



Molecular Characterization of *Enterococcus faecium* Clinical Isolates Harboured *erm* (T) from an Italian Hospital

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Abstract

The presence of *erm*(T) gene conferring resistance to macrolides, lincosamides and streptogramin B (MLS_B), was screened in 296 enterococci collected from clinical samples in a central Italy hospital and seven *Enterococcus faecium* isolates resulted positive to *erm*(T) by PCR. All isolates were resistant to erythromycin, tetracycline, ciprofloxacin and ampicillin but susceptible to vancomycin and chloramphenicol. Whole Genome Sequencing analysis revealed that in five *E. faecium* isolates, all belonging to the sequence type ST80 included in the clonal complex CC17 responsible of nosocomial infections, *erm* (T) gene was chromosome-located, in different genetic contexts. In *E. faecium* 735,236, *erm* (T) was on a 4,159-bp region flanked by two *IS1216* and inserted at the 3' end of the *mp* gene. In *E. faecium* 711,448 and 739,437, *erm* (T) was found in a 4,463-bp region identical to that detected in *E. faecium* 735,236 except for 319 bp. In *E. faecium* 713,729 and 757,415, *erm* (T) was on a 7,038-bp region flanked by *IS1251* and *ISEfm2* transposases and encompassed between the genes encoding a recombinase and three hypothetical proteins. *erm*(T)-carrying minicircles were detected in all isolates by inverse PCR assays demonstrating that *erm*(T) was included in mobile elements. However, in conjugation assays by filter mating, the *erm*(T) transferability was unsuccessful. Although macrolides are not used to treat enterococcal infections, the resistance is nonetheless widespread. These antibiotics are critically important in human medicine, but only few studies focused on *erm* (T)-harbouring clinical enterococci. The emergence of *erm* (T)-mediated erythromycin resistance among enterococci, potentially transferable to other nosocomial pathogens, should be constantly monitored.

Introduction

Macrolide antibiotics inhibit bacterial protein synthesis by binding to the 50S ribosomal subunit. They are effective against Gram-positive and some Gram-negative bacteria [1].

To date, two most common mechanisms responsible for acquired macrolide resistance are active-drug efflux and target-site modification [2].

Active efflux reduces the intracellular antibiotic to sub-lethal concentrations through membrane transporters. Target-site modification is mediated by methylases encoded by *erm* class genes which dimethylate the 23S rRNA nucleotide A2058 near the peptidyl transferase center. The A2058-dimethylation confers cross-resistance to macrolide, lincosamide, and streptogramin B (MLS_B) antibiotics [1].

It is well established that the MLS_B resistance is encoded by five *erm* class genes: The widespread *erm*(B) determinant usually conferring high-level resistance expressed either constitutively or inducibly, and the less common *erm*(A),

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erm(TR), *erm*(C) and *erm*(T) genes encoding an inducible erythromycin resistance [1, 3].

The *erm*(T) gene, commonly found in clinical as well as animal erythromycin-resistant lactobacilli, streptococci and staphylococci, is rarely detected in enterococci [4–11]. Very recently this gene was also found in *Clostridium perfringens* of poultry origin, in *Mannheimia haemolytica* from calf, in *Bhargavaea beijingensis* from soil and even in a *Klebsiella pneumoniae* clinical isolate [12–16].

Moreover, a non-functional chromosomal *erm*(T) gene, due to alterations both in the coding region and leader peptide, was detected in an *Enterococcus faecium* clinical isolate [17].

The *erm*(T) gene is mainly carried by small plasmids but it can also have a chromosomal location usually on Integrative and Conjugative Elements (ICEs) [3, 6, 7, 11–16].

However, only few studies focused on *erm*(T)-harbouring clinical enterococci, on their genomic characterization and on the *erm*(T) transferability.

The aim of this study was to investigate the occurrence and frequency of the *erm*(T) gene among a collection of *E. faecalis* and *E. faecium* clinical isolates from the Ancona Regional Hospital; the *erm*(T) genetic contexts, the gene transferability and the clonal relationships between isolates have been also indagated.

Material and Methods

Bacterial Strains

From October 2021 to June 2022, 296 enterococci (*Enterococcus faecalis* $n = 210$ and *E. faecium* $n = 86$) were recovered from different specimens collected from patients hospitalized in several wards of the Ancona regional hospital. To prevent duplicate isolates, only one strain for each patient was included in the study. The isolates were identified by MALDI-TOF MS (Vitek MS, bioMérieux, France).

Amplification Assays

All isolates were tested by PCR for the presence of the *erm*(T) gene using the primer pair *erm*T-fw 5'—ATAGTT TTATGTATTCTTTGTTA—3' and *erm*T-rv 5'- ATTGAG ATTGGTTCAGGGA—3' found through the online tool PREMIER Biosoft (<https://www.premierbiosoft.com/crm/jsp/com/pbi/crm/clientside/EligibleForDiscountLoginForm.jsp?LoginForFreeTool=true&PID=3>). To investigate the excision of the *erm*(T) genetic context, PCR mapping was performed using outward-directed primer pairs targeting the resistance gene (*erm*T3 5' –TAACAAAGA

ATACATAAAACTAT—3' and *erm*T4 5'—TCCCTGAAC CAATCTCAAT—3') as previously described [17].

Susceptibility Tests

erm(T)-positive enterococci were tested for their susceptibility to erythromycin, vancomycin, chloramphenicol, tetracycline, ciprofloxacin and ampicillin (Sigma Aldrich, St. Louis, MI) by broth microdilution assay according to the CLSI standards [18]. Susceptibility tests were interpreted according to CLSI clinical breakpoints [18]. *E. faecalis* ATCC 29212 was used as quality control.

Pulsed-Field Gel Electrophoresis (PFGE) Analysis

Macrorestriction with SmaI endonuclease (New England Biolabs, Beverly, MA) and pulsed-field gel electrophoresis (PFGE) analysis were performed as described elsewhere [19]. The banding pattern was interpreted according to the criteria of Tenover et al. [20].

Whole Genome Sequencing (WGS) and Sequence Analysis

Bacterial genomic DNA was extracted by the QIAcube automated extractor using DNeasy PowerLyzer PowerSoil Kit according to manufacturer's instructions (Qiagen, Germany). Extracted DNA was subjected to WGS using both short-read Illumina MiSeq platform (MicrobesNG, Birmingham, UK) with a 2 × 250 bp paired end technology and a long-read sequencing approach (MinION, Oxford Nanopore Technologies, Oxford, UK). Unicycler v. 0.4.8 software was used for the hybrid assembly of short and long reads (<https://github.com/rrwick/Unicycler>). RAST tool (<https://rast.nmpdr.org/>) was used for the annotation of DNA sequences.

In silico identification of acquired antimicrobial resistance genes, molecular typing, and virulome were carried out using dedicated tools available at the Center for Genomic Epidemiology available at <http://www.genomicepidemiology.org/> (v.2.0, ResFinder v.3.2, MLST 2.0, CSI Phylogeny 1.4 and VirulenceFinder 2.0) and by the BLAST suite (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The *E. faecium* 709,555, a laboratory strain, was used as the reference genome.

The Easyfig tool was used to compare relevant genetic elements (<https://mjsull.github.io/Easyfig/>). Figtree v1.4.4 was used to show the phylogenetic relationships between the strains resulting in a phylogenetic tree.

Conjugation Experiments

The conjugative transfer of the *erm*(T) gene was assessed by filter mating experiments as previously described [21]. The erythromycin-susceptible and fusidic acid- and rifampicin-resistant *E. faecium* 64/3 was used as recipient [22].

Both the recipient and the donors were grown to an optical density of 0.4 ± 0.05 units at 540 nm and then mixed at a donor/recipient ratio of 1:5. The filter, placed on a pre-warmed plate of brain heart infusion agar (BHIA) (Oxoid, Basingstoke, UK), was incubated at 37 °C for 18 h. Cells were resuspended in 1 mL of sterile saline and plated onto BHIA supplemented with erythromycin and fusidic acid (all, 10 mg/L). Plates were incubated at 37 °C for 48 to 72 h and then examined for the presence of transconjugants. The transfer frequency was expressed as the ratio of the cell number (CFU/mL) of the transconjugant to that of the recipient.

