

# Novel macrolide-lincosamide-streptogramin B resistance gene *erm(56)* in *Trueperella pyogenes*

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**ABSTRACT** Whole-genome sequence analysis of a macrolide, lincosamide, streptogramin B (MLS<sub>B</sub>)-resistant *Trueperella pyogenes* from a dog revealed a new 23S ribosomal RNA methylase gene *erm(56)*. Expression of the cloned *erm(56)* confers resistance to MLS<sub>B</sub> in *T. pyogenes* and *Escherichia coli*. The *erm(56)* gene was flanked by two IS6100 integrated on the chromosome next to a *sul1*-containing class 1 integron. GenBank query revealed additional *erm(56)*-containing elements in another *T. pyogenes* and in *Rothia nasimurium* from livestock.

**IMPORTANCE** A novel 23S ribosomal RNA methylase gene *erm(56)* flanked by insertion sequence IS6100 was identified in a *Trueperella pyogenes* isolated from the abscess of a dog and was also present in another *T. pyogenes* and in *Rothia nasimurium* from livestock. It was shown to confer resistance to macrolide, lincosamide, streptogramin B antibiotics in *T. pyogenes* and *E. coli*, indicating functionality in both Gram-positive and Gram-negative bacteria. The detection of *erm(56)* on different elements in unrelated bacteria from different animal sources and geographical origins suggests that it has been independently acquired and likely selected by the use of antibiotics in animals.

**KEYWORDS** macrolides-lincosamides-streptogramin B, mechanisms of resistance, DNA sequencing, gene expression

*Trueperella pyogenes*, a commensal Gram-positive bacterium of the skin and mucous membranes of animals, can cause suppurative infections in multiple animal species and rarely humans (1). Despite macrolides and lincosamides being antibiotics used as second-line treatment for these infections, their usage may contribute to the selection of antimicrobial resistances. So far, acquired resistances to macrolide, lincosamide, and streptogramin B (MLS<sub>B</sub>) antibiotics in *T. pyogenes* have been associated with the presence of erythromycin ribosome methylase (*erm*) genes, specifically *erm(B)* and *erm(X)*, that prevent the binding of the MLS<sub>B</sub> antibiotics to the 23S rRNA (2–4). In *T. pyogenes*, these genes have been reported within mobile genetic elements as either interposed between insertion sequence (IS) elements (5) or integrated in a class 1 integron together with other antimicrobial resistance genes (6).

## Detection and characterization of *erm(56)*

*T. pyogenes* strain 09KM1269, isolated from an abscess of a dog in 2009 in Switzerland, exhibited constitutive resistance to erythromycin and clindamycin as determined by Clinical and Laboratory Standards Institute (CLSI) criteria (7), suggesting the presence of an MLS<sub>B</sub> methylase (*Erm*) (Table 1). The absence of *erm(B)* and *erm(X)* as determined using previously described PCR assays (8) prompted us to search for the underlying resistance mechanism by whole-genome sequence analysis. Genomic DNA was extracted using MasterPure Complete DNA and RNA Purification Kit (Lucigen, Middleton, WI), sequenced on a PacBio Sequel IIe system (Next-Generation Sequencing Platform,

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University of Bern), and the resulting reads were *de novo* assembled using Flye 2.9.1 (9). Analysis of the complete genome using ResFinder 4.1 (Center for Genomic Epidemiology, Denmark) and blast search showed the absence of any so-far known *erm* gene listed in the Nomenclature Center for MLS<sub>B</sub> Genes (<https://faculty.washington.edu/marilynr/>) (10). GenBank query using tblastx (<https://blast.ncbi.nlm.nih.gov>) and *erm(X)* as subject sequence (GenBank accession number [NC\\_005206](https://www.ncbi.nlm.nih.gov/nuccore/NC_005206)) identified a novel putative *erm* gene. This gene encoded a 267-aa 23S rRNA methylase and showed the closest relatedness to the Erm(X) determinant of plasmid pAP2 from *T. pyogenes* with 58% amino acid (aa) and 54% nucleotide (nt) identity (Fig. 1) and was designated *erm(56)* (<http://faculty.washington.edu/marilynr/>) (10). Putative -35 (TTGACC) and -10 (TGCTAATGT) promoter sequences were identified using BPPROM (11) 31 and 11 bp upstream of the putative guanine transcription start located 153 bp upstream of *erm(56)*. This 153 bp upstream region contained the ribosomal binding sites and imperfect inverted repeats capable of folding into stem-loops which may play a role in the translational attenuation of the Erm(56) methylase (12) (Fig. S1).

