



HAL
open science

Small RNA-mediated regulation of the tet(M) resistance gene expression in *Enterococcus faecium*

Killian Le Neindre, Loren Dejoies, Sophie Reissier, François Guérin, Brice Felden, Vincent Cattoir

► **To cite this version:**

Killian Le Neindre, Loren Dejoies, Sophie Reissier, François Guérin, Brice Felden, et al.. Small RNA-mediated regulation of the tet(M) resistance gene expression in *Enterococcus faecium*. *Research in Microbiology*, 2022, 173 (4-5), pp.103941. 10.1016/j.resmic.2022.103941 . hal-03660105

HAL Id: hal-03660105

<https://hal.science/hal-03660105>

Submitted on 11 May 2022

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

1 **Small RNA-mediated regulation of the *tet(M)* resistance gene**
2 **expression in *Enterococcus faecium***

3 **Killian Le Neindre^{a,b}, Loren Dejoies^{a,b}, Sophie Reissier^a, François Guérin^{b,c}, Brice Felden^{a†},**
4 **Vincent Cattoir^{a,b,c*}**

5 ^aUnité Inserm U1230, Université de Rennes 1, Rennes, France

6 ^bService de Bactériologie-Hygiène hospitalière, CHU de Rennes, Rennes, France

7 ^cCNR de la Résistance aux Antibiotiques (laboratoire associé 'Entérocoques'), Rennes, France

8 †Deceased author

9
10 ***Correspondence and reprints:** Prof. Vincent Cattoir, CHU de Rennes, Service de Bactériologie-
11 Hygiène hospitalière, 2 rue Henri Le Guilloux, 35033 Rennes Cedex, France. +33-2-99-28-98-28,
12 Fax: +33-2-99-28-41-59, E-mail: vincent.cattoir@chu-rennes.fr.

13
14 E-mail address: killian.le-neindre@parisdescartes.fr (K. Le Neindre), loren.dejoies@chu-rennes.fr
15 (L. Dejoies), reissier.sophie@chu-amiens.fr (S. Reissier), francois.guerin@chu-rennes.fr (F.
16 Guerin), bfelden@univ-rennes1.fr (B. Felden), vincent.cattoir@chu-rennes.fr (V. Cattoir)

17

18 **Abstract**

19 We investigated the role of a novel small RNA expressed in *Enterococcus faecium* (named
20 Ern0030). We revealed that *ern0030* was encoded within the 5' untranslated region of *tet(M)*, a
21 gene conferring tetracycline resistance through ribosomal protection. By RACE mapping, we
22 accurately determined the boundaries of *ern0030*, which corresponded to *Ptet*. This upstream
23 sequence of *tet(M)*, *Ptet*, was previously described within transcriptional attenuation mechanism.
24 Here, Northern blot analyses revealed three transcripts of different lengths (ca. 230, 150 and 100
25 nucleotides) expressed from *Ptet*. Phenotypically, the total deletion of *ern0030* conferred a decrease
26 in tetracycline MICs that was consistent with gene expression data showing no significant *tet(M)*
27 induction under tetracycline SIC in *ern0030*-deleted mutant as opposed to a 10-fold increase of
28 *tet(M)* expression in the wild-type strain. We investigated the transcriptional attenuation
29 mechanism by toeprint assay. Whereas the expected *tet(M)* RBS was detected, the RBS of the
30 putative leader peptide was not highlighted by toeprint assay, suggesting the transcriptional
31 attenuation was unlikely. Here, we demonstrate that Ern0030 has a role in regulation of *tet(M)*
32 expression and propose a novel model of *tet(M)* regulation alternative or complementary to
33 transcriptional attenuation.

34

35 **Keywords:** *E. faecium*; tetracycline resistance; 5' untranslated region; sRNA

36 1. Introduction

37 The worldwide spread of multidrug-resistant (MDR) bacterial pathogens has become a major
38 public health issue and there is an urgent need to find new tools and approaches to control it. The
39 most critical MDR microorganisms have been grouped as “ESKAPE” pathogens that comprise
40 *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*,
41 *Pseudomonas aeruginosa* and *Enterobacter* spp. [1,2]. Among them, *E. faecium* is a leading cause
42 of nosocomial infections and hospital outbreaks, especially vancomycin-resistant *E. faecium*
43 (VREF) isolates for which limited therapeutic options are available [3,4]. The epidemiological
44 success of VREF mainly results from the worldwide dissemination of a specific clonal complex
45 (CC17) comprising clinical isolates that share a common MDR phenotype with high-level
46 resistance to both ampicillin and fluoroquinolones [5]. CC17 is part of a human hospital-adapted
47 lineage (clade A1) that emerged from the animal-associated lineage (clade A2) after the
48 introduction of antibiotics, and which differs genetically from the human community-associated
49 lineage (clade B) [4]. Thanks to a remarkable genomic plasticity, *E. faecium* is particularly adept
50 at acquiring new genes through horizontal gene transfer of a large panel of mobile genetic elements
51 [6,7] while it also exhibits a versatile metabolism allowing it to cope with many environmental
52 stresses [3,8].

53 Post-transcriptional control by non-coding RNAs (ncRNAs) enables a rapid and tight regulation to
54 environmental cues, with gene expression directly influenced by metabolic variations [9]. ncRNAs
55 can be divided into small RNAs (sRNAs) and RNA leaders, the latter including riboswitches and
56 attenuators [10]. Regulation of the expression of antibiotic resistance genes by attenuators is
57 widespread, particularly for ribosome-targeting antibiotics such as tetracyclines, aminoglycosides
58 or MLS (macrolides-lincosamides-streptogramins) antibiotics [10–13]. The attenuation
59 mechanism is based on the presence of a short open reading frame (ORF) coding for a leader

60 peptide immediately upstream of the resistance gene, which recruits translating ribosomes to the
61 regulatory 5'-untranslated region (5'-UTR) element. In the absence of antibiotics, there is an
62 inhibition of the resistance gene expression through a premature transcriptional terminator
63 (transcriptional attenuation) or the ribosome binding site (RBS) masking (translational attenuation)
64 [13,14]. When the antibiotic is present in the cell, there is a RNA leader conformational switch due
65 to the ribosome stalling resulting in alternative base-pairing patterns that promote an anti-
66 termination conformation or a RBS release, which consequently activate the expression of the
67 regulated gene [13,14].

68 Recently, we identified the first set of transcripts expressed from non-annotated regions in *E.*
69 *faecium* using the reference strain Aus0004 [15] and provided a list of 61 sRNA candidates [16].
70 Out of them, 10 were experimentally validated by Northern blots and by qPCR experiments as
71 expressed along bacterial growth [16]. However, their biological functions as well as their
72 molecular targets and associated gene networks remain unknown. Among them, a putative sRNA
73 (initially designated sRNA_0030 and renamed here Ern0030 for *Enterococcus* ribonucleotide acid)
74 was located upstream of the *tet(M)* gene, which codes for a ribosomal protection protein involved
75 in tetracycline resistance [17–20]. This gene is carried by the widespread Tn916-like conjugative
76 transposons [21–24], and its expression is inducible by low concentrations of tetracycline [11,25].
77 The regulatory region upstream of *tet(M)*, known as *Ptet*, has been described as RNA leader
78 sequence encoding a putative peptide leader (Orf12), and it has been suggested by structure
79 prediction that *tet(M)* regulation might be mediated by transcriptional attenuation with the
80 hypothetical implication of the availability of rare aminoacyl-tRNAs [11,26]. Interestingly, it has
81 also been demonstrated in *Enterococcus faecalis* that partial deletions of *Ptet* could be responsible
82 for an upregulation of *tet(M)* expression associated with resistance to tigecycline, antibiotic usually
83 effective despite Tet(M) production [25].

84 Since the regulation mechanism of *tet(M)* expression has been poorly studied, the aim of this study
85 was to molecularly characterize a small *cis*-regulatory RNA (Ern0030) expressed within the 5'
86 UTR of *tet(M)* and to mechanistically investigate its role in regulation of *tet(M)* expression and
87 associated tetracycline resistance.

Journal Pre-proof

88 2. Materials and methods

89 2.1. Bacterial strains, plasmids and growth conditions

90 The reference strain *E. faecium* Aus0004 (GenBank accession number CP003351.1) and
91 isogenic mutants (Table S1) were grown under ambient air at 37°C in brain heart infusion (BHI;
92 Oxoid) broth or on BHI agar (Oxoid). Strains of *Escherichia coli* (Table S1) were grown under
93 ambient air and agitation (160 rpm) at 37°C in Luria-Bertani (LB; ThermoFischer Scientific)
94 medium or on LB agar (ThermoFischer Scientific). For some experiments, these media were
95 supplemented with spectinomycin (Sigma) and/or tetracycline (Sigma) (see below). The plasmids
96 pAT29 and pWS3 (Table S2) were used for cloning and gene disruption, respectively.

97 2.2. Antimicrobial susceptibility testing

98 Minimal inhibitory concentration (MICs) of tetracycline and tigecycline were determined by
99 the microdilution reference method in Mueller-Hinton (MH; ThermoFischer Scientific) broth in
100 accordance with EUCAST guidelines (www.eucast.org). The sub-inhibitory concentration (SIC)
101 of tetracycline was determined in BHI broth and was defined as the highest antibiotic concentration
102 that did not altered significantly the bacterial growth kinetics.

103 2.3. Construction of deleted mutants and trans-complemented strains

104 *E. faecium* Aus0004 contain a large sequence within *tet(M)* gene. The restoration of a
105 functional *tet(M)* gene (named *E. faecium* Aus0004^{TR}) was obtained from *E. faecium* Aus0004 (as
106 represented in Fig. 1A) by homologous recombination and excision of the large sequence using the
107 pWS3 suicide vector and specific primers, as previously described [27] (Tables S1-S3). Using the
108 same approach, the entire *ern0030* gene (from 5' to 3' position; see Fig. 1B) was deleted in *E.*
109 *faecium* Aus0004^{TR} and the corresponding deleted mutant was named Δ *ern0030*-Aus0004^{TR}

110 (Tables S1-S3). Both deletions were confirmed by PCR and sequencing using specific primers
111 (Table S3).

112 *Trans*-complemented strains were constructed by cloning the *ern0030* gene (with its native
113 promoter and rho-independent terminator) in the pAT29 shuttle vector [28] using specific primers
114 (Tables S1-S3). The recombinant plasmid was transformed by electroporation into *E. coli* EC1000
115 then into both *E. faecium* Aus0004^{TR} and Δ *ern0030*-Aus0004^{TR} strains (Table S1). For
116 investigation of the Ern0030 function, different recombinant plasmids were generated using the
117 pAT29 vector in which was cloned the functional *tet*(M) gene. The different recombinant plasmids
118 contained wild-type sequence of *ern0030* or several mutated *ern0030* sequences. These plasmids
119 were introduced into *E. coli* EC1000 strain and then into *E. faecium* Aus0004 (Tables S1-S3).
120 Transformants were selected on agar plates containing spectinomycin at the concentration of 100
121 mg/L (*E. coli*) or 300 mg/L (*E. faecium*). Inserts of recombinant plasmids were sequenced using
122 specific primers (Table S3).