Nucleotide Sequence Accession Numbers

The WGS data are available under the BioProject ID PRJNA1136823.

Results and Discussion

Genotypic and Phenotypic Characterization

Seven *E. faecium* strains (2.36% of all enterococcal isolates), mostly from urine and blood, were *erm*(T)-positive (Table 1). The 7 enterococci were resistant to erythromycin (MIC range, 16- > 128 mg/L), tetracycline (MIC range, 16–128 mg/L), ciprofloxacin (MIC range, 64- > 128 mg/L) and ampicillin (MIC range, 64- > 128 mg/L). All isolates were susceptible to chloramphenicol (MIC range, 8–16 mg/L) and vancomycin (MIC range, 1–2 mg/L) except *E. faecium* 711,448 (MIC, > 128 mg/L). Further data on these strains are shown in Table 1.

SmaI-PFGE Experiments

The 7 *erm*(T)-positive *E. faecium* isolates were typed by SmaI-PFGE to establish their genetic relatedness (Table 1). PFGE clustering showed three main clones: (i) the first one included the 711,448 isolate showing the pulsotype A; (ii) the second encompassed the 757,415 and 713,729 isolates displaying the pulsotype B and the closely related pulsotype B1, respectively; (iii) the third comprised the 728,813, 739,437, and 757,304 isolates exhibiting the pulsotype C and the 735,236 stain showing the closely related pulsotype C1.

Genome Analysis

Five isolates, one for each pulsotype, were subjected to WGS (Table 2). ResFinder analysis revealed the presence, besides *erm*(T), of several acquired macrolide, lincosamide, streptogramin A and tetracycline resistance genes. The *E. faecium* 711,448 was resistant to vancomycin for the presence of the *vanHAX* genetic cluster (Table 2). The virulomes of all *E. faecium* isolates shared three acquired virulence genes encoding bacterial adhesins; *E. faecium* 711,448 also showed a gene coding for a hyaluronidase (Table 2).

All sequenced enterococci exhibited the sequence type ST80 (Table 2), a widespread *E. faecium* clone belonging to the clonal complex CC17 associated to nosocomial infections [23]. Phylogenetic analysis indicated that the *E. faecium* isolates differ from a min of 16 SNPs and a max of 3268 SNPs (Fig. 1) (Table S1).

erm(T) Genetic Contexts

An in-depth WGS analysis revealed that, in all sequenced isolates, *erm*(T) was chromosome-located and the relevant genetic contexts were not carried by ICEs.

Table 1 General features of the *erm*(T)-positive *E. faecium* clinical isolates

Strain	Isolation Data	Source	Ward	MIC (mg/L)						PFGE Pulsotype
				ERY	CHL	TET	VAN	CIP	AMP	
713,729	Nov-2021	Blood	ICU	> 128	8	128	2	64	64	B1
711,448	Nov-2021	Blood	Emergency room	> 128	16	16	> 128	> 128	> 128	A
728,813	Jan-2022	Urine	ICU	> 128	8	128	2	> 128	> 128	C
735,236	Feb-2022	Urine	Nephrology unit	16	8	128	2	> 128	> 128	C1
739,437	Mar-2022	Urine	Cardiology unit	> 128	8	128	2	> 128	> 128	C
757,304	Jun-2022	Urine	Neurologic unit	> 128	8	128	1	> 128	> 128	C
757,415	Jun-2022	Wound swab	ICU	> 128	8	128	2	128	128	B

ERY erythromycin; CHL chloramphenicol; TET tetracycline; VAN vancomycin; CIP ciprofloxacin; AMP ampicillin. ICU Intensive Care Unit

Table 2 Molecular typing, resistance and virulence of the five *erm*(T)-positive *E. faecium* subjected to WGS