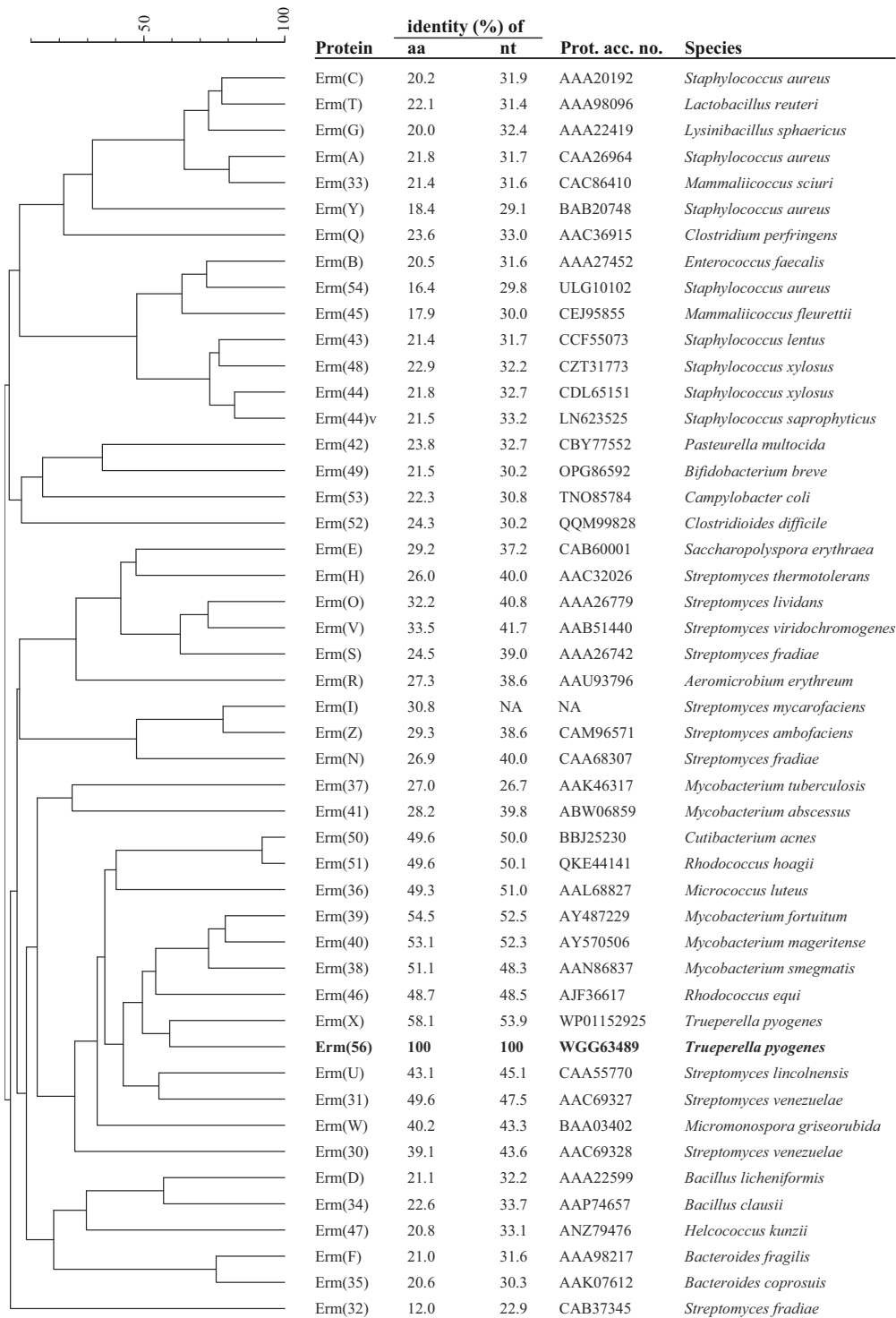
### Cloning and expression of *erm(56)*

To test the functionality of *erm(56)*, a 1,078 bp region of strain 09KM1269 including *erm(56)* and promoter sequences was amplified by PCR using Q5 High-Fidelity DNA Polymerase (New England Biolabs, MA, USA) and primers *erm56-Sall-F3* (5'-cacatgtcgacGCCATCACACTGCTTGTTC AACGA) and *erm56-SpeI-R* (5'-cacagactagtATTTTCTTGGCCCGCTCCCA GAG) (annealing temperature, 58°C; extension time, 1 min). The primers contained overhangs (lowercase) with restriction site sequences (underlined) to facilitate cloning into the Sall and SpeI restriction sites of pJRD215 (15). The resulting *erm(56)*-containing plasmid pJEM1269 was obtained in *Escherichia coli* DH5α after ligation and heat shock transformation and selection on LB agar plates containing 30 µg/mL kanamycin. Plasmid pJEM1269 was subsequently transformed by electroporation into susceptible strains of *E. coli* AG100A ( $\Delta$ *acrAB::KAN<sup>R</sup>*) (14) using electroporation cuvettes of 0.1 cm and settings of 2.3 kV/cm, 25 µF, 200 Ω and of *T. pyogenes* 13OD0707 (GenBank accession number [CP123403](https://www.ncbi.nlm.nih.gov/nuccore/CP123403)) using settings of 18 kV/cm, 50 µF, 246 Ω and a time constant of 10 ms (16). *E. coli* AG100A and *T. pyogenes* 13OD0707 electrotransformants were selected on LB containing 10 µg/mL erythromycin and BHI agar containing 5% sheep blood and 30 µg/mL kanamycin, respectively.

MIC values of erythromycin (macrolide), clindamycin (lincosamide) (Sigma-Aldrich, St-Louis, MO, USA), pristinamycin IA (streptogramin B), and pristinamycin IIA (streptogramin A) (Molcan Corporation, Richmond Hill, ON, Canada) of *E. coli* and *T. pyogenes* strains were determined by broth microdilution using Mueller-Hinton broth supplemented with 5% lysed horse blood and 48 h incubation for *Trueperella* following CLSI recommendations (7) (Table 1). When *erm(56)* was expressed from plasmid pJEM1269 in *T. pyogenes* 13OD0707, the MIC of MLS<sub>B</sub> antibiotics increased by more than 256-fold for erythromycin and clindamycin and by 16-fold for pristinamycin IA, while no difference was seen for the streptogramin A pristinamycin IIA as assumed for an Erm methylase. Increased MICs of erythromycin (64-fold) and clindamycin (8-fold) were also measured for AG100A containing pJEM1269, but no conclusion could be drawn for pristinamycin IA, for which the MIC was already high for the recipient strain and remained unchanged in the presence of *erm(56)* (Table 1).