123 2.4. RNA extraction

124 Total RNAs were isolated at three different time points during growth: mid exponential (ME,
125 OD_{600nm} 1), late exponential (LE, OD_{600nm} 2.2) and early stationary (ES, OD_{600nm} 3) phase. In some
126 experiments, tetracycline was added in the growth medium at the SIC (equivalent to 1/32 MIC).
127 Extractions were performed as previously described [29]. Briefly, cell pellets were dissolved into
128 500 μ L of lysis buffer, and cells were broken by acid-treated glass beads and acidic phenol (pH 4)
129 using a FastPrep-24 cell disruptor (MP biomedical). Following acid phenol extraction, total RNAs
130 were cleaned with chloroform and absolute ethanol precipitated overnight at -20°C with sodium
131 acetate. Residual chromosomal DNA was removed by treating all total RNA samples with the
132 Turbo DNA-free kit (Life Technologies).

133 2.5. RACE mapping

134 RACE mapping was performed using bacterial cells grown in BHI broth and collected at ME
135 growth phase, as previously described [30]. Briefly, Total RNAs (5 µg) from *E. faecium* Aus0004
136 were circularized using T4 RNA Ligase (Promega). After precipitation, the reaction was reverse
137 transcribed by the M-MLV RT (Promega) using primer sRNA0030R1 and then two PCR reactions
138 were performed with Taq polymerase (Invitrogen) using sRNA0030R2-F1 and sRNA0030R3-F2
139 primers (Table S2). The PCR products were cloned in pGEM-T vector (Promega) and recombinant
140 plasmids were transformed into *E. coli* XL1blue (Table S1), and sequenced with M13 reverse
141 vector primer (Table S3).

142 2.6. Northern Blot

143 Northern blots were done using total RNAs extracted at the three different time points during
144 growth (see above). RNA samples (5 to 20 µg) were loaded on denaturing 8% polyacrylamide/8M
145 urea gels for separation of smaller RNA transcripts (100-600 nt) or denaturing 0.8% agarose gel
146 for separation of larger RNA transcripts (0.2-3 kb), and transferred onto membrane Hybond N+
147 (GE Healthcare). Membranes were hybridized with specific ³²P 5' end-labeled probes (Table S3)
148 in ExpressHyb solution (Clontech), washed, exposed, and scanned with a Typhoon FLA 9500
149 scanner (GE Healthcare). tmRNA levels were used as internal loading controls.

150 2.7. RT-PCR and RT-qPCR

151 For RT-PCR, DNase-treated RNAs isolated from *E. faecium* Aus0004 grown at the ME phase
152 (OD_{600nm} 1) in BHI broth supplemented with tetracycline SIC were used. cDNA was synthesized
153 using the High Capacity cDNA reverse transcription kit (ThermoFischer Scientific) with
154 qPCRTETMR or qPCRsRNA0030R primers (Supplementary Table S3). PCRs were then carried
155 out under standard conditions using specific primers (Table S3). Each PCR amplification was

156 performed on cDNA, chromosomal DNA (positive control), water or RNAs with no RT step
157 (negative controls).

158 From 40 ng of DNase-treated total RNA samples, RT-qPCR was performed using the Power
159 SYBR Green RNA-to-CT 1-Step kit (ThermoFischer Scientific) and a StepOnePlus Real-Time
160 PCR system (Applied Biosystems). Transcript levels (expressed as copy numbers per ng of RNA)
161 in each sample were determined by absolute quantification using serial dilutions of PCR products.
162 The primers efficiencies mainly ranged between 95 to 120%. Expression of the *adk* gene as a
163 housekeeping control gene was also measured to control expression homogeneity between the
164 samples. Each experiment was performed in triplicate. For statistical analysis, two-way ANOVA
165 and Tukey tests were employed using the GraphPad Prism 7.0 software.

166 2.8. Half-life determination

167 *E. faecium* Aus0004^{TR} cells were cultured overnight, diluted 1:50, then grown to ME phase
168 (OD_{600nm} 1) at 37°C in BHI broth supplemented or not with tetracycline SIC, and then incubated
169 with 150 mg/L of rifampicin. Total RNAs were extracted at different time intervals and transcript
170 levels of *Ern0030* and *tet(M)* RNA were quantified by RT-qPCR with specific primers (Table S3).
171 Half-lives of both RNAs were calculated by non-linear regression curve fitting (one phase decay)
172 using GraphPad Prism 7.0 software as previously described [31].

173 2.9. Toeprints assays

174 *In vitro* transcription was conducted from PCR-amplified templates using Aus0004 genomic
175 DNA and the MEGAscript T7 kit (Ambion) with specific primers (Table S3). RNAs were gel-
176 purified, eluted and precipitated with ethanol in the presence of 0.3M sodium acetate. Toeprint
177 assays were performed as previously described [32] with some minor modifications. Briefly,
178 annealing mixtures containing 5 pmol 5'UTR-*tet(M)* RNA with 1 pmol ^γ32P 5' end-labeled primers
179 (Table S3) were incubated in a buffer (20 mM Tris-HCl, pH7.5, 60 mM NH₄Cl) for 1 min at 90°C.

180 Renaturation was done in the presence of 10 mM MgCl₂ for 20 min at 20°C. 70S ribosomes were
181 reactivated for 15 min at 37°C, diluted in the reaction buffer and incubated again for 15 min at
182 37°C. Various concentrations (0 to 1.9 pmol) of purified 70S ribosomes were added to each sample,
183 and these were incubated for 5 min at 37°C, after which MgCl₂ was adjusted to 10 mM. After 15
184 min at 37°C, an amount of 10 pmol of uncharged tRNA^{fMet} was added and incubated for 5 min at
185 37°C. The cDNAs were synthesized with 10 U of AMV RT (New England Biolabs) for 20 min at
186 42°C. Reactions were stopped by the addition of 15 µl of loading buffer II (Ambion), and cDNAs
187 were loaded and separated onto 8% polyacrylamide/8M urea gels. Sequencing ladders were
188 generated with the same 5' end-labeled primer for toeprint.

189 3. Results and discussion

190 3.1. Characterization of *ern0030* transcripts in *E. faecium* Aus0004

191 In a previous study, we identified a set of novel transcripts in the ESKAPE pathogen *E. faecium*
192 strain Aus0004 [16]. Among them, *ern0030* was found to be expressed upstream from the initiation
193 codon of the *tet(M)* tetracycline resistance gene. In *E. faecium* Aus0004, *tet(M)* is chromosomally
194 encoded but interrupted by a large insertion sequence (from EFAU004_00064 to
195 EFAU004_00066) containing the *dfrG* trimethoprim resistance gene (Fig. 1A) related to Tn5801
196 ‘type A5’ transposons [33]. Due to an overlap between *ern0030* and *Ptet*, we hypothesized a
197 functional relationship between *ern0030* and *tet(M)*. Therefore, we repaired the *tet(M)* gene of *E.*
198 *faecium* Aus0004 producing *E. faecium* strain Aus0004^{TR} and confirmed its functionality by
199 Northern blot (Fig. S1) and determination of tetracycline MICs (Table 1).

200 By RACE mapping, the 5’ end of *ern0030* was determined to be located at the genomic position
201 65144 on *E. faecium* Aus0004 chromosome (T₁; Fig. 1B). This position corresponds to the
202 transcription initiation site (TSS) previously reported for *tet(M)* [11]. Its 3’ end corresponded to a
203 predicted rho-independent terminator (A₂₃₃; Fig. 1B). This revealed that *ern0030* corresponded to
204 *Ptet*, the RNA leader sequence upstream *tet(M)* previously reported [11,26]. Surprisingly, by using
205 the same probe previously published to reveal *Ern0030* [16], we identified three transcripts of
206 different lengths (ca. 230, 150 and 100 nucleotides; Fig. 2) expressed from *Ptet*, which has not been
207 previously described [11,16]. Using other *ern0030*-specific probes, we demonstrated that the two
208 shorter forms were located in the 5’ end of the *Ptet* sequence between positions G₂₉ and C₁₃₃ (Fig.
209 1B and Fig. 2), suggesting RNA processing or premature transcription termination. Note that Su *et*
210 *al.* were not able to detect these shorter transcripts since they used a probe targeting the 3’ moiety
211 of the *Ptet* sequence [11]. The study of RNA level of these three different transcripts showed
212 significant variations during bacterial growth, with high-level expression of 230- and 150-nt

213 transcripts and weak expression of the 100-nt transcript at the ME phase whereas an opposite
214 pattern was observed at the ES phase (Fig. 2). The high-level expression of the 100-nt transcript at
215 the ES phase suggests post-transcriptional regulation of *Ern0030* expression allowing its
216 accumulation.

217 To complete *Ern0030* characterization, the half-life of 233-nt form RNA was measured in *E.*
218 *faecium* Aus0004^{TR} in the absence of tetracycline. The half-life of *Ern0030* (mean = 4 minutes and
219 18 seconds) was slightly higher than that of *tet(M)* (mean = 1 minute and 48 seconds) (Fig. 3). The
220 *Ern0030* half-life is shorter than those of conventional *trans*-acting sRNAs while the very short
221 half-life of *tet(M)* mRNA is consistent with a dynamic transcriptional adaptation [34]. Half-life
222 measurement with a SIC of tetracycline was not calculable since transcript levels did not differ
223 between different time points. This may be related to the fact that tetracycline SIC may result in a
224 global mRNA stabilization, as previously described in *Bacillus subtilis* [35].

225 3.2. Role of *ern0030* in *tet(M)* expression

226 We firstly investigated the functional link between *ern0030*, *tet(M)* and tetracycline resistance
227 by using the *ern0030*-deleted Aus0004^{TR} mutant. Phenotypically, the *ern0030* deletion caused an
228 eight-fold decrease in MICs of tetracycline (from 32 to 4 mg/L) while no change was observed for
229 tigecycline (Table 1). This observation was consistent with variations of *tet(M)* expression between
230 the two strains with a ~10-fold increase of *tet(M)* expression in Aus0004^{TR} upon addition of
231 tetracycline and no change in the steady state mRNA level in Δ *ern0030*-Aus0004^{TR} (Fig. 4A).
232 Noteworthy, tetracycline did not affect *ern0030* expression in Aus0004^{TR} (Fig. 4B). This decrease
233 in tetracycline MICs is quite surprising since in a previous study bioreactor-adapted *E. faecalis*
234 mutants with partial deletions in *Ptet* were resistant to higher levels of tetracycline resistance,
235 especially towards tigecycline [25]. In the latter study, two types of *tet(M)*-overexpressing

236 chromosomal mutants were obtained, one with a partial deletion of 3' end of *Ptet* (named BTR87b)
237 and another with a partial deletion of 5' end of *Ptet* (named BTR22). Since the complete deletion
238 of *ern0030* seems to confer a phenotype different from those obtained by partial deletions, it
239 suggests a complex mechanism of regulation.

