 2565 bp (pMiCAN82d). The initial software-based search for antibiotic resistance genes only identified the chloramphenicol resistance gene *flexB* on plasmid pMiCAN82a that shared 99% nucleotide identity with *flexB* of *Enterococcus faecium* strain EFM-1 (GenBank accession number JN201336). Further individual analysis of the remaining ORFs of pMiCAN82a revealed the presence of an operon containing two adjacent genes related to macrolide resistance operons of the *mef*–*msr* type.^{11,13,16}

The putative new *mef* gene found in pMiCAN82a encodes a 397 amino acid MFS protein that contains the PROSITE profile PS50850 and exhibits 71% amino acid identity with the Mef(D) protein of *M. canis* (GenBank accession number MN728681) and less than 40% amino acid identity with all other known macrolide efflux proteins (Figure 1a). The second gene located in the operon is the putative new *msr* gene, which encodes a 485 amino acid ABC-F protein that contains two nucleotide-binding domains (PS50893) separated by

Table 1. MICs of 14-, 15- and 16-membered ring macrolides erythromycin, azithromycin, tylosin and the streptogramin B pristinamycin IA for *Staphylococcus aureus* and *Macrococcus canis* as determined by the broth microdilution method

Strain/plasmid	Origin and characteristics ^a	Reference	Antibiotic resistance genes ^b	MIC (mg/L)					
				ERY	AZM	TYL	iTYL	PIA	iPIA
<i>S. aureus</i>									
RN4220	Recipient strain for electrotransformation, plasmid free.	19		≤0.125	0.5	1	NA	8	NA
RN4220/pTSSCm	RN4220 with <i>S. aureus</i> - <i>E. coli</i> shuttle vector pTSSCm.	18	<i>tet</i> (L)	≤0.125	0.25	1	NA	8	NA
RN4220/pJEFMEF	RN4220 with <i>mef</i> (F) and its regulatory region cloned into pTSSCm.	This study	<i>mef</i> (F), <i>tet</i> (L)	4	8	0.5	1 ^c	8	8 ^c
RN4220/pJEFMSR	RN4220 with <i>msr</i> (G) and upstream 113 bp intergenic region cloned into pTSSCm.	This study	<i>msr</i> (G), <i>tet</i> (L)	≤0.125	0.5	0.5	NA	8	NA
RN4220/pJEFMSR2	RN4220 with <i>msr</i> (G) and the regulatory region upstream of <i>mef</i> (F) cloned into pTSSCm.	This study	<i>msr</i> (G), <i>tet</i> (L)	32	64	0.5	1	8	32
RN4220/pJEFBG	RN4220 with <i>mef</i> (F), its regulatory region and <i>msr</i> (G) cloned into pTSSCm.	This study	<i>mef</i> (F), <i>msr</i> (G), <i>tet</i> (L)	16	64	0.5	1	8	16
<i>M. canis</i>									
KM45013	<i>M. canis</i> type strain.	5	<i>mecB</i>	≤0.125	0.5	0.5	NA	2	NA
Epi0082	<i>M. canis</i> strain with plasmid pMiCAN82a.	4	<i>mef</i> (F), <i>msr</i> (G), <i>fexB</i>	8	16	0.5	0.25	2	2

ERY, erythromycin; AZM, azithromycin; TYL, tylosin; PIA, pristinamycin IA; iPIA and iTYL, tylosin and pristinamycin IA induced with erythromycin 2 mg/L; NA, not applicable.

^apTSSCm is a promoterless cloning vector.

^bAntibiotic resistance genes and function: *tet*(L), tetracycline efflux; *mef*(F), macrolide efflux; *msr*(G), ABC-F ribosomal protection for macrolide and inducible streptogramin B resistance; *fexB*, phenicol exporter; *mecB*, alternative penicillin-binding protein PBP 2a for methicillin resistance.