### Genomic location of *erm(56)* and detection in additional bacteria

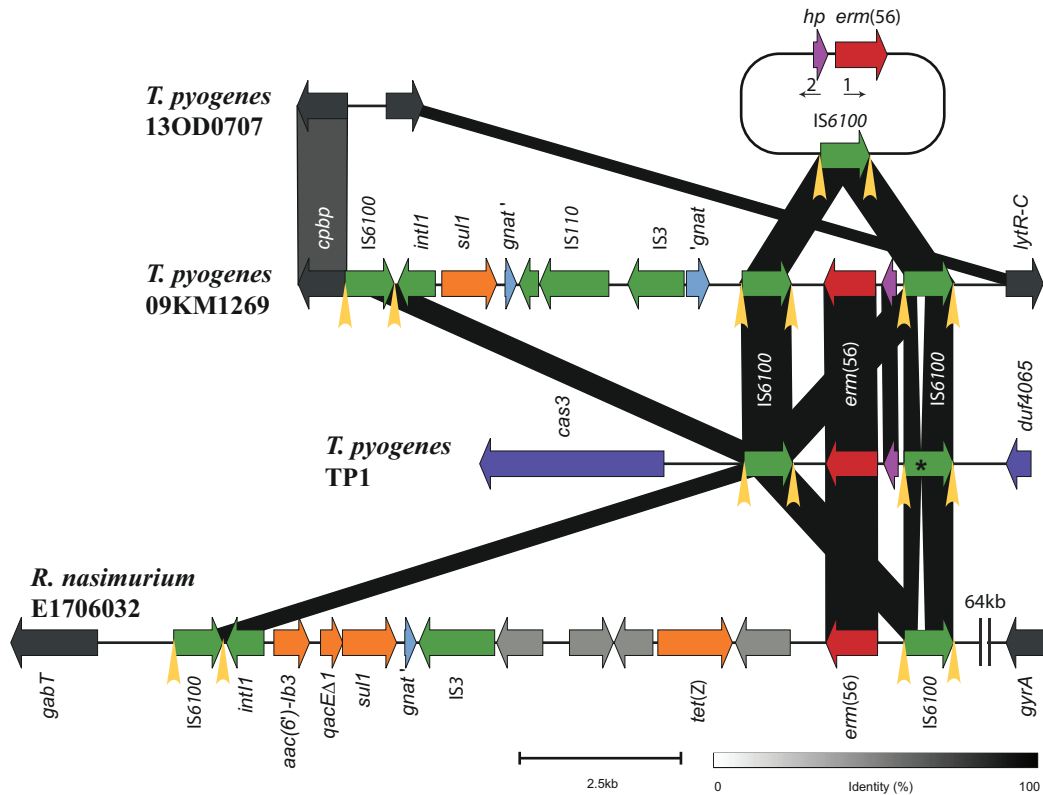
The *erm(56)* gene of *T. pyogenes* 09KM1269 was preceded by a hypothetical protein (hp) and integrated into the chromosome between two identical IS6100 elements located in the same orientation, each flanked by 14 bp inverted repeats (IR-L, GGCTCTGTTGCAAA; IR-R, TTTGCAACAGAGCC). The IS6100-hp-*erm(56)*-IS6100 element was situated next to a class 1 integron containing the sulfonamide resistance gene *sul1*, which was also delimited by a copy of IS6100 (Fig. 2). GenBank searches also identified *erm(56)* associated with IS6100 in *T. pyogenes* strain TP1 isolated from bovine lung in China (GenBank



**FIG 1** Relationship tree of all known Erm methylases, including the novel Erm(56) detected in *T. pyogenes* 09KM1269. Amino acid (aa) and nucleotide (nt) identity was obtained by sequence alignment using Clustal Omega 1.2.2 (Geneious Prime 2022.2.2, Biomatters Ltd., Auckland, New Zealand). The tree was constructed using BioNumerics 8.1.1 (BioMérieux, Marcy-l'Étoile, France) and the following settings: standard algorithm for pairwise alignment; open gap penalty, 100%; unit gap penalty, 0%; and unweighted pair group method using average linkages. The protein sequences are indicated by their GenBank accession numbers. The Erm(I) amino acid (aa) sequence is not available (NA) in the GenBank and was obtained from its original publication (13).

