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Research paper

Molecular basis for the emergence of a new hospital endemic tigecycline-resistant *Enterococcus faecalis* ST103 lineage

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ABSTRACT

Enterococcus faecalis are a major cause of nosocomial infection worldwide, and the spread of vancomycin resistant strains (VRE) limits treatment options. Tigecycline-resistant VRE began to be isolated from inpatients at a Brazilian hospital within months following the addition of tigecycline to the hospital formulary. This was found to be the result of a spread of an ST103 *E. faecalis* clone. Our objective was to identify the basis for tigecycline resistance in this lineage. The genomes of two closely related tigecycline-susceptible (MIC = 0.06 mg/L), and three representative tigecycline-resistant (MIC = 1 mg/L) ST103 isolates were sequenced and compared. Further, efforts were undertaken to recapitulate the emergence of resistant strains *in vitro*. The specific mutations identified in clinical isolates in several cases were within the same genes identified in laboratory-evolved strains. The contribution of various polymorphisms to the resistance phenotype was assessed by *trans*-complementation of the wild type or mutant alleles, by testing for differences in mRNA abundance, and/or by examining the phenotype of transposon insertion mutants. Among tigecycline-resistant clinical isolates, five genes contained non-synonymous mutations, including two genes known to be related to enterococcal tigecycline resistance (*tetM* and *rpsJ*). Finally, within the *in vitro*-selected resistant variants, mutation in the gene for a MarR-family response regulator was associated with tigecycline resistance. This study shows that *E. faecalis* mutates to attain tigecycline resistance through the complex interplay of multiple mechanisms, along multiple evolutionary trajectories.

1. Introduction

Enterococci are opportunistic pathogens and one of the major causes of nosocomial infection worldwide. The emergence of vancomycin-resistant enterococci (VRE) has significantly reduced available treatment options (Cetinkaya et al., 2000). Tigecycline is an antibiotic of last resort for infections caused by a number of multidrug-resistant organisms, including VRE (Cunha et al., 2017). This glycylcycline antibiotic, synthetic derivate from tetracycline, was approved for the treatment of complicated skin and soft-structure infections, complicated intra-abdominal infections, and community-acquired bacterial pneumonia in United States (Food and Drug Administration, 2005) and Brazil (Agência Nacional de Vigilância Sanitária, n.d.) in 2005, and in Europe (European Medicines Agencies, n.d.) in 2006.

Drug efflux is the most frequently reported mechanism of resistance to tigecycline (Zhong et al., 2014; Peleg et al., 2007; Ruzin et al., 2007). In *Staphylococcus aureus*, tigecycline resistance has been ascribed to mutations in the *mepRAB* efflux pump (McAleese et al., 2005; Dabul et al., 2017) and response regulator (Dabul et al., 2017). Monooxygenases capable of modifying the tigecycline molecule have been identified in *Acinetobacter baumannii* (Costello et al., 2016) and *Bacillus subtilis* (Bartha et al., 2011). Tigecycline resistance in multiple

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organisms has also been associated with mutations in the gene encoding the ribosomal protein S10 (Beabout et al., 2015). Tigecycline-resistant isolates of *E. faecalis* have been reported previously (Tyson et al., 2018; Werner et al., 2008), but resistance mechanisms have not been fully explored in this pathogen.

In the process of infection control surveillance at Risoleta Tolentino Neves Hospital in Belo Horizonte, Brazil in 2009, 63 VRE were isolated from sites of infection and colonization (Merlo et al., 2015). Of these VRE, 14 strains were identified as *E. faecalis* (22.2%) and none exhibited resistance to tigecycline (Merlo et al., 2015). In 2011, tigecycline was introduced in this hospital for treatment of soft tissue infections caused by multidrug-resistant *Acinetobacter* sp., and also for abdominal infections caused by VRE. Subