

SUPPLEMENTARY MATERIAL

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

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Figures S1 to S3

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	[1]
Aus0004 ^{TR}	Mutant derived from Aus0004 with functional <i>tet</i> (M) (see Figure 1A)	This study
$\Delta ern0030$ -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 $\Omega ern0030$	Aus0004 ^{TR} carrying recombinant plasmid pAT29 $\Omega ern0030$	This study
$\Delta ern0030$ -Aus0004 ^{TR} _pAT29	$\Delta ern0030$ -Aus0004 ^{TR} carrying empty pAT29 vector	This study
$\Delta ern0030$ -Aus0004 ^{TR} _pAT29 $\Omega ern0030$	$\Delta ern0030$ -Aus0004 ^{TR} carrying recombinant plasmid pAT29 $\Omega ern0030$	This study
Aus0004_pAT29	Aus0004 carrying empty pAT29 vector	This study
Aus0004_pAT29 Ωtet (M)	Aus0004 carrying recombinant plasmid pAT29 Ωtet (M)	This study
Aus0004_pAT29 Ωtet (M)- $\Delta_{205-239} ern0030$	Aus0004 carrying pAT29 Ωtet (M)- $\Delta_{205-239} ern0030$	This study
Aus0004_pAT29 Ωtet (M)-TGA ₁₇₂₋₁₇₄	Aus0004 carrying pAT29 Ωtet (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	[2]

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 [®]

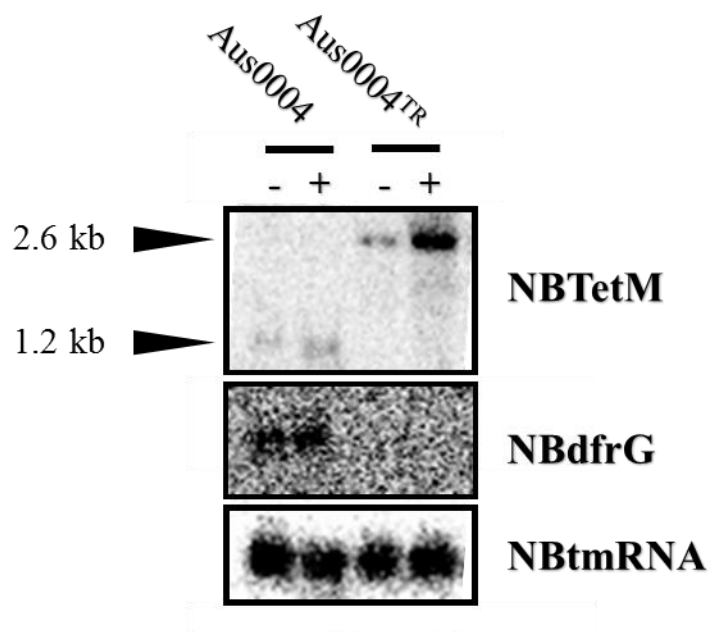
pWS3	Temperature-sensitive pG(+)host9-derived shuttle vector used for gene disruption (Spc ^r)	[3]
pAT29	High-copy-number promoterless shuttle vector used for cloning (Spc ^r)	[4]
pAT29Ω <i>ern0030</i>	pAT29 containing <i>ern0030</i> with its native promoter and rho-independent terminator (Spc ^r)	This study
pAT29Ω <i>tet</i> (M)	pAT29 containing both <i>ern0030</i> and <i>tet</i> (M) genes (see Figure 1A) (Spc ^r , Tet ^r)	This study
pAT29Ω <i>tet</i> (M)-Δ ₂₀₅₋₂₃₉ <i>ern0030</i>	pAT29Ω <i>tet</i> (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 <i>tet</i> (M)-TGA ₁₇₂₋₁₇₄	pAT29Ω <i>tet</i> (M) plasmid with an unique mutation (ATG → TGA) in the translation initiation codon of the putative peptide leader (see Figure 1B) (Spc ^r , Tet ^r)	This study

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

Supplementary Table S3 | Oligonucleotides used in the study.