240 To assess whether Ern0030 could act in *trans* on Tet(M) expression, we expressed
241 pAT29 Ω *ern0030* in Aus0004^{TR} and Δ *ern0030*-Aus0004^{TR} strains (Tables S1 and S2). No
242 difference in MICs of tetracycline and tigecycline was observed between *trans*-complemented
243 strains and those harboring the pAT29 empty vector (Table 1), indicating that Ern0030 does not
244 Tet(M) in a *cis-trans* manner [36].

245 3.3. Investigation of transcriptional attenuation mechanism

246 The upstream sequence of the *tet*(M) gene, *Ptet*, was defined as a transcriptional attenuator only
247 based on predicted secondary structures and on the presence a putative peptide leader ORF (*orf*₁₂)
248 [11,26]. This mechanism hypothetically relies on peptide leader translation and ribosome stalling
249 that may cause an alternative structure of the rho-independent transcriptional terminator located at
250 3' end of *ern0030* (Fig. 1B). The role of these two elements (i.e. rho-independent transcriptional
251 terminator and putative peptide leader ORF) was then investigated.

252 First, we verified that there was a transcriptional read-through of *ern0030* and *tet*(M) promoted
253 by tetracycline as previously described [11,26]. For this, we used Aus0004^{TR} strain and its isogenic
254 mutant Δ *ern0030*-Aus0004^{TR} and we confirmed the read-through after tetracycline addition (Fig.
255 5). The co-transcription of *ern0030* and *tet*(M) was also observed by RT-PCR (Fig. S2). The role
256 of the rho-independent transcriptional terminator was investigated by studying the
257 Aus0004_pAT29 Ω *tet*(M)- Δ ₂₀₅₋₂₃₉*ern0030* strain that carries a pAT29 Ω *tet*(M) (that contained both
258 *ern0030* and *tet*(M) genes) derivative with a 35-nt deletion covering the rho-independent terminator

259 in *ern0030* (Tables S1 and S2). MICs of tetracycline and tigecycline were similar (only a one-
260 dilution difference) between the two strains (Table 1), suggesting a minor effect of the rho-
261 independent transcriptional terminator.

262 The second important element in transcriptional attenuation mechanism is the ribosomal
263 binding to the 5' UTR and the peptide leader translation. Based on structural predictions, it has
264 been suggested that the RNA leader termination conformation was induced by the ribosome stalling
265 during peptide leader translation [11]. According to this hypothesis, under non-induced conditions,
266 the translation of the leader peptide is delayed since it is composed of codons corresponding to rare
267 aminoacyl-tRNAs, and then the expression of Tet(M) is limited only to what can be generated as a
268 result of transcriptional read-through of the terminator. When bacterial cells are exposed to
269 tetracycline SIC, which delays translation, there is an increased availability of those rare
270 aminoacyl-tRNAs, which allows leader peptide translation to more easily keep up with
271 transcription, disrupt the terminator stem-loop base pairing and enable continuing *tet(M)*
272 transcription. To determine whether the peptide leader had a function in regulation of *tet(M)*
273 expression, we investigated the ribosomal loading onto the predicted peptide leader RBS by
274 toeprint assays. This *in vitro* assay relies on the inhibition of reverse transcription due to stalled
275 ribosome onto the initiation codon. Recognition of a RBS sequence by ribosomes induces a toeprint
276 during primer extension that usually occurs 16-19 nucleotides downstream of the initiation codon
277 [37]. We used the two different radiolabeled primers TETMPRINT and RBSTETM-R targeting
278 the RBS of *tet(M)* and that of the putative leader peptide [11], respectively (Fig. 1B). With the
279 TETMPRINT primer, three different toeprints (A₃₀₀, G₂₉₂ and U₂₆₀) were detected (Fig. 6A). The
280 position G₂₉₂ corresponds to the expected *tet(M)* RBS (Fig. 1B). Being close to G₂₉₂, A₃₀₀ may be
281 the result of structural changes due to ribosome binding. For U₂₆₀, it is located ~15-20 nucleotides
282 downstream of an AUG that has a putative RBS (GGGA) suggesting that another leader peptide

283 might exist (Fig. 1B). Note that the stop at the position A₂₃₃ was independently observed regardless
284 of the ribosome concentration and corresponded to the end of the stem-loop of the Ern0030 rho-
285 independent transcription terminator, which is undoubtedly a very robust structure (Fig. 6A).

286 The research of the putative peptide leader RBS was also performed with RBSTETM-R primer.
287 According to Su *et al.* [11], the expected theoretical ribosome toeprint should have been detected
288 between positions U₁₉₁ and U₁₉₄ (Fig. 1B). However, none toeprint was observed suggesting that
289 this putative leader peptide does not exist or is not involved in ribosomal recruitment and regulation
290 of *tet(M)* expression (Fig. 6B). The role of this putative peptide leader was also challenged by
291 studying the Aus0004_pAT29Ω*tet(M)*-TGA₁₇₂₋₁₇₄ strain that carries a pAT29Ω*tet(M)* (that
292 contained both *ern0030* and *tet(M)* genes) derivative with a unique mutation (ATG → TGA) in the
293 translation initiation codon of the putative peptide leader (Tables S1 and S2). Despite the absence
294 of leader peptide translation, tetracycline MICs were similar (only one-dilution difference) between
295 Aus0004_pAT29Ω*tet(M)* and Aus0004_pAT29Ω*tet(M)*-TGA₁₇₂₋₁₇₄ strains (Table 1), indicating
296 that this putative leader peptide (if it does exist) has no major role in tetracycline-mediated
297 regulation of *tet(M)* regulation.

298 The *tet(M)* regulation by transcriptional attenuation was unlikely with the peptide leader
299 ORF12 previously described [11]. Another more likely hypothetic mechanism could be a
300 regulation mediated by regulatory protein included within Tn916-like transposon. In fact, recent
301 study demonstrated that *tet(M)* induction by tetracycline was Tn916-dependent [38]. In absence
302 to ribosome recruitment on RBS of ORF12 supported by our toeprint assay (Fig. 6), the
303 antitermination conformation was likely the most stable [11]. We could suspect a read-through, in
304 accordance with basal expression of *tet(M)* without tetracycline (Fig. 4), to regulatory region
305 containing genes coding for some proteins with unknown function [23,26]. One of these proteins

306 could interact with *Ptet* and promote the termination conformation, corresponding to Ern0030. The
307 addition of tetracycline could inhibit the regulatory protein translation and indirectly induce the
308 antitermination conformation.

309 Finally, it has been demonstrated that tetracycline could interact with double-stranded RNAs
310 [39] and hypothetically might interfere with ribosomal interaction. This ability to alter ribosome
311 binding onto the mRNA was also evaluated by toeprint assays, but we did not detect differences
312 regardless of tetracycline concentration (Fig. S3). Another argument suggesting another regulation
313 mechanism is that it has been previously demonstrated that a partial deletion of 5' *Ptet* (mutant
314 BTR22), which does not include the peptide leader *orf12*, is more resistant to tetracyclines with
315 upregulation of *tet(M)* [25].

316 4. Conclusion

317 Here, we have for the first time investigated the role of a regulatory RNA (Ern0030) expressed
318 in *E. faecium*. We have demonstrated that Ern0030 had a role in regulation of *tet(M)* expression.
319 Even though further investigations are needed, results obtained here indicate the existence of
320 alternative or complementary mechanism(s) of *tet(M)* regulation other than transcriptional
321 attenuation, and we propose a new hypothetical model where it may be envisaged the existence of a
322 putative regulatory protein encoded by Tn916-like transposon that might interact with *Ptet* and
323 promote the termination conformation. Indeed, a recent study demonstrated that the induction of
324 *tet(M)* expression by tetracycline was Tn916-dependent [38] and in our hypothesis, tetracycline
325 may inhibit translation of this regulatory protein and indirectly induce the anti-termination
326 conformation. Nevertheless, this hypothesis needs further investigations for the identification of
327 this putative regulatory protein and the demonstration of its direct interaction with *Ptet*. The
328 complexity of regulation *tet(M)* expression has also been pointed out since it has been demonstrated
329 that the transcription of *tet(M)* from Tn916 was induced by SICs of other antibiotics (such as

330 spectinomycin and MLS) or biocides (such as ethanol) [38,40]. Besides its role in the regulation of
331 the *tet(M)* resistance gene, Ern0030 may also be involved in the control of the transfer of Tn916
332 and then the dissemination of antibiotic resistance genes [23,25].

Journal Pre-proof

333 **Conflict of Interest:** The authors declare that the research was conducted in the absence of any
334 commercial or financial relationships that could be constructed as a potential conflict of interest.

Journal Pre-proof

335 **Acknowledgments**

336 This work was supported by the the “Institut National de la Santé et de la Recherche Médicale” and
337 University of Rennes 1.

338 The authors thank Yoann Augagneur (BRM, Rennes, France) for his critical reading of the
339 manuscript, Stéphane Dréano (IGDR, Rennes, France) for the DNA sequencing of all constructs in
340 this study and Marie-Laure Pinel-Marie and Noëlla Amiot (BRM, Rennes, France) for ribosome
341 gift.