^cInduced with 1 mg/L erythromycin.

a 99 amino acid linker. This protein shares <67% amino acid identity with Msr(F) and Msr(H) of *M. canis* and <42% amino acid identity with all other characterized Msr proteins (Figure 1b). The new genes were assigned the names *mef*(F) and *msr*(G) according to the nomenclature for MLS genes, which defines a new gene if the deduced amino acid sequence shares <80% identity to any of the previously characterized genes (<https://faculty.washington.edu/marilynr>).³⁹

The *mef*(F) and *msr*(G) genes were organized in an operon [*mef*(F)-*msr*(G)] with *msr*(G) situated 117 bp downstream of *mef*(F). The presence of a PCR product that spans the *mef*(F) and *msr*(G) genes in the RT-PCR analysis confirmed co-transcription of both genes (Figure S2). A promoter search predicted a putative TSS 443 bp upstream of the *mef*(F) start codon with -10 (5'-ATATATAAT) and -35 (5'-TTGACT) promoter sequences located 14 and 33 bp upstream of the TSS, respectively. Analysis of the mRNA secondary structure of the 443 bp leader region identified several stem-loops, two putative transcription terminators, two ribosomal binding sites (RBSs) and a 14 codon leader peptide (MTHSMRLRSLSLNK) (Figure S3). The similarity of these features to those found in the leader region of inducible expressed *erm* and (*mef*)-*msr* genes suggests that *mef*(F)-*msr*(G) expression depends on the presence of erythromycin.⁴⁰ Our RT-PCR results further showed that the *mef*(F)-*msr*(G) transcript signal was stronger in the presence of erythromycin while the signal of the leader region was less affected by the presence or absence of the inducer (Figure S2). This observation suggests that expression of *mef*(F) and *msr*(G) is controlled by transcriptional

attenuation similar to the mechanism described for *erm*(K) and *mef*(A)-*msr*(D) genes.^{41,42}

Functional characterization of *mef*(F) and *msr*(G)

M. canis strain Epi0082 showed an at least 64-fold higher MIC of erythromycin and a 32-fold higher MIC of azithromycin compared with the type strain KM45013^T (Table 1). To demonstrate the association of the *mef*(F)-*msr*(G) operon with macrolide resistance, the genes were inserted individually or together into the shuttle vector pTSSCm¹⁸ and expressed in *S. aureus* RN4220 (Table 1). Four different pTSSCm constructs were generated, each containing either *mef*(F)-*msr*(G) (pJEFBG), *mef*(F) (pJEFMEF) or *msr*(G) under the control of the regulatory sequence of the operon (pJEFMSR2) [including 761 bp of DNA upstream of *mef*(F)] or *msr*(G) with only 113 bp of upstream DNA (pJEFMSR) corresponding to the intergenic region between *mef*(F) and *msr*(G) (Figure S1). The MIC of the 14-membered ring erythromycin for RN4220 harbouring the complete *mef*(F)-*msr*(G) operon on pJEFBG was 16 mg/L, corresponding to an at least 128-fold increased resistance compared with the control strains RN4220 and RN4220/pTSSCm (Table 1). A lower MIC of 4 mg/L was measured for RN4220 expressing only *mef*(F) from pJEFMEF, whereas a higher MIC of 32 mg/L was observed when *msr*(G) alone was expressed from the regulatory sequence upstream of *mef*(F) on plasmid pJEFMSR2. However, when *msr*(G) was cloned with the intergenic sequence upstream of *msr*(G) in pJEFMSR (Table 1), the MIC remained unchanged, indicating that the promoter sequence upstream of *mef*(F) is also