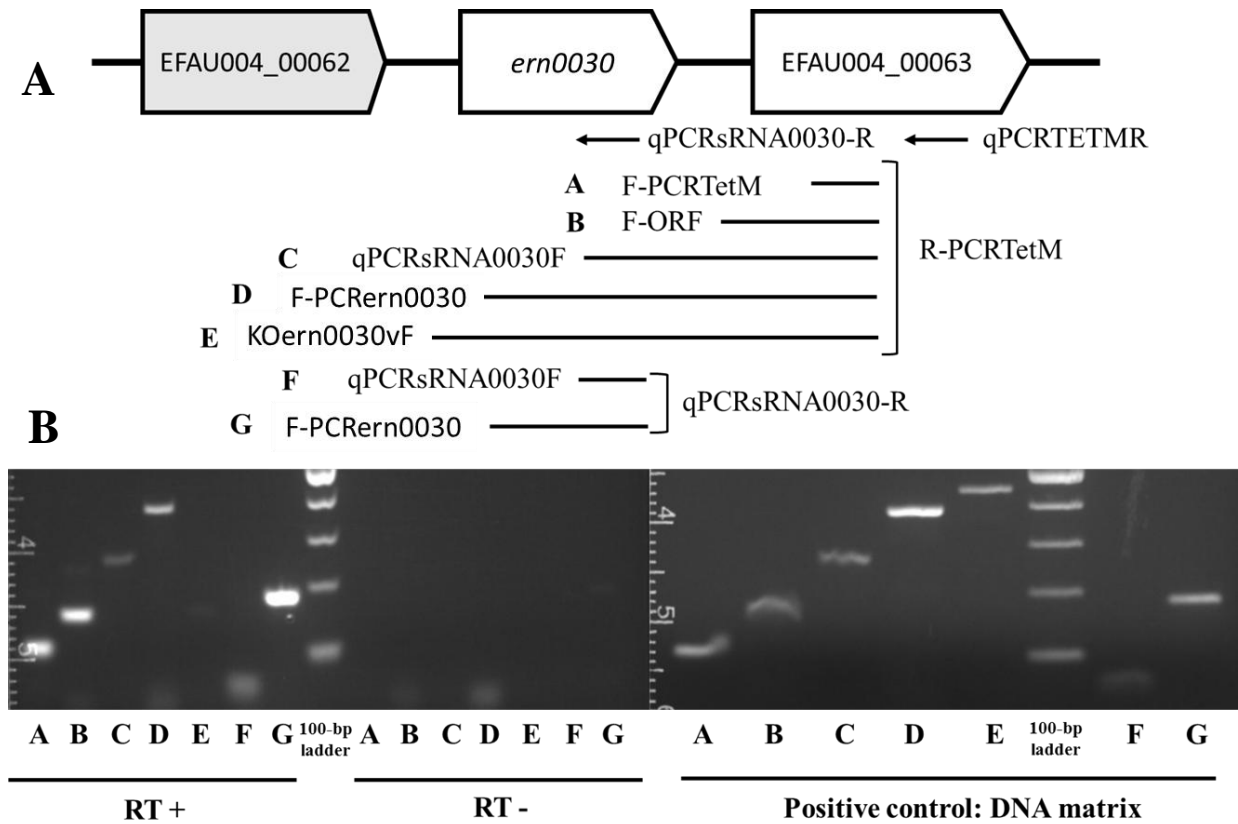
Name	Nucleotide sequence (5'-3')	Purpose
sRNA0030F1	CATGTGATTCTAAAGTATCCAG	5'3'RACE mapping of <i>ern0030</i>
sRNA0030F2	CTGTATGCTTTGTATACCTATGG	
sRNA0030R1	AAAGGTATTCTTAAACTGGG	
sRNA0030R2	CTAAGCCCTCCTAAAAAAGGACATC	
sRNA0030R3	CCCCTATCAAATGACAGTT	
KOern0030F1	CTATGAATTCAGCTTTCATGTGGTTGTCCATATAG	Knockout deletion of <i>ern0030</i>
KOern0030F2	TTTTTATGCCCTTTTGGGTTTTTGAATGG	
KOern0030R1	CCAAAAGGGCATAAAAACTTAAATAATAGCACGTAAGAGC	
KOern0030R2	GTTTCGAATTCCTGTTTGATTACAATTCGGCAG	
KOern0030vF	TTGGAGATTCTTTACAAATATGC	
KOern0030vR	TTTCTGCATCAACATGAGC	
KOdfR-GF1	AGAAGGAATTCCAAAGGTACAACGAGGACGG	Restoration of <i>tet(M)</i> function
KOdfR-GF2	ATACGCCCTTTATAGTGGAGTACTACATTTACGAGATTTCG	
KOdfR-GR1	CTCCACTATAAAGGCGTATATATGCAAGACGTTGTCTTT	
KOdfR-GR2	GTTCCAGAATTCCTAAAGGCTTGCTCCAGTAC	
vF-KOdfR-G	CATCAACACATCGAGGTCCG	
vR-KOdfR-G	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	GTAAAACGACGGCCAGTG	Verification of recombinant plasmid constructions