342 The work is dedicated to the memory of Prof. Brice Felden.

343 **References**

- 344 [1] Rice LB. Federal funding for the study of antimicrobial resistance in nosocomial pathogens: No
345 ESKAPE. *J Infect Dis* 2008;197:1079–81. <https://doi.org/10.1086/533452>.
- 346 [2] Santajit S, Indrawattana N. Mechanisms of antimicrobial resistance in ESKAPE pathogens. *Biomed*
347 *Res Int* 2016;2016. <https://doi.org/10.1155/2016/2475067>.
- 348 [3] Arias CA, Murray BE. The rise of the Enterococcus: Beyond vancomycin resistance. *Nat Rev*
349 *Microbiol* 2012;10:266–78. <https://doi.org/10.1038/nrmicro2761>.
- 350 [4] Lebreton F, Schaik W van, McGuire AM, Godfrey P, Griggs A, Mazumdar V, et al. Emergence of
351 epidemic multidrug-resistant *Enterococcus faecium* from animal and commensal strains. *MBio*
352 2013;4:e00534-13. <https://doi.org/10.1128/mBio.00534-13>.
- 353 [5] Lee T, Pang S, Abraham S, Coombs GW. Antimicrobial-resistant CC17 *Enterococcus faecium*: The
354 past, the present and the future. *J Glob Antimicrob Resist* 2019;16.
355 <https://doi.org/10.1016/j.jgar.2018.08.016>.
- 356 [6] Hegstad K, Mikalsen T, Coque TM, Werner G, Sundsfjord A. Mobile genetic elements and their
357 contribution to the emergence of antimicrobial resistant *Enterococcus faecalis* and *Enterococcus*
358 *faecium*. *Clin Microbiol Infect* 2010;16:541–54. <https://doi.org/10.1111/j.1469-0691.2010.03226.x>.
- 359 [7] Zhong Z, Kwok L-Y, Hou Q, Sun Y, Li W, Zhang H, et al. Comparative genomic analysis revealed
360 great plasticity and environmental adaptation of the genomes of *Enterococcus faecium*. *BMC*
361 *Genomics* 2019;20:602. <https://doi.org/10.1186/s12864-019-5975-8>.
- 362 [8] Gaca AO, Lemos JA. Adaptation to adversity: The intermingling of stress tolerance and pathogenesis
363 in enterococci. *Microbiol Mol Biol Rev* 2019;83. <https://doi.org/10.1128/MMBR.00008-19>.
- 364 [9] Repoila F, Darfeuille F. Small regulatory non-coding RNAs in bacteria: Physiology and mechanistic
365 aspects. *Biol Cell* 2009;101:117–31. <https://doi.org/10.1042/BC20070137>.
- 366 [10] Dersch P, Khan MA, Mühlen S, Görke B. Roles of regulatory RNAs for antibiotic resistance in
367 bacteria and their potential value as novel drug targets. *Front Microbiol* 2017;8.
368 <https://doi.org/10.3389/fmicb.2017.00803>.
- 369 [11] Su YA, He P, Clewell DB. Characterization of the *tet(M)* determinant of Tn916: Evidence for
370 regulation by transcription attenuation. *Antimicrob Agents Chemother* 1992;36:769.
- 371 [12] Chancey ST, Bai X, Kumar N, Drabek EF, Daugherty SC, Colon T, et al. Transcriptional attenuation
372 controls macrolide inducible efflux and resistance in *Streptococcus pneumoniae* and in other Gram-
373 positive bacteria containing *mef/mel(msr(D))* elements. *PLoS One* 2015;10.
374 <https://doi.org/10.1371/journal.pone.0116254>.
- 375 [13] Lovett PS, Rogers EJ. Ribosome regulation by the nascent peptide. *Microbiol Rev* 1996;60:366–85.
- 376 [14] Gollnick P, Babitzke P. Transcription attenuation. *Biochim Biophys Acta* 2002;1577:240–50.
377 [https://doi.org/10.1016/S0167-4781\(02\)00455-4](https://doi.org/10.1016/S0167-4781(02)00455-4).
- 378 [15] Lam MMC, Seemann T, Bulach DM, Gladman SL, Chen H, Haring V, et al. Comparative Analysis
379 of the first complete *Enterococcus faecium* genome. *J Bacteriol* 2012;194:2334–41.
380 <https://doi.org/10.1128/JB.00259-12>.
- 381 [16] Sinel C, Augagneur Y, Sassi M, Bronsard J, Cacaci M, Guérin F, et al. Small RNAs in vancomycin-
382 resistant *Enterococcus faecium* involved in daptomycin response and resistance. *Sci Rep* 2017;7.
383 <https://doi.org/10.1038/s41598-017-11265-2>.

- 384 [17] Burdett V. Purification and characterization of Tet(M), a protein that renders ribosomes resistant to
385 tetracycline. *J Biol Chem* 1991;266:2872–7.
- 386 [18] Dantley KA, Dannelly HK, Burdett V. Binding interaction between Tet(M) and the ribosome:
387 Requirements for binding. *J Bacteriol* 1998;180:4089–92.
- 388 [19] Dönhöfer A, Franckenberg S, Wickles S, Berninghausen O, Beckmann R, Wilson DN. Structural basis
389 for TetM-mediated tetracycline resistance. *Proc Natl Acad Sci U S A* 2012;109:16900–5.
390 <https://doi.org/10.1073/pnas.1208037109>.
- 391 [20] Grossman TH. Tetracycline antibiotics and resistance. *Cold Spring Harb Perspect Med* 2016;6.
392 <https://doi.org/10.1101/cshperspect.a025387>.
- 393 [21] Burdett V. Nucleotide sequence of the *tet(M)* gene of Tn916. *Nucleic Acids Res* 1990;18:6137.
- 394 [22] Rice LB. Tn916 family conjugative transposons and dissemination of antimicrobial resistance
395 determinants. *Antimicrob Agents Chemother* 1998;42:1871–7.
- 396 [23] Roberts AP, Mullany P. Tn916-like genetic elements: a diverse group of modular mobile elements
397 conferring antibiotic resistance. *FEMS Microbiol Rev* 2011;35:856–71.
398 <https://doi.org/10.1111/j.1574-6976.2011.00283.x>.
- 399 [24] Ciric L, Jasni A, Vries LE de, Agersø Y, Mullany P, Roberts AP. The Tn916/Tn1545 family of
400 conjugative transposons. In: Roberts, AP and Mullany, P, (eds.) *Bacterial integrative mobile genetic*
401 *elements*. Landes Bioscience, Austin, US, 2013, p. 153–70.
- 402 [25] Beabout K, Hammerstrom TG, Wang TT, Bhatti M, Christie PJ, Saxer G, et al. Rampant parasexuality
403 evolves in a hospital pathogen during antibiotic selection. *Mol Biol Evol* 2015;32:2585.
404 <https://doi.org/10.1093/molbev/msv133>.
- 405 [26] Celli J, Trieu-Cuot P. Circularization of Tn916 is required for expression of the transposon-encoded
406 transfer functions: Characterization of long tetracycline-inducible transcripts reading through the
407 attachment site. *Mol Microbiol* 1998;28:103–17. <https://doi.org/10.1046/j.1365-2958.1998.00778.x>.
- 408 [27] Zhang X, Vrijenhoek JEP, Bonten MJM, Willems RJL, van Schaik W. A genetic element present on
409 megaplasmids allows *Enterococcus faecium* to use raffinose as carbon source. *Environ Microbiol*
410 2011;13:518–28. <https://doi.org/10.1111/j.1462-2920.2010.02355.x>.
- 411 [28] Trieu-Cuot P, Carlier C, Poyart-Salmeron C, Courvalin P. A pair of mobilizable shuttle vectors
412 conferring resistance to spectinomycin for molecular cloning in *Escherichia coli* and in Gram-positive
413 bacteria. *Nucleic Acids Res* 1990;18:4296.
- 414 [29] Bronsard J, Pascreau G, Sassi M, Mauro T, Augagneur Y, Felden B. sRNA and *cis*-antisense sRNA
415 identification in *Staphylococcus aureus* highlights an unusual sRNA gene cluster with one encoding
416 a secreted peptide. *Sci Rep* 2017;7:4565. <https://doi.org/10.1038/s41598-017-04786-3>.
- 417 [30] Pinel-Marie M-L, Brielle R, Felden B. Dual toxic-peptide-coding *Staphylococcus aureus* RNA under
418 antisense regulation targets host cells and bacterial rivals unequally. *Cell Rep* 2014;7:424–35.
419 <https://doi.org/10.1016/j.celrep.2014.03.012>.
- 420 [31] Ratnadiwakara M, Änkö M-L. mRNA stability assay using transcription inhibition by actinomycin D
421 in mouse pluripotent stem cells. *Bio Protoc* 2018;8:e3072. doi: 10.21769/BioProtoc.3072
- 422 [32] Riffaud C, Pinel-Marie M-L, Pascreau G, Felden B. Functionality and cross-regulation of the four
423 SprG/SprF type I toxin-antitoxin systems in *Staphylococcus aureus*. *Nucleic Acids Res*
424 2019;47:1740–58. <https://doi.org/10.1093/nar/gky1256>.
- 425 [33] León-Sampedro R, Novais C, Peixe L, Baquero F, Coque TM. Diversity and evolution of the Tn5801-
426 *tet(M)*-like integrative and conjugative elements among *Enterococcus*, *Streptococcus*, and

- 427 *Staphylococcus*. Antimicrob Agents Chemother 2016;60:1736–46.
428 <https://doi.org/10.1128/AAC.01864-15>.
- 429 [34] Felden B, Augagneur Y. Diversity and Versatility in Small RNA-Mediated Regulation in Bacterial
430 Pathogens. Front Microbiol. 2021 Aug 10;12:719977. doi: 10.3389/fmicb.2021.719977.
- 431 [35] Wei Y, Bechhofer DH. Tetracycline induces stabilization of mRNA in *Bacillus subtilis*. J Bacteriol
432 2002;184:889–94. <https://doi.org/10.1128/jb.184.4.889-894.2002>.
- 433 [36] Sayed N, Jousselin A, Felden B. A cis-antisense RNA acts in trans in *Staphylococcus aureus* to control
434 translation of a human cytolytic peptide. Nat Struct Mol Biol. 2011 Dec 25;19(1):105-12. [https://doi:](https://doi.org/10.1038/nsmb.2193)
435 10.1038/nsmb.2193.
- 436 [37] Nilsen TW. Toeprinting. Cold Spring Harb Protoc 2013;2013:896–9.
437 <https://doi.org/10.1101/pdb.prot077180>.
- 438 [38] Scornec H, Bellanger X, Guilloteau H, Groshenry G, Merlin C. Inducibility of Tn916 conjugative
439 transfer in *Enterococcus faecalis* by subinhibitory concentrations of ribosome-targeting antibiotics. J
440 Antimicrob Chemother 2017;72:2722–8. <https://doi.org/10.1093/jac/dkx202>.
- 441 [39] Chukwudi CU, Good L. Interaction of the tetracyclines with double-stranded RNAs of random base
442 sequence: New perspectives on the target and mechanism of action. J Antibiot 2016;69:622–30.
443 <https://doi.org/10.1038/ja.2015.145>.
- 444 [40] Seier-Petersen MA, Jasni A, Aarestrup FM, Vigre H, Mullany P, Roberts AP, et al. Effect of
445 subinhibitory concentrations of four commonly used biocides on the conjugative transfer of Tn916 in
446 *Bacillus subtilis*. J Antimicrob Chemother 2014;69:343–8. <https://doi.org/10.1093/jac/dkt370>.
- 447 [41] Leenhouts K, Buist G, Bolhuis A, ten Berge A, Kiel J, Mierau I, et al. A general system for generating
448 unlabelled gene replacements in bacterial chromosomes. Mol Gen Genet 1996;253:217–24.
449

450 **Legends to figures**

451 **Figure 1** | Genetic environment of chromosomal *ern0030* and *tet(M)* genes in *E. faecium* strains.