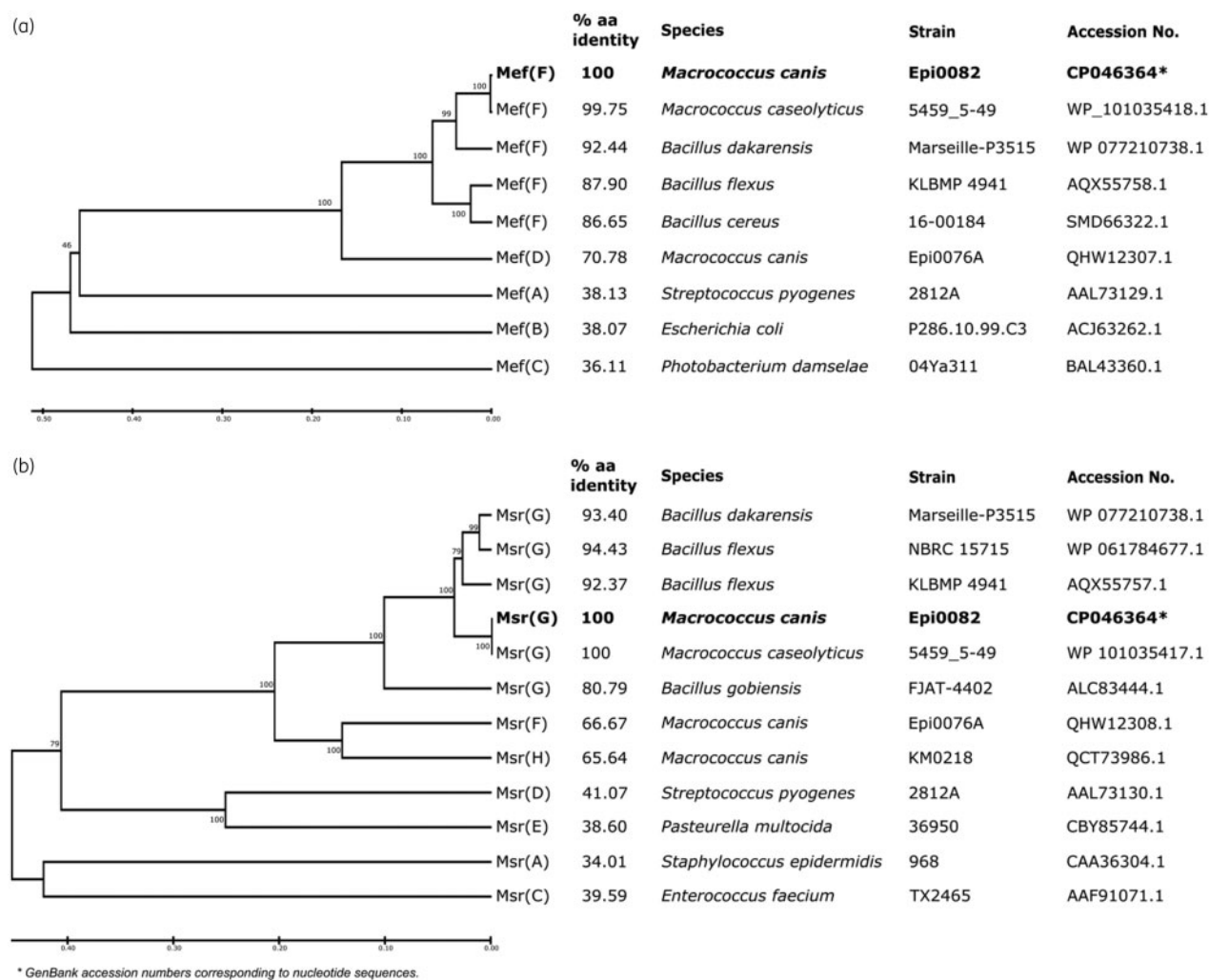


Figure 1. Evolutionary analysis of the amino acid (aa) sequences of Mef and Msr proteins from different families. (a) Clustering of Mef protein. (b) Clustering of Msr protein. The clustering of aa sequences was performed using MEGA v10.1.5.³² The percentage aa identity between the novel determinants Mef(D) and Msr(G) and related determinants is indicated.

required for expression of *msr(G)*. Determination of the MICs of 15- and 16-membered ring macrolides for the same constructs indicated that both genes can confer resistance to the 15-membered ring azithromycin but not to the 16-membered tylosin (Table 1). For *S. aureus*, MIC values increased 32-fold for RN4220 cells expressing *mef(F)* from pJEFMEF (MIC 8 mg/L) and 256-fold for cells expressing *msr(G)* from pJEFMSR2 (MIC 64 mg/L) compared with cells harbouring the empty vector pTSSCm (MIC 0.25 mg/L). The results also indicate that azithromycin functions as an inducer. Resistance to tylosin was not observed and could also not be induced with erythromycin (Table 1). Resistance against pristinamycin IA (with and without 2 mg/L erythromycin induction) was not observed for *M. canis* Epi0082. However, for RN4220 containing pJEFMSR2 and pJEFBG, an up to 2-fold higher MIC was observed under erythromycin induction, indicating that *msr(G)* also has an ability to mediate low-level streptogramin B resistance (Table 1).