accession number CP033902) and in *Rothia nasimurium* strain E1706032 isolated from duck brain in China (GenBank accession number CP056080) (Fig. 2). In *T. pyogenes* TP1, the IS6100-hp-erm(56)-IS6100 element was found on a large 27.4 kb fragment which was related to the *Lactobacillus* phage PLE2 (GenBank accession number NC031036) using PHASTER (17). The sequence of the IS6100 situated upstream of erm(56) in *T. pyogenes* TP1 had a cytosine deletion at position 164 leading to a frameshift and an early stop codon in the IS6100 transposase. In *R. nasimurium* E1706032, erm(56) was only preceded by one IS6100 and integrated next to tet(Z) and a class 1 integron containing aac(6')-Ib3, qacEA1, and sul1; this resistance element was delimited by two copies of IS6100 (Fig. 2).

Although a circular conformation containing one copy of IS6100 was detected by PCR using primers pointing outward from erm(56) [erm56(159)-F, 5'-GGGACGATCTCTCAC-AGCTG and hp-erm56(45)-R, 5'-GAGAAGCTCGACCCAAACGAGGATCA; annealing temperature, 60°C; extension time, 2 min] and subsequent Sanger sequencing (Fig. 2), the erm(56) gene could not be transferred by either filter mating (19) into MLS<sub>B</sub> susceptible



**FIG 2** Schematic gene map showing the erm(56)-containing elements and flanking region of *T. pyogenes* 09KM1269 (GenBank accession number CP123393) compared to the chromosomal region of erm(56)-negative strain 13OD0707 (GenBank accession number CP123403), as well as the integration of erm(56) in *T. pyogenes* TP1 (GenBank accession number CP033902) and *R. nasimurium* E1706032 (GenBank accession number CP056080). Black and dark-gray areas represent regions showing 100% and 85% similarity at nucleotide level, respectively. Arrows represent open reading frames (ORFs). The ORF of erm(56) is indicated by a red arrow, and the erm(56)-preceding ORF (hypothetical protein gene, hp) is in purple. ORFs of other antibiotic resistance genes are shown in orange: sul1, dihydropteroate synthase gene for sulfonamide resistance; aac(6')-Ib3, aminoglycoside N-acetyltransferase gene; qacEA1, quaternary ammonium compound efflux transporter gene; tet(Z), tetracycline efflux transporter gene. Other ORFs of hypothetical proteins are represented by gray arrows. ORFs of transposase genes associated with IS and integrase (intl1) are indicated with green arrows. Asterisk (\*) within IS6100 of *T. pyogenes* TP1 indicates a cytosine deletion at position 164 leading to a frameshift of the transposase of IS6100. ORFs of phage protein genes are indicated in dark blue. The 3'-end truncated part (gnat') and the 5'-end truncated part ('gnat') of the GNAT N-acetyltransferase gene are indicated in light blue. ORFs of core genome genes are indicated as dark gray arrows. Inverted repeats of the IS6100 elements are indicated by yellow arrow heads (IR-L, GGCTCTGTTGCAAA; IR-R, TTTGCAACAGAGCC). Circular conformation of the IS6100-erm(56) element obtained by PCR using primers erm56(159)-F and hp-erm56(45)-R (indicated by small arrows 1 and 2) is represented by a circle containing the respective ORFs. The figure was generated using Clinker (18) and Adobe Illustrator.