M13 Reverse short	CAGGAAACAGCTATGAC	
pWS3F1	TTTCCCAGTCACGACGTTGT	
pWS3R1	ACTGACAGCTTCCAAGGAGC	
pAT29vF	GTAAAACGACGGCCAG	
pAT29vR	CAGGAAACAGCTATGAC	
qPCRsRNA0030F	CATGTGATTCTAAAGTATCCAGAGA	RT-qPCR
qPCRsRNA0030R	TGCATAACCATAGGTATACAAAGCA	
qPCRtETMF	CGATTACAGAATTAGGAAGCG	
qPCRtETMR	GATGTTACCTTCGTATTTTCC	
adk-F	CCGCACATCTCAACAGGAGA	
adk-R	AGGCCAAGAGCTGTTTCGTT	
T7ern0030F	TAATACGACTCACTATAGGGTGACTATTTAAAAGGAGTTAATAA ATATGCG	<i>In vitro</i> transcription
R-PCRtETM	GTCCTCGTTGTACCTTTGTC	
F-PCRtETM	GGAGTTTTAGCTCATGTTGATGC	RT-PCR
F-ORF	CCCTTTTGGGTTTTTGAATGGAG	
F-PCRern0030	GGAGTTAATAAATATGCGGCAAGG	
NBtmRNA	TCGCTAGTCTGATGTTCTCTTTTTTTC	Probes for Northern blot
NBTetM	CGCTTCCTAATTCTGTAATCGC	
NBern0030*	GGTATTCTTAAACTGGGTACAAAAA	
NBRBSputPLern0030	AGGTATACAAAGCATAACAGATATTCTCTGG	
NBS'ern0030	CAGTTTATTTAAGAATACCTTGCCCG	
NBdfR-G	GGTTCTTCTACCTAATATTATCGG	
TETMPRINT	CTCCACTGTTATATAATAAGC	Toeprint assays
RBSTETM-R	CATGTGATTTTCTCCAT	