Susceptibility testing performed with *S. aureus* strains clearly confirmed that the *mef(F)*-*msr(G)* operon from *M. canis* Epi0082

mediates resistance to macrolides. It also showed that both genes can function independently, although they are expressed together from the same operon promoter. The *msr(G)* gene appears to contribute more to the resistance phenotype than *mef(F)*, similar to observations made before for *mef*-*msr* operons in *M. canis* and *S. pyogenes*.^{12,16}

Characterization of genetic elements containing *mef(F)* and *msr(G)*

In *M. canis* Epi0082, the *mef(F)*-*msr(G)* operon was located on pMiCAN82a (Figure 2). This plasmid contained 42 ORFs and its G + C content was 28.3 mol% which was lower than that of the chromosome (36.5 mol%), except for a region containing the *mef(F)*-*msr(G)* operon and *fexB* that exhibited a higher GC content (32.3 mol%). This aforementioned 9317 bp region was bordered by 25 bp terminal inverted repeats (IRs) and flanking 6 bp direct repeats (DRs) (GAATCG), and contained ORFs similar to the

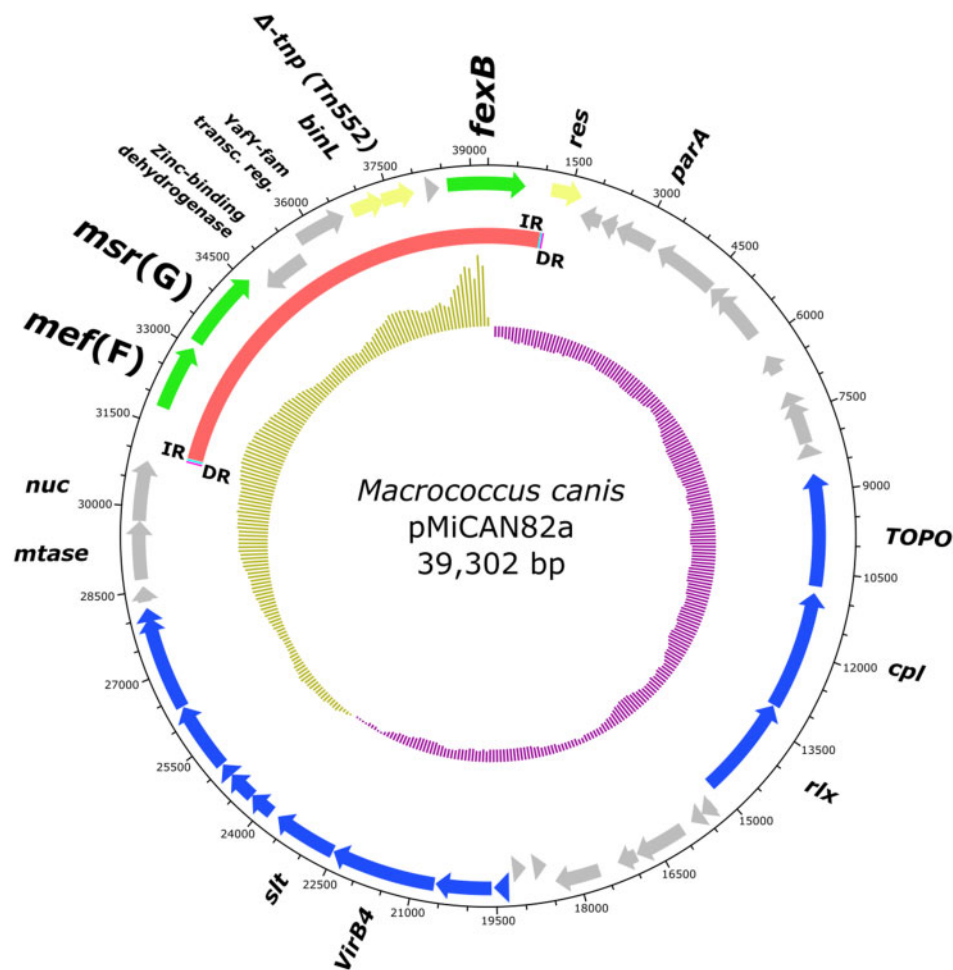


Figure 2. Genomic circular representation of plasmid pMiCAN82a including the *fexB* gene and the macrolide resistance operon containing the novel genes *mef(F)* and *msr(G)*. The inner circle represents GC% content, with the colour code corresponding to above/below average. Genes are represented by arrows and coloured by putative function: resistance genes are shown in green; putative conjugation-associated gene arrays are in blue; and recombinases are in yellow. Inverted repeats (IRs) [TTCTCATATATC(G/A)AGTAAAGTGACA] and direct repeats (DRs) (GAATCG) flanking the resistance region are indicated. Abbreviations: mtase, modification methylase; nuc, endonuclease; for other abbreviations, see text. The map was drawn using DNAPlotter (Release 18.1.0)³⁵ and the sequence of *M. canis* Epi0082 (CP046364). This figure appears in colour in the online version of JAC and in black and white in the printed version of JAC.