452 (A) Genetic organization of *ern0030* and *tet(M)* genes in *E. faecium* Aus0004 and derivative strains
453 (Table S1). Open reading frames (ORFs) are indicated by horizontal arrows. Putative transcription
454 terminators, predicted with ARNold finding terminator software (Neville et al., 2011), were
455 indicated by loops. The *tet(M)* gene actually corresponds to both EFAU004_00063 and
456 EFAU0004_00067 annotated genes (Genbank accession no. CP003351.1).

457 (B) Annotated nucleotide sequence of the 5' untranslated region of *tet(M)*. The -35 and -10
458 promoter boxes are in bold as well as the transcription start site (+1) from which the numbering
459 starts. The white triangle corresponds to the 3' end of *ern0030*. The sequence deleted within
460 $\Delta ern0030$ -Aus0004^{TR} correspond to G₂ to 3' indicated by white triangle. Translation initiation
461 codons of *ern0030*, the putative leader peptide and *tet(M)* are indicated by ATG in bold. The
462 position of mutation within pAT29 $\Omega tetM$ -TGA₁₇₂₋₁₇₄ was indicated by TGA under the translation
463 initiation codon of putative leader peptide previously described by Su an al. The rho-independent
464 transcriptional terminator (comprising the stem-loop between positions 204 and 233) was
465 underlined and correspond to deleted sequence within pAT29 $\Omega tet(M)$ - $\Delta_{205-239} ern0030$. The
466 putative leader peptide sequence are represented. Positions of Northern blot probes and toeprint
467 primers (Table S3) are indicated. Toeprint positions (Figure 6A) are symbolized by black triangles.

468 **Figure 2** | Expression of *Ern0030* transcripts with or without induction by tetracycline SIC in *E.*
469 *faecium* Aus0004.

470 Northern Blots were performed on total RNAs extracted from Aus0004 cells collected at an
471 OD_{600nm} of 1, 2.2 and 3 corresponding to mid (ME), late (LE) exponential and early stationary (ES)
472 phases of growth. The RNAs were extracted on cells grown in the absence (-tet) or presence (+tet)

473 of tetracycline SIC (equivalent 1/32 of MIC). The detection of transcripts was performed by using
474 three different probes (Figure 1B, Table S3). tmRNA levels were used as internal loading controls.
475 The data show a representative experiment among three independent biological replicates.

476 **Figure 3** | Half-life measurement of Ern0030 (A) and *tet(M)* (B) transcripts in *E. faecium*
477 Aus0004^{TR} in the absence of tetracycline.

478 After reaching the ME phase, rifampicin was added at $t = 0$. RNA abundance was measured by
479 absolute RT-qPCR (number of copies by ng of total RNAs) and half-lives (in seconds) were
480 determined by nonlinear one phase decay.

481 **Figure 4** | Expression levels of *tet(M)* (panel A) and Ern0030 (panel B) in *E. faecium* strains in the
482 presence or absence of tetracycline SIC.

483 Total RNA samples were collected from bacterial cells grown until mid-exponential (ME) phase
484 in BHI broth supplemented (grey bar) or not (white bar) with tetracycline SIC (equivalent to 1/32
485 of MIC). Transcript levels (expressed as copy numbers per ng of RNA) in each sample were
486 determined by absolute quantification. The multiple comparison between each sample was done
487 by the Tukey test (p-value adjusted: * < 0.05, *** < 0.001, **** < 0.0001). Error bar shows 95%CI
488 between three independent biological replicates.

489 **Figure 5** | Expression of Ern0030 and *tet(M)* transcripts with or without induction by tetracycline
490 SIC in Aus0004^{TR} and $\Delta ern0030$ -Aus0004^{TR} strains.

491 Northern Blots were performed on total RNAs extracted from Aus0004^{TR} and $\Delta ern0030$ -
492 Aus0004^{TR} cells collected at an OD_{600nm} of 1 corresponding to mid exponential (ME) phase of
493 growth. The RNAs were extracted on cells grown in the absence (-) or presence (+) of tetracycline
494 SIC (equivalent 1/32 of MIC). The detection of transcripts was performed on two different probes,
495 one with a *ern0030*-specific probe and another with a *tet(M)*-specific probe (Table S3). tmRNA

496 levels were used as internal loading controls. The data show a representative experiment among
497 three independent biological replicates.

498 **Figure 6** | Translational toeprint assays on 5' untranslated region (5' UTR) of *tet(M)*.

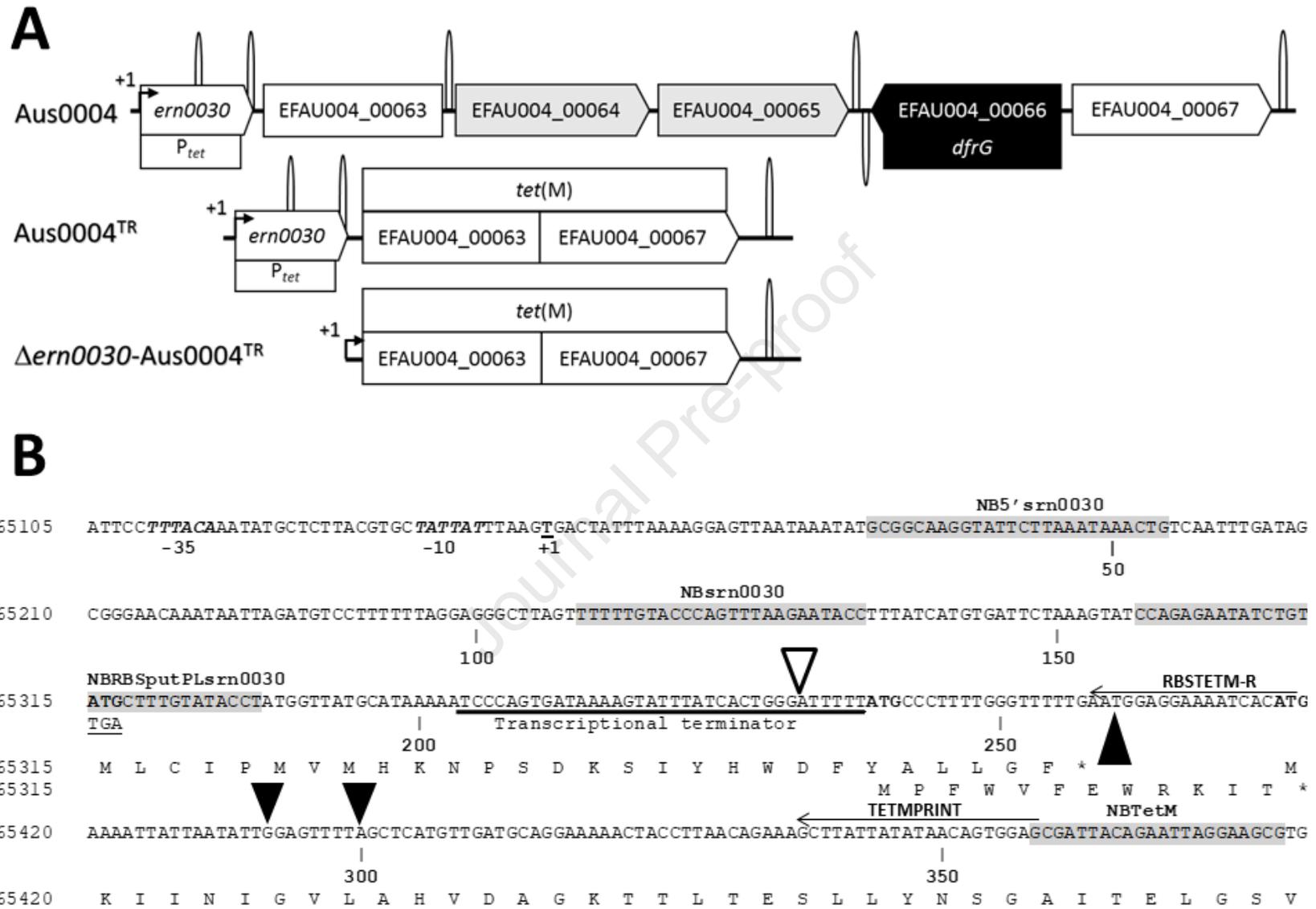
499 The cDNAs were synthesized using primers TETMPRINT (A) and RBSTETM-R (B) that target
500 the RBS of *tet(M)* and that of the putative leader peptide, respectively. Concentrations of 70S
501 ribosomes were indicated as follows: R0: 0 nM, R1: 0.25 nM, R2: 0.5 nM, R3: 1 nM, R4: 1.9 nM.
502 Positions of toeprints are represented by black triangles according to *ern0030* numbering (Figure
503 1B). The position A₂₃₃ corresponds to the end of the stem-loop of the *Ern0030* rho-independent
504 transcription terminator.

505

506 **Table 1.** MIC values (mg/L) of tetracycline and tigecycline against the different *E. faecium* strains.