**TABLE 1** MIC of erythromycin, clindamycin, pristinamycin IA, and pristinamycin IIA for different *T. pyogenes* and *E. coli* strains, as determined by broth microdilution

| Strain                      | Characteristic(s) or origin   | Reference or source | Antibiotic resistance gene(s) <sup>a</sup> | MIC (μg/mL) <sup>b</sup> |       |     |       |
|-----------------------------|---|---------------------|--|--------------------------|-------|-----|-------|
|                             |   |                     |  | ERY                      | CLI   | PIA | PIIA  |
| <i>T. pyogenes</i> 09KM1269 | Dog abscess sample  | This study          | <i>erm(56)</i> , <i>sul1</i>               | >256                     | 128   | 32  | ≤0.25 |
| <i>E. coli</i> AG100A       | Recipient strain, plasmid free, Δ <i>acrAB</i> ::KAN <sup>R</sup>                                     | Reference 14        | <i>aph(3')-II</i>                          | 2                        | 2     | 128 | 2     |
| <i>T. pyogenes</i> 13OD0707 | Recipient strain, plasmid free  | This study          |  | ≤0.25                    | ≤0.25 | 2   | ≤0.25 |
| 13OD0707/pJRD215            | 13OD0707 with cloning vector pJRD215 (KAN <sup>R</sup> )  | This study          | <i>aph(3')-II</i>                          | ≤0.25                    | ≤0.25 | 2   | ≤0.25 |
| 13OD0707/pJEM1269           | 13OD0707 with <i>erm(56)</i> and promoter region from <i>T. pyogenes</i> 09KM1269 cloned into pJRD215 | This study          | <i>erm(56)</i> , <i>aph(3')-II</i>         | >256                     | 128   | 32  | ≤0.25 |
| AG100A/pJEM1269             | AG100A with <i>erm(56)</i> and promoter region from <i>T. pyogenes</i> 09KM1269 cloned into pJRD215   | This study          | <i>erm(56)</i> , <i>aph(3')-II</i>         | 128                      | 16    | 128 | 128   |

<sup>a</sup>Antibiotic resistance genes and functions: *erm(56)*, 23S rRNA methylase; *sul1*, sulfonamide-resistant dihydropteroate synthase; *aph(3')-II*, aminoglycosides phosphotransferase.

<sup>b</sup>ERY, erythromycin; CLI, clindamycin; KAN, kanamycin; PIA, pristinamycin IA; PIIA, pristinamycin IIA.

and plasmid-free *T. pyogenes* strain 13KM1326 (STR<sup>R</sup>; RpsL mutation K43R) (GenBank accession number [CP123400](#)) or electroporation of genomic DNA (16) into *T. pyogenes* 13OD0707 (GenBank accession number [CP123403](#)) using BHI agar containing 5% sheep blood (BHI-S) added of 50 μg/mL of streptomycin and 10 μg/mL of erythromycin for the selection of transconjugants and 10 μg/mL of erythromycin for the selection of electrotransformants.

Although *erm(56)* could not be transferred *in vitro*, its detection on different elements in unrelated bacteria suggests that it has a potential for broader dissemination. Given its detection in bacteria from different animal sources and geographical origins, it is likely that it has been independently selected by the use of antibiotics in animals.

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## AUTHOR CONTRIBUTIONS

Emma Marchionatti, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft | Vincent Perreten, Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – review and editing

## DATA AVAILABILITY

The nucleotide sequence of *erm*(56) of *T. pyogenes* 09KM1269 was deposited into the GenBank/ENA/DDBJ databases under accession number [OQ326498](https://doi.org/10.1093/nar/nzq123). The complete genome sequences of *T. pyogenes* 09KM1269, 13OD0707, and 13KM1326 were deposited into the GenBank under accession numbers [CP123393](https://doi.org/10.1093/nar/nzq123), [CP123403](https://doi.org/10.1093/nar/nzq123), and [CP123400](https://doi.org/10.1093/nar/nzq123).

## ADDITIONAL FILES

The following material is available [online](#).

## Supplemental Material

**Figure S1 (mSphere00239-23 S0001.pdf).** Schematic minimum free energy (MFE) structure encoding base pair probabilities and predicting secondary structure of the 153-bp upstream DNA sequence upstream of the start codon of *erm*(56).

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