resolvase *binL* (99% nt identity) and part of the replicative transposase of Tn552 (89% nt identity) (GenBank accession number X52734) (Figure 2). The remaining part of pMiCAN82a (29 985 bp) was additionally found (with 97% coverage and >99% identity) in the genome of *M. caseolyticus* strain 5798_EF375 deposited in the GenBank database (accession numbers PIXG01000020.1 and PIXG01000024.1).⁴³ Interestingly, *M. caseolyticus* 5798_EF375 also carries the *mef(F)*–*msr(G)* operon (>99% nt identity), but in the chromosome (accession number PIXG01000018.1) (see below).

In pMiCAN82a, it was not possible to identify any proteins containing the classical domain of replication initiation proteins for theta-mode replication [RepA_N (pfam06970), Rep_3 (pfam01051) and PriCT_1 (pfam08708)].⁴⁴ Other features typically found in plasmids were present, including plasmid resolvase (Res) and partitioning proteins (ParA) as well as key proteins possibly

involved in conjugative transfer, such as DNA relaxase (Rlx) (pfam03389: MobA_MobL), coupling protein (Cpl) (COG3505: VirD4), conjugative transfer ATPase (VirB4) (TIGR00929: VirB4_CagE) and lytic transglycosylase (Slr) (COG2951: MltB) (Figure 2).

In the *M. caseolyticus* strain 5798_EF375 and in another three *M. caseolyticus* strains, 5459_5_49, 5781_EF64 and 5782_EF_83, all macrolide resistant from bovine sources in the UK,⁴³ the *mef(F)*–*msr(G)* operon was located in the chromosome downstream of the glutamine-fructose-6-phosphate transaminase gene (*glmS*) as part of a 13 820 bp or 13 894 bp element flanked by IRs (Figure 3). Recombinase genes unrelated to those found on plasmid pMiCAN82a were present, encoding proteins structurally related to transposition proteins TnsABCDE of Tn7 from *E. coli* (GenBank accession number KX117211).⁴⁵ The chromosomal location and the presence of TnsABCDE-related products suggest