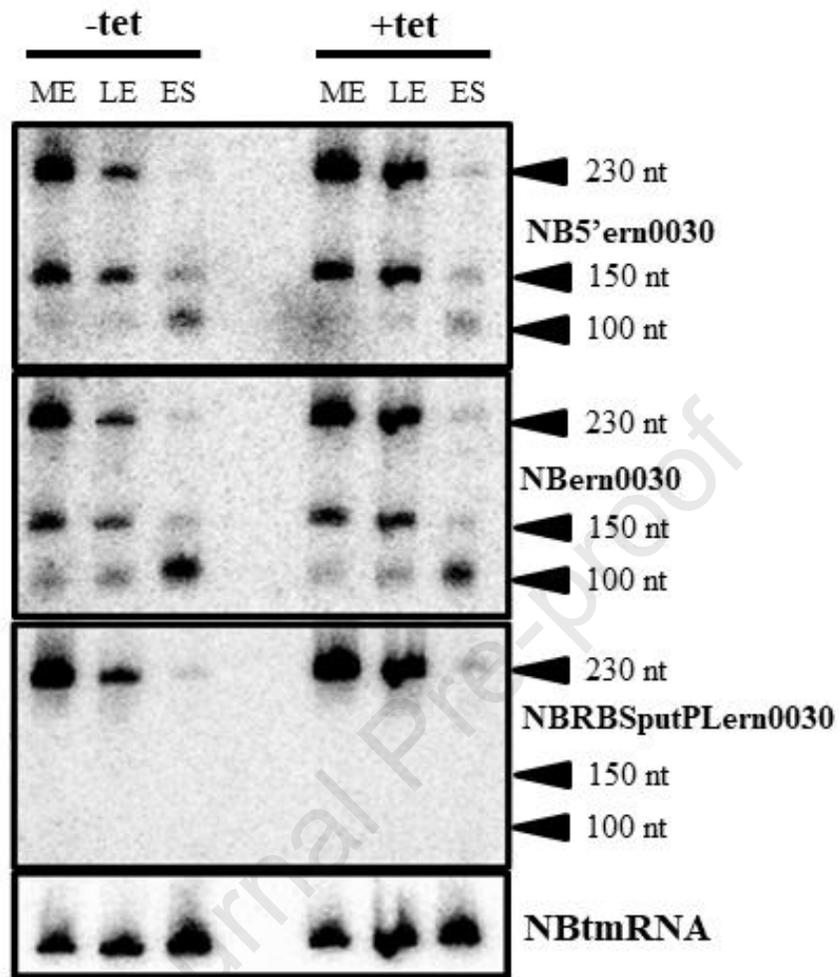
<i>E. faecium</i>	Tetracycline	Tigecycline
Aus0004	0.12	0.01
Aus0004 ^{TR}	32	0.03
$\Delta ern0030$ -Aus0004 ^{TR}	4	0.03
Aus0004 ^{TR} _pAT29	32	0.03
Aus0004 ^{TR} _pAT29 $\Omega ern0030$	32	0.03
$\Delta ern0030$ -Aus0004 ^{TR} _pAT29	4	0.03
$\Delta ern0030$ -Aus0004 ^{TR} _pAT29 $\Omega ern0030$	4	0.03
Aus0004_pAT29	0.12	0.06
Aus0004_pAT29 $\Omega tetM$	64	0.25
Aus0004_pAT29 $\Omega tetM$ - $\Delta_{205-239} ern0030$	32	0.12
Aus0004_pAT29 $\Omega tetM$ -TGA ₁₇₂₋₁₇₄	32	0.03