Isolate	PFGE Pulsotype	Sequence Type	Acquired Resistance Genes					Acquired virulence Genes		
			Macrolides	Lincosamides	Tetracyclines	Streptogramins Group B	Diaminopyrimidines	Glycopeptides	Adhesins and Surface proteins	Hyaluronidase
711,448	A	ST80	<i>erm</i> (T) <i>erm</i> (B) <i>msr</i> (C)	<i>erm</i> (T) <i>erm</i> (B)	<i>tet</i> (L) <i>tet</i> (M)	<i>erm</i> (T) <i>erm</i> (B) <i>msr</i> (C)	<i>vanHAX</i>	<i>acm</i> <i>efaAfm</i> <i>espfm</i>	<i>hylEfm</i>	
757,415	B	ST80	<i>erm</i> (T) <i>erm</i> (B) <i>msr</i> (C)	<i>erm</i> (T) <i>erm</i> (B) <i>lnu</i> (B)	<i>tet</i> (L) <i>tet</i> (M)	<i>erm</i> (T) <i>erm</i> (B) <i>msr</i> (C)		<i>acm</i> <i>efaAfm</i> <i>espfm</i>		
739,437	C	ST80	<i>erm</i> (T) <i>msr</i> (C)	<i>erm</i> (T)	<i>tet</i> (L) <i>tet</i> (M)	<i>erm</i> (T) <i>msr</i> (C)	<i>dfr-G</i>	<i>acm</i> <i>efaAfm</i> <i>espfm</i>		
735,236	C1	ST80	<i>erm</i> (T) <i>msr</i> (C)	<i>erm</i> (T)	<i>tet</i> (L) <i>tet</i> (M)	<i>erm</i> (T) <i>msr</i> (C)	<i>dfr-G</i>	<i>acm</i> <i>efaAfm</i> <i>espfm</i>		
713,729	B1	ST80	<i>erm</i> (T) <i>erm</i> (B) <i>msr</i> (C)	<i>erm</i> (T) <i>erm</i> (B) <i>lnu</i> (B)	<i>tet</i> (L) <i>tet</i> (M)	<i>erm</i> (T) <i>erm</i> (B) <i>msr</i> (C)		<i>acm</i> <i>efaAfm</i> <i>espfm</i>		

In the *E. faecium* 735,236, *erm* (T) was found on a 4,159-bp DNA region flanked by two *IS1216*, belonging to IS6 family, with the same polarity. This genetic environment was inserted between the *pts* gene, encoding a phosphotransferase, and a truncated *mp* gene, encoding a membrane protein. Within the *erm* (T) genetic context the *ISBce13* transposase, belonging to the IS3 family, and two hypothetical proteins were found (Fig. 2). BLASTN analysis showed that the *erm* (T) genetic environment was 99% identical (coverage 99%) to a chromosomal region of the *E. faecium* 13-009 (accession no. CP025389.1) from a rectal swab in France.

In *E. faecium* 711,448 and 739,437 isolates, the *erm* (T) gene was on a 4,463-bp DNA region—inserted between the *pts* gene and a truncated *rep* gene—identical to the *erm* (T) genetic context of *E. faecium* 735,236 except for 319 bp bounded by *IS1216* and *ISBce13* transposases (Fig. 2).

Finally, in *E. faecium* 713,729 and 757,415 isolates the *erm* (T) gene was located on a 7,038-bp DNA region flanked by the *IS1251* and *ISEfm2* transposases (belonging to the *ISL3* and *IS256* families, respectively) with the same orientation. This region also showed the *IS1216* and *ISEf1* transposases (both belonging to the *IS256* family), the *tk* gene encoding a thymidine kinase and three hypothetical proteins. This *erm* (T) genetic environment was encompassed between a chromosomal gene encoding a recombinase and three hypothetical proteins (Fig. 2). BLASTN analysis revealed that this region was identical to that found in the chromosome of the *E. faecium* ICU-1-2 clinical isolate (accession no. CP063549.1) from a rectal swab in Germany.

Instability and Conjugation Assays

Erm (T)-carrying minicircles were detected in all enterococcal isolates. These results could suggest an intracellular mobility of the *erm* (T)-carrying genetic contexts due to the IS-mediated recombination.

Despite numerous attempts, filter mating experiments failed to demonstrate the transferability of the *erm* (T) gene to erythromycin-susceptible *E. faecium* 64/3 recipient.

Conclusions

This 8-month local surveillance showed that the prevalence of *erm* (T) gene in clinical enterococci is 2.36%. Interestingly, this gene only occurred in *E. faecium* isolates, all belonging to ST80 clonal lineage.