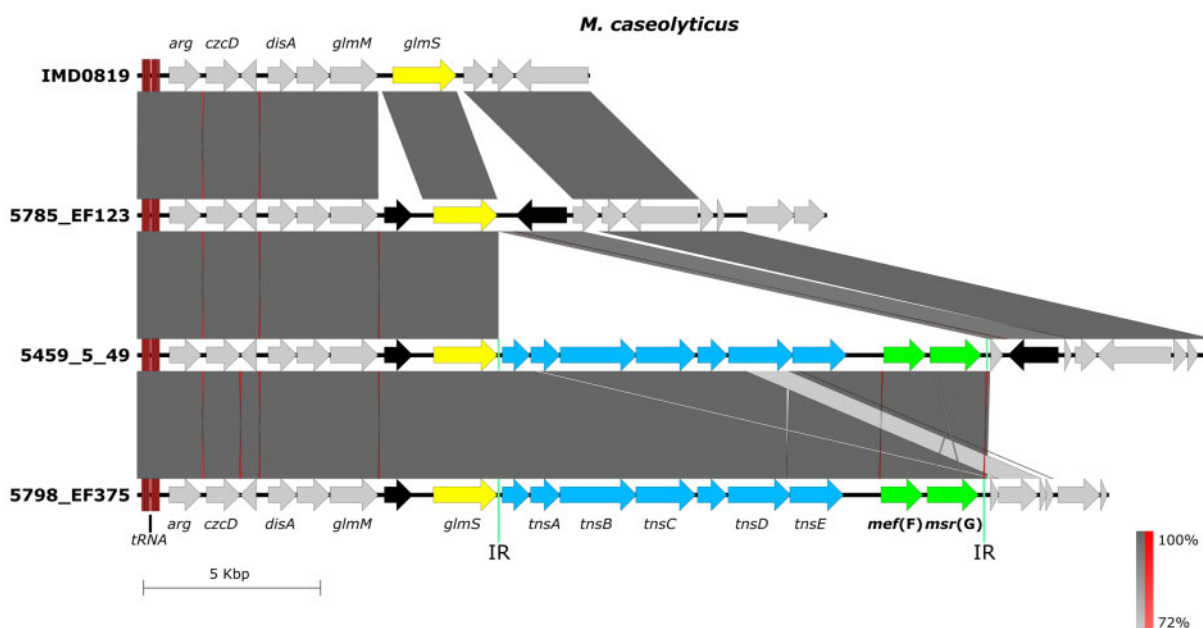


Figure 3. Transposon Tn6776 containing the *mef(F)*–*msr(G)* operon in *M. caseolyticus*. The transposon was inserted into the chromosome downstream of the glutamine-fructose-6-phosphate transaminase gene (*glmS*). Genes distantly related to those of Tn7 of *E. coli* (*tnsABCDE*) as well as the flanking inverted repeat [IR: 5'-TGTTGGTTTA(T/A)AATAAAG(A/T)TTGAT] are indicated. The figure was generated using Easyfig (v2.1)³⁶ and *M. caseolyticus* sequences of strains without the transposon IMD0819 (GenBank accession number CP021058.1) and 5785_EF123 (PIWZ00000000.1) and with the transposon 5459_5_49 (PIWU00000000.1) and 5798_EF375 (PIXG00000000.1). This figure appears in colour in the online version of JAC and in black and white in the printed version of JAC.

that *mef(F)*–*msr(G)* are carried in *M. caseolyticus* by a novel transposon distantly related to Tn7 that was assigned the name Tn6776 (<https://transposon.lstmed.ac.uk/>).⁴⁶ Furthermore, operons similar to *mef(F)*–*msr(G)* encoding proteins that share around 90% amino acid identities to Mef(F) and Msr(G) were identified in different *Bacillus* species (GenBank accession numbers CP016790.1, NZ_LT707408.1 and FWYT01000069.1) (Figure 1). These operons in *Bacillus* were located in the chromosome, and no further similarities to pMiCAN82a and Tn6776 were observed in their vicinity.

The identification of an operon containing the new macrolide resistance genes *mef(F)* and *msr(G)* in different species of the Staphylococcaceae and Bacillaceae families, indicates that these genes might be widespread in the environment. Both elements carrying *mef(F)*–*msr(G)* in *Macrococcus*, the plasmid pMiCAN82a of *M. canis* and the transposon Tn6776 of *M. caseolyticus*, pose a risk of further dissemination of macrolide resistance across bacterial populations.

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Transparency declarations

None to declare.

Supplementary data

Tables S1 and Figures S1 to S3 are available as [Supplementary data](#) at JAC Online.

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