507



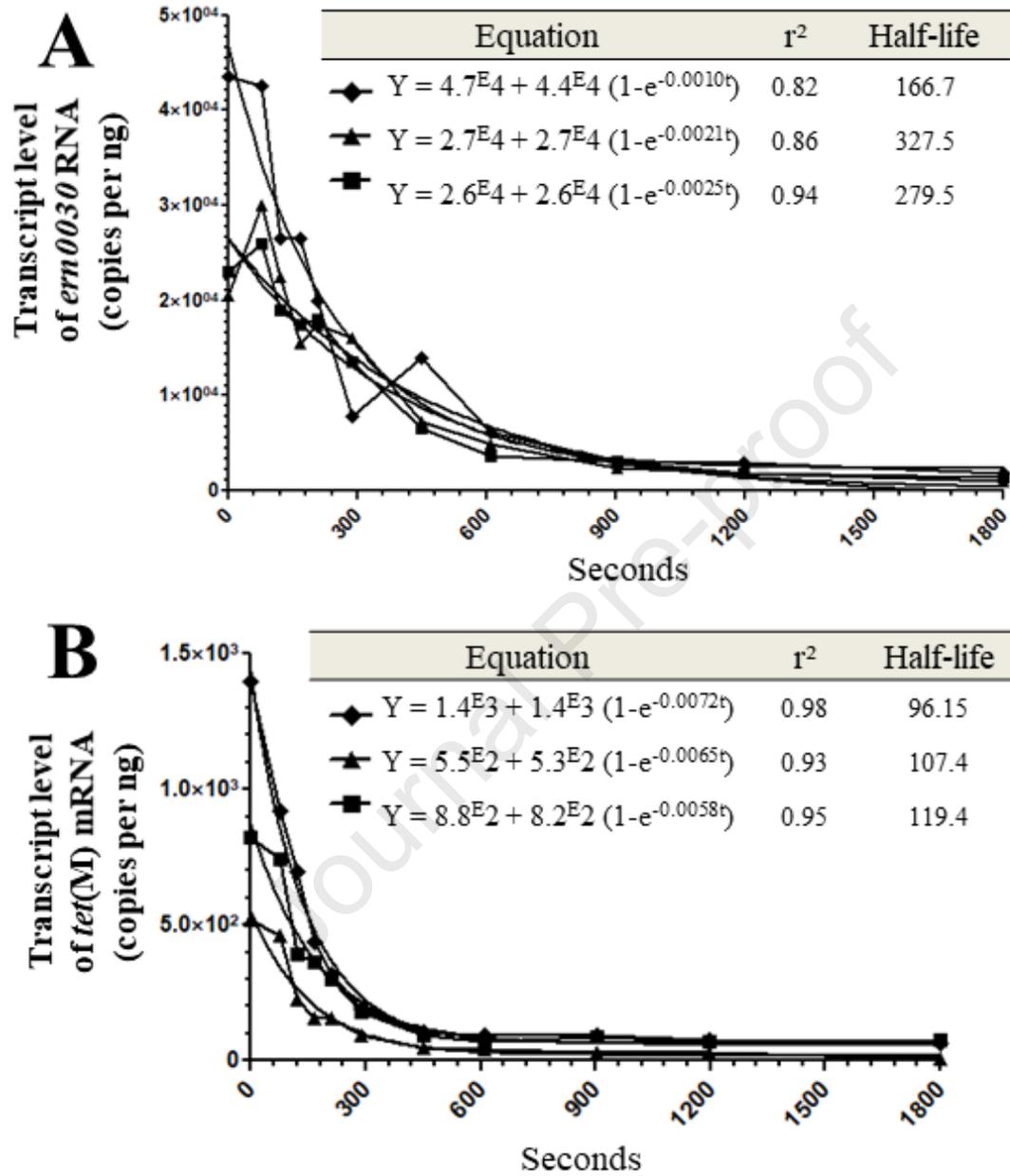
508

509 Figure 1



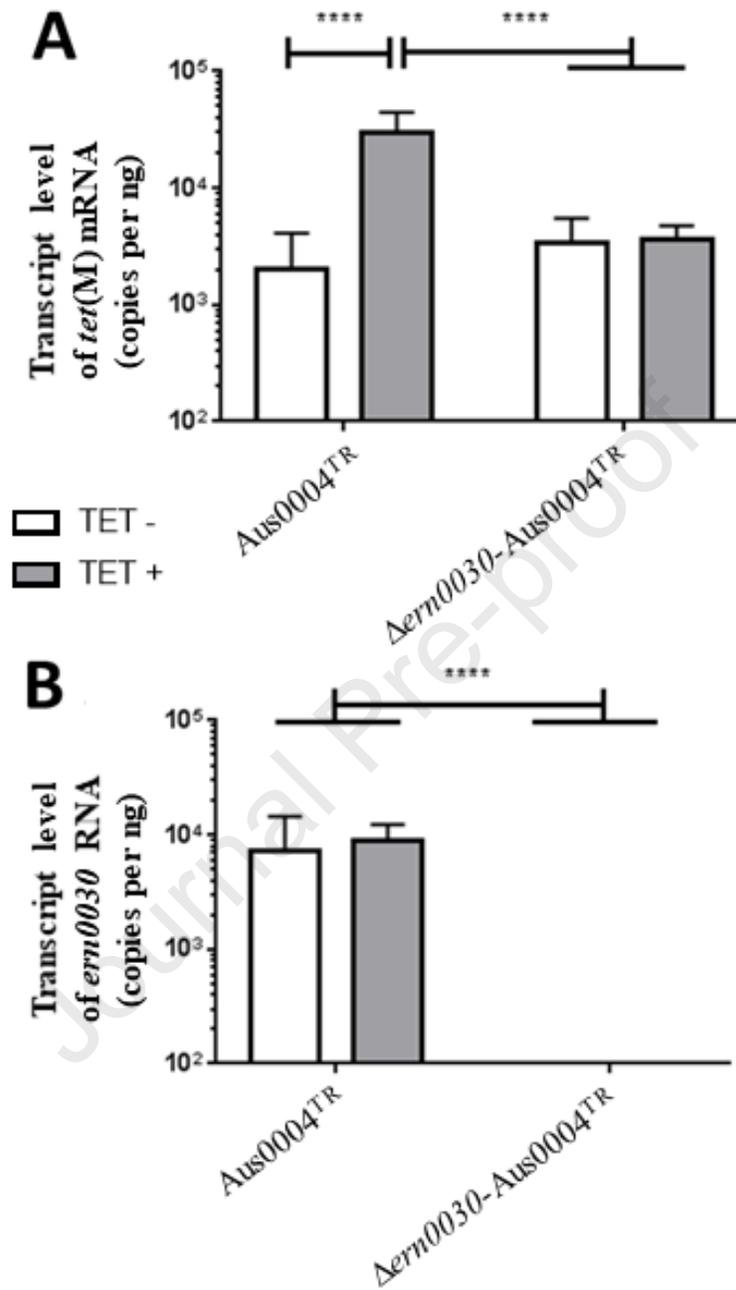
510

511 **Figure 2**



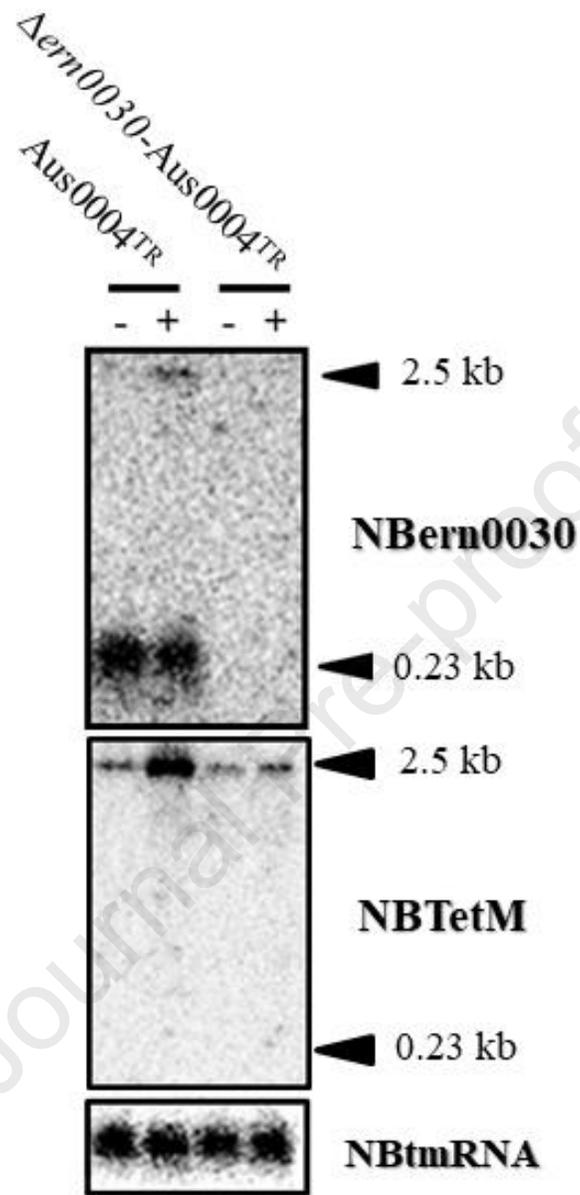
512

513 Figure 3



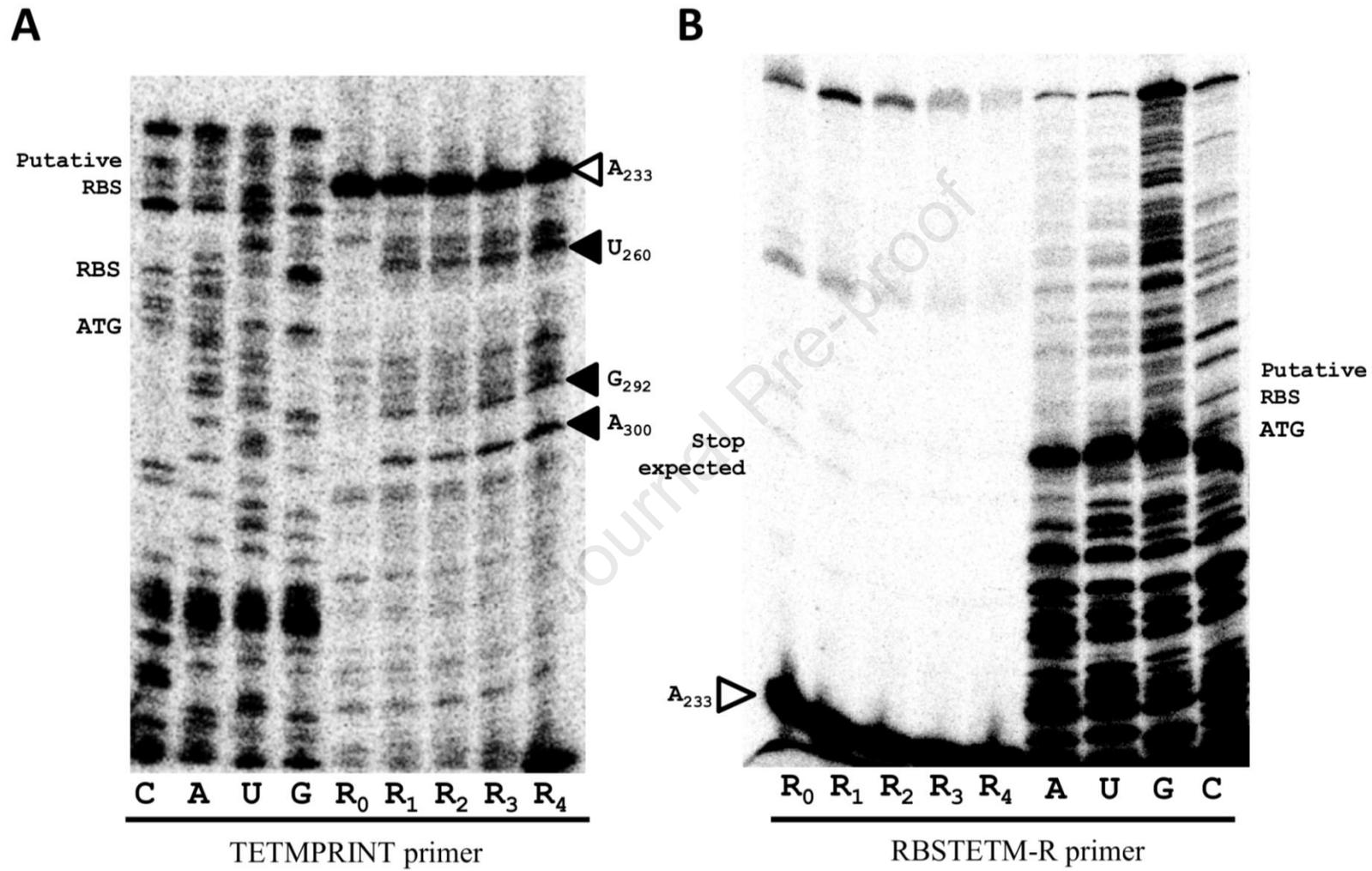
514

515 **Figure 4**



516

517 **Figure 5**



518

519 **Figure 6**

520 **Supplementary material**521 **Supplementary Table S1** | Bacterial strains used in the study.

Strains	Relevant characteristics	Reference
<i>E. faecium</i>		
Aus0004	<i>vanB</i> -positive reference strain, isolated in Australia, 1998	[15]
Aus0004 ^{TR}	Mutant derived from Aus0004 with functional <i>tet</i> (M) (see Figure 1A)	This study
Δ <i>ern0030</i> -Aus0004 ^{TR}	Aus0004 ^{TR} deleted for <i>ern0030</i> (see Figure 1A)	This study
Aus0004 ^{TR} _pAT29	Aus0004 ^{TR} carrying empty pAT29 vector	This study
Aus0004 ^{TR} _pAT29 Ω <i>ern0030</i>	Aus0004 ^{TR} carrying recombinant plasmid pAT29 Ω <i>ern0030</i>	This study
Δ <i>ern0030</i> -Aus0004 ^{TR} _pAT29	Δ <i>ern0030</i> -Aus0004 ^{TR} carrying empty pAT29 vector	This study
Δ <i>ern0030</i> -Aus0004 ^{TR} _pAT29 Ω <i>ern0030</i>	Δ <i>ern0030</i> -Aus0004 ^{TR} carrying recombinant plasmid pAT29 Ω <i>ern0030</i>	This study
Aus0004_pAT29	Aus0004 carrying empty pAT29 vector	This study
Aus0004_pAT29 Ω <i>tet</i> (M)	Aus0004 carrying recombinant plasmid pAT29 Ω <i>tet</i> (M)	This study
Aus0004_pAT29 Ω <i>tet</i> (M)- Δ ₂₀₅₋₂₃₉ <i>ern0030</i>	Aus0004 carrying pAT29 Ω <i>tet</i> (M)- Δ ₂₀₅₋₂₃₉ <i>ern0030</i>	This study
Aus0004_pAT29 Ω <i>tet</i> (M)-TGA ₁₇₂₋₁₇₄	Aus0004 carrying pAT29 Ω <i>tet</i> (M)-TGA ₁₇₂₋₁₇₄	This study
<i>E. coli</i>		
XL1blue	Strain used for 5'3'RACE mapping and cloning	Stratagene®
EC1000	Strain used for cloning	[41]

522 **Supplementary Table S2** | Plasmids used in the study.

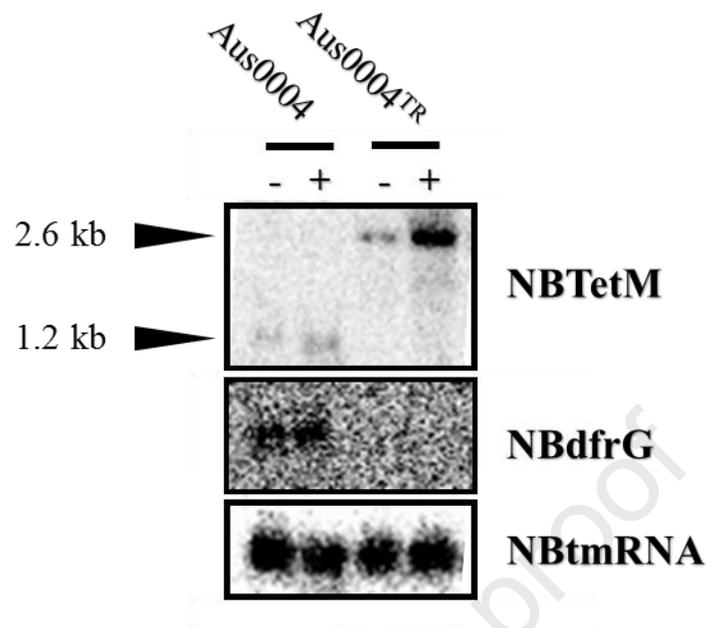
Plasmids	Relevant characteristics ^a	Reference
pGEM-T	High-efficiency TA cloning vector used for 5'3'RACE mapping (Amp ^r)	Promega [®]
pWS3	Temperature-sensitive pG(+) _{host9} -derived shuttle vector used for gene disruption (Spc ^r)	[27]
pAT29	High-copy-number promoterless shuttle vector used for cloning (Spc ^r)	[28]
pAT29Ω _{ern0030}	pAT29 containing <i>ern0030</i> with its native promoter and rho-independent terminator (Spc ^r)	This study
pAT29Ω _{tet(M)}	pAT29 containing both <i>ern0030</i> and <i>tet(M)</i> genes (see Figure 1A) (Spc ^r , Tet ^r)	This study
pAT29Ω _{tet(M)} -Δ ₂₀₅₋₂₃₉ _{ern0030}	pAT29Ω _{tet(M)} with a 35-nt deletion in <i>ern0030</i> comprising its rho-independent terminator (see Figure 1B) (Spc ^r , Tet ^r)	This study
pAT29Ω _{tet(M)} -TGA ₁₇₂₋₁₇₄	pAT29Ω _{tet(M)} plasmid with a unique mutation (ATG → TGA) in the translation initiation codon of the putative peptide leader (see Figure 1B) (Spc ^r , Tet ^r)	This study

523 ^aAmp^r, ampicillin resistance; Spc^r, Spectinomycin resistance; Tet^r, tetracycline resistance.

524 **Supplementary Table S3** | Oligonucleotides used in the study.