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



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

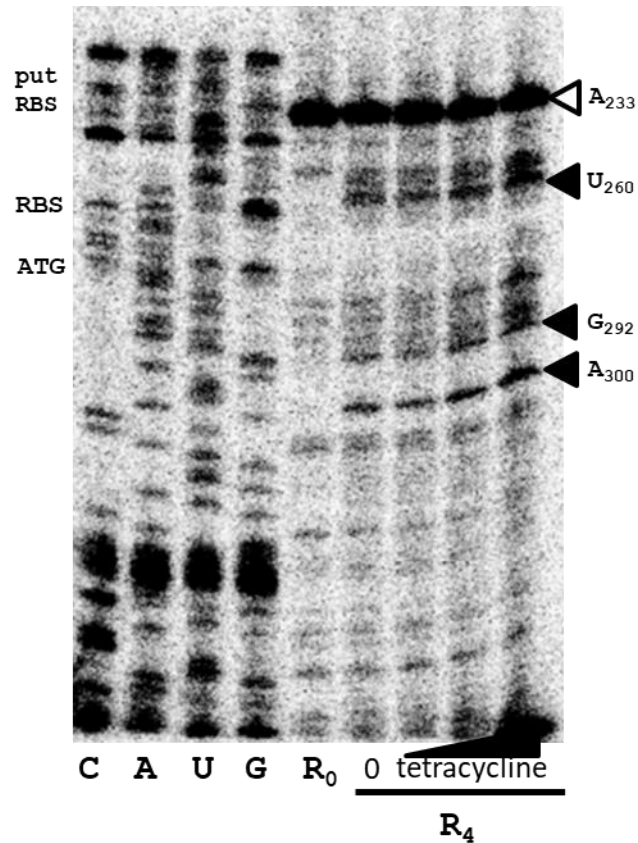
Northern Blots were performed on total RNAs extracted from Aus0004^{TR} cells collected at an OD_{600nm} of 2.1 corresponding to the late exponential phase of growth. The RNAs were extracted on cells grown in the absence (-) or presence (+) of tetracycline SIC (equivalent 1/32 of MIC). The detection of transcripts was performed by using *tet(M)*-specific and *dfrG*-specific probes (Supplementary Table S3). tmRNA levels were used as internal loading controls. The data show a representative experiment among three independent biological replicates.



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

(A) Schematic representation of the genetic environment of *ern0030* in *E. faecium* Aus0004 (Genbank accession no. CP003351.1). Black labeled lines (A-G) indicate the regions of interest for amplification. The two primers used for reverse transcription are represented by arrows.

(B) Visualization of PCR-amplified products using cDNAs as matrix (RT +). Genomic DNA was used as positive control and DNase-treated RNAs as negative control (RT -).



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

The cDNAs were synthesized using the TETMPRINT primer that target the RBS of *tet(M)*. Concentrations of 70S ribosomes were indicated as follows: R0: 0 nM, R4: 1.9 nM. Concentrations of tetracycline were from 0 to 288 μM (0 M; 281 nM; 9 μM ; 288 μM). Positions of toeprints are represented by black triangles according to *ern0030* numbering (Figure 1B). The position A_{233} corresponds to the end of the stem-loop of the *ern0030* rho-independent transcription terminator.

References

- [1] Lam MMC, Seemann T, Bulach DM, Gladman SL, Chen H, Haring V, et al. Comparative Analysis of the first complete *Enterococcus faecium* genome. *J Bacteriol* 2012;194:2334–41. <https://doi.org/10.1128/JB.00259-12>.
- [2] Leenhouts K, Buist G, Bolhuis A, ten Berge A, Kiel J, Mierau I, et al. A general system for generating unlabelled gene replacements in bacterial chromosomes. *Mol Gen Genet* 1996;253:217–24.
- [3] Zhang X, Vrijenhoek JEP, Bonten MJM, Willems RJL, van Schaik W. A genetic element present on megaplasmids allows *Enterococcus faecium* to use raffinose as carbon source. *Environ Microbiol* 2011;13:518–28. <https://doi.org/10.1111/j.1462-2920.2010.02355.x>.
- [4] Trieu-Cuot P, Carlier C, Poyart-Salmeron C, Courvalin P. A pair of mobilizable shuttle vectors conferring resistance to spectinomycin for molecular cloning in *Escherichia coli* and in Gram-positive bacteria. *Nucleic Acids Res* 1990;18:4296.