E. faecium is one of the main causes of healthcare-associated infections in many countries and ST80 vancomycin-resistant *E. faecium* lineage is increasingly involved

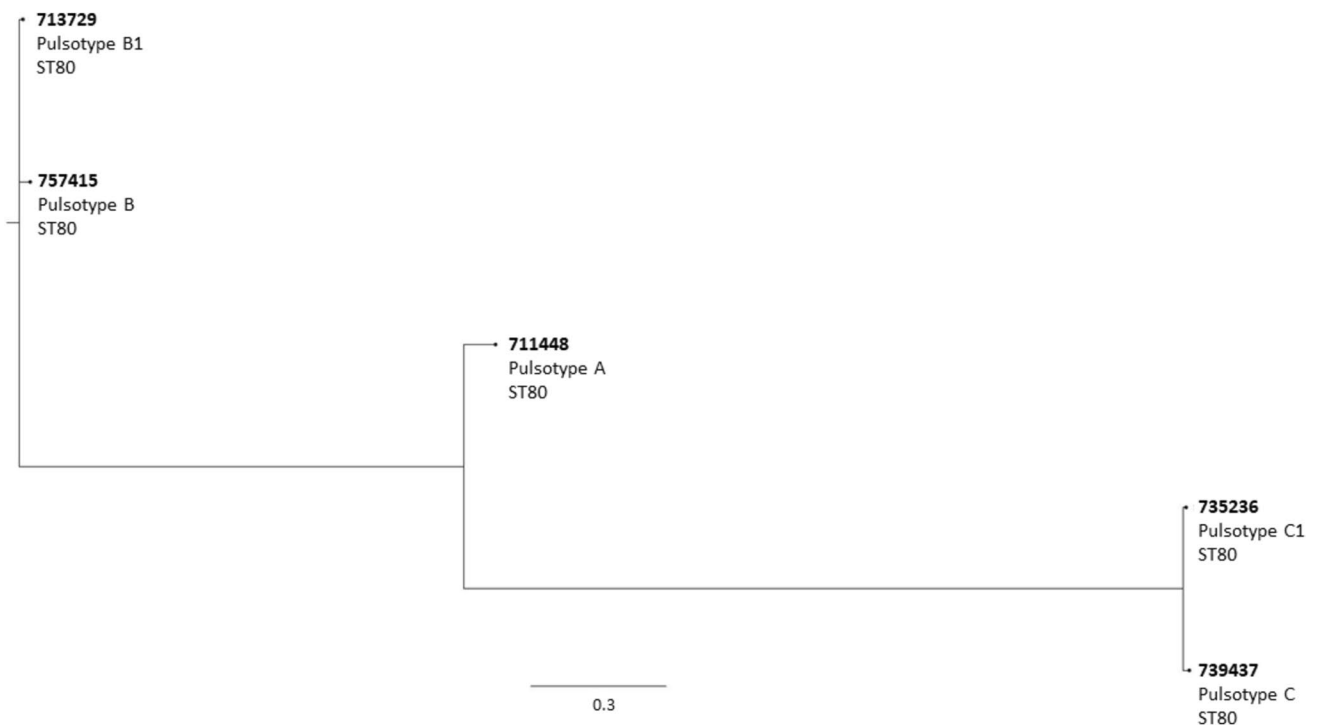


Fig. 1 Maximum likelihood phylogenetic tree of the five strains sequenced in this study. Pulsotype and the sequence type (ST) of each isolate are shown

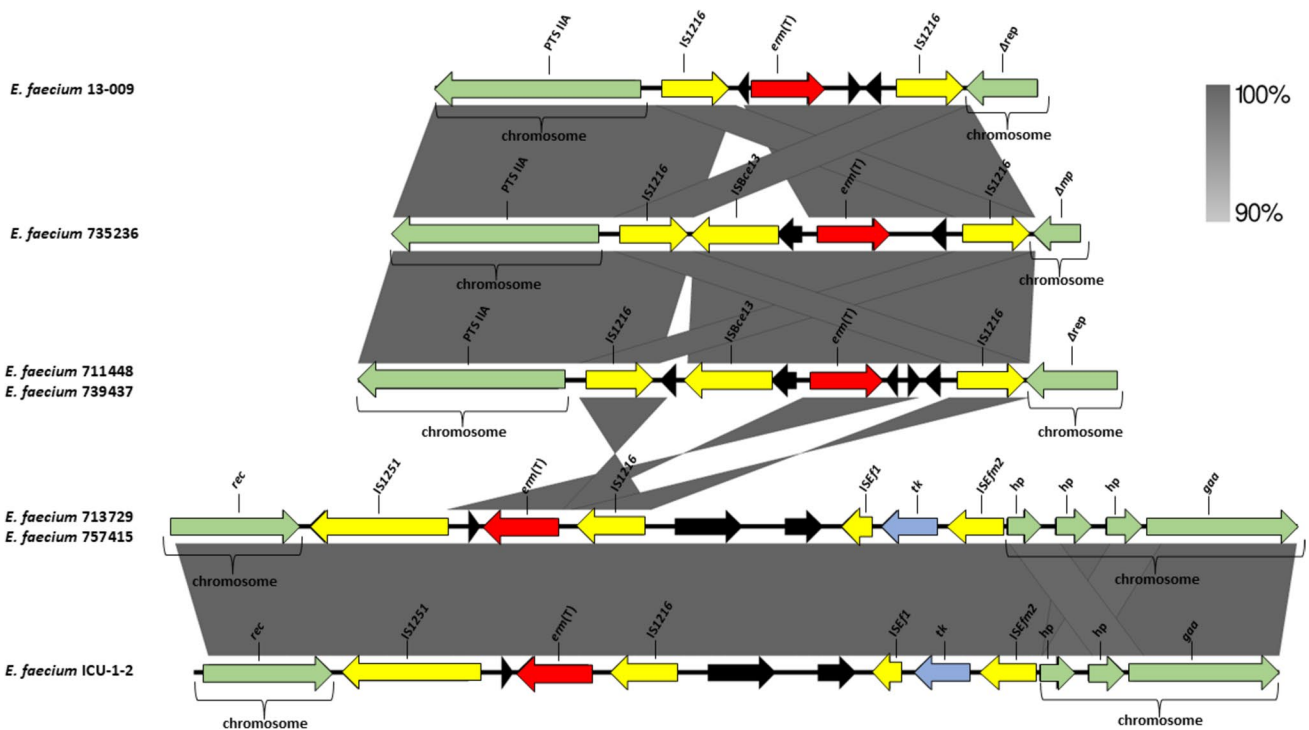


Fig. 2 Map of the *erm*(T) genetic contexts of the five *E. faecium* characterized in this study, their insertion sites and two *erm*(T) genetic contexts from *E. faecium* 13–009 and *E. faecium* ICU-1–2 strains using Easyfig tool (<https://mjsull.github.io/Easyfig/>). The positions and transcriptional direction of the ORFs are represented with arrows.

The green, red, and yellow ORFs indicate chromosomal genes, *erm*(T) and insertion sequences, respectively. The other ORFs are represented in black. Truncated genes are indicated with the symbol Δ

in hospital outbreaks in Europe, Australia, and America [24–28] and recently also in China [29].

Macrolides have been categorized as critically important for human medicine by the World Health Organization (<https://iris.who.int/bitstream/handle/10665/255027/9789241512220-eng.pdf?sequence=1&isAllowed=y>) [1] and although they are not used to treat enterococcal infections, the resistance is nonetheless widespread in this genus.

Despite *erm* (T) was not transferable by conjugation, the gene was able to mobilize intracellularly, therefore its insertion into mobile genetic elements such as conjugative plasmids, ICEs and prophages cannot therefore be excluded. Such events might originate highly versatile mobile genetic elements able to promote the spread of the MLS_B resistance to other nosocomial pathogens. In addition, the *erm* (T) gene could integrate into a vancomycin resistance element promoting its spread among the enterococcal population due to co-selection events likely occurring in hospital setting. Therefore, the emergence of *erm* (T)-mediated erythromycin resistance among clinical enterococci should be constantly monitored.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00284-024-03968-3>.

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Data Availability Whole Genome Sequencing data are available in GenBank under BioProject ID PRJNA1136823.

Declarations

Conflicts of interest The authors declare that they have no relevant conflicts of interest.

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