Name	Nucleotide sequence (5'-3')	Purpose
sRNA0030F1	CATGTGATTCTAAAGTATCCAG	5'3'RACE mapping of <i>ern0030</i>
sRNA0030F2	CTGTATGCTTTGTATACCTATGG	
sRNA0030R1	AAAGGTATTCTTAAACTGGG	
sRNA0030R2	CTAAGCCCTCCTAAAAAAGGACATC	
sRNA0030R3	CCCGCTATCAAATTGACAGTT	
KOern0030F1	CTATGAATTCAGCTTTCATGTGGTTGTCCATATAG	Knockout deletion of <i>ern0030</i>
KOern0030F2	TTTTATGCCCTTTTGGGTTTTTGAATGG	
KOern0030R1	CCAAAAGGGCATAAAAACTTAAATAATAGCACGTAAGAGC	
KOern0030R2	GTTTGAATTCCTGTTTATTGATTACAATTCGGCAG	
KOern0030vF	TTGGAGATTCCCTTACAAATATGC	
KOern0030vR	TTCTCTGCATCAACATGAGC	
KOdfGF1	AGAAGGAATTCCAAAGGTACAACGAGGACGG	Restoration of <i>tet(M)</i> function
KOdfGF2	ATACGCCTTTATAGTGGAGTACTACATTTACGAGATTTCG	
KOdfGR1	CTCCACTATAAAGGCGTATATATGCAAGACGTTGTCTTT	
KOdfGR2	GTTCCAGAATTCCTAAAGGCTTGTCCAGTAC	
vF-KOdfG	CATCAACACATCGAGGTCGG	
vR-KOdfG	CTCCAGAATAAGCTCTATCAATCTTAC	
pCern0030F	GGTGGTGGATCCCAAATATTGGTACATGATTACAG	Construction of recombinant plasmids
pCern0030R	GGTGGTGAATTCGTGATTTTCTCCATTCAAAAAC	
F-Dloopern0030	ATGCCCTTTTGGGTTTTTGA	
R-Dloopern0030	AACCCAAAAGGGCATTTTTTATGCATAACCATAGGTATAC	
R-StopPLtetM	TACAAAGTCAACAGATATTCTCTGGATACTT	
F-StopPLtetM	TACTGTTGACTTTGTATACCTATGGTTATGC	
R-pCtetM	GGTGGTGAATTCCAAAGAGCCGATAAGATGAG	
M13F	GTA AACGACGGCCAGTG	Verification of recombinant plasmid constructions
M13 Reverse short	CAGGAAACAGCTATGAC	
pWS3F1	TTCCAGTCACGACGTTGT	
pWS3R1	ACTGACAGCTTCCAAGGAGC	
pAT29vF	GTA AACGACGGCCAG	
pAT29vR	CAGGAAACAGCTATGAC	
qPCRsRNA0030F	CATGTGATTCTAAAGTATCCAGAGA	RT-qPCR
qPCRsRNA0030R	TGCATAACCATAGGTATACAAAGCA	
qPCRTEtMF	CGATTACAGAATTAGGAAGCG	
qPCRTEtMR	GATGTTACCTTCGTATTTTCC	
adk-F	CCGCACATCTCAACAGGAGA	
adk-R	AGGCCAAGAGCTGTTTCGTT	
T7ern0030F	TAATACGACTCACTATAGGGTGACTATTTAAAAGGAGTTAATA AATATGCG	<i>In vitro</i> transcription
R-PCRTEtM	GTCTCGTTGTACCTTTGTC	
F-PCRTEtM	GGAGTTTTAGCTCATGTTGATGC	RT-PCR
F-ORF	CCTTTTGGGTTTTTGAATGGAG	
F-PCRern0030	GGAGTTAATAAATATGCGCAAGG	
NBtmRNA	TCGCTAGTCTGATGTTCTCTTTTTTC	Probes for Northern blot
NBTetM	CGCTTCCTAATTCTGTAATCGC	
NBern0030*	GGTATTCTTAAACTGGGTACAAAAA	
NBRBSputPLern0030	AGGTATACAAAGCATAACAGATATTCTCTGG	
NB5'ern0030	CAGTTTATTTAAGAATACCTTGCCGC	
NBdfG	GGTCTCTCCTACCTAATATTATCGG	
TETMPRINT	CTCCACTGTTATATAATAAGC	Toeprint assays
RBSTETM-R	CATGTGATTTTCTCCAT	

*Probe for *ern0030* used previously [16].



526

527

528 **Supplementary Figure S1** | Validation of the Aus0004^{TR} strain by Northern Blot.

529 Northern Blots were performed on total RNAs extracted from Aus0004^{TR} cells collected at an

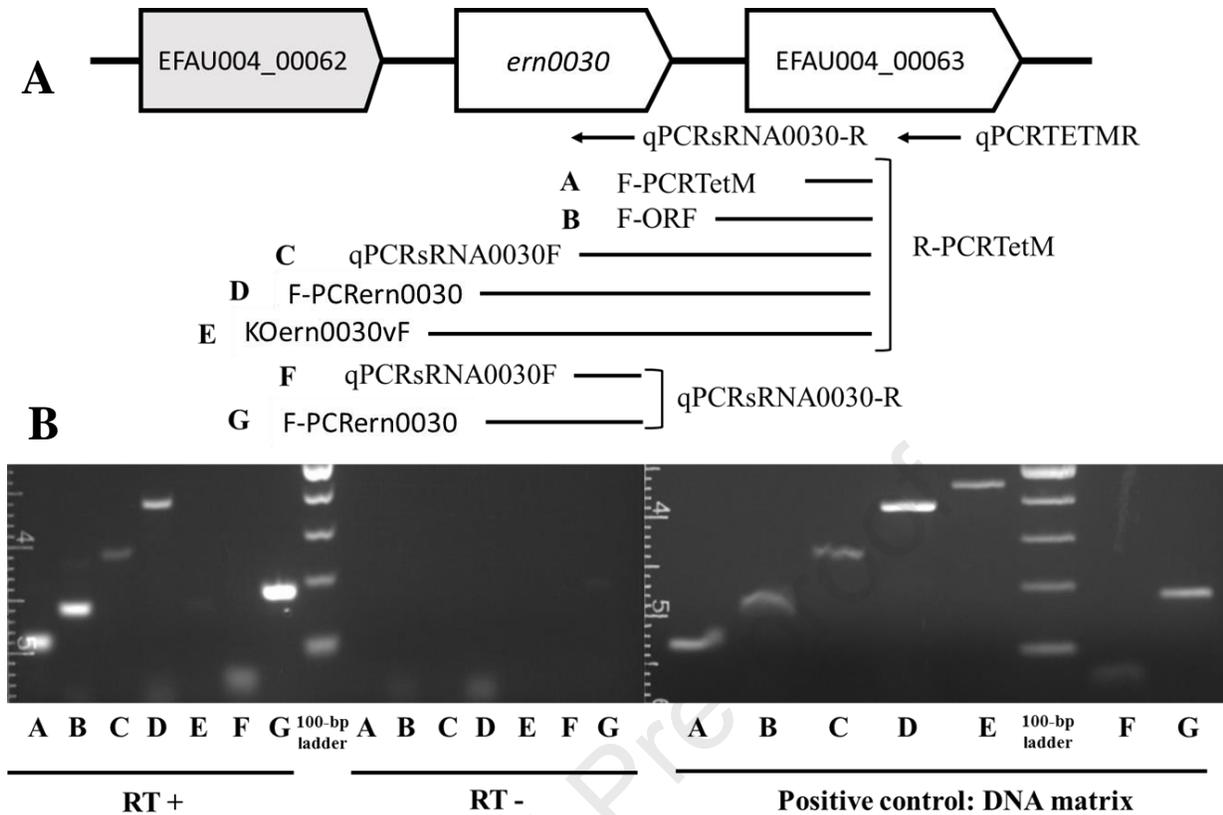
530 OD_{600nm} of 2.1 corresponding to the late exponential phase of growth. The RNAs were extracted

531 on cells grown in the absence (-) or presence (+) of tetracycline SIC (equivalent 1/32 of MIC).

532 The detection of transcripts was performed by using *tet(M)*-specific and *dfrG*-specific probes

533 (Supplementary Table S3). tmRNA levels were used as internal loading controls. The data show

534 a representative experiment among three independent biological replicates.



535

536 **Supplementary Figure S2** | Mapping of the 5' untranslated region (5' UTR) of *tet(M)* by RT-
 537 PCR.

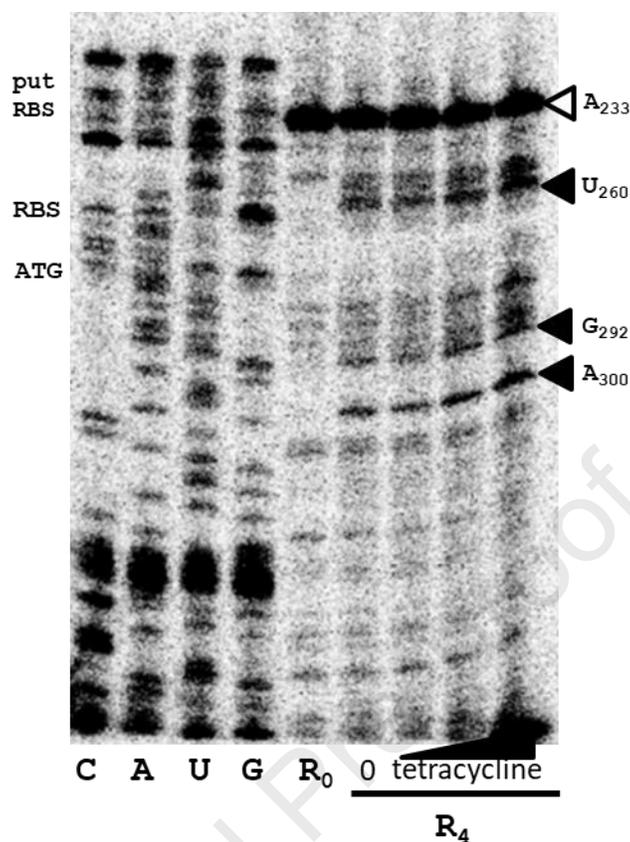
538 (A) Schematic representation of the genetic environment of *ern0030* in *E. faecium* Aus0004

539 (Genbank accession no. CP003351.1). Black labeled lines (A-G) indicate the regions of interest

540 for amplification. The two primers used for reverse transcription are represented by arrows.

541 (B) Visualization of PCR-amplified products using cDNAs as matrix (RT +). Genomic DNA was

542 used as positive control and DNase-treated RNAs as negative control (RT -).



543

544 **Supplementary Figure S3** | Translational toeprint assays on 5' untranslated region (5' UTR) of

545 *tet(M)*.

546 The cDNAs were synthesized using the TETMPRINT primer that target the RBS of *tet(M)*.

547 Concentrations of 70S ribosomes were indicated as follows: R0: 0 nM, R4: 1.9 nM.

548 Concentrations of tetracycline were from 0 to 288 μ M (0 M; 281 nM; 9 μ M; 288 μ M). Positions

549 of toeprints are represented by black triangles according to *ern0030* numbering (Figure 1B). The

550 position A₂₃₃ corresponds to the end of the stem-loop of the *ern0030* rho-independent

551 transcription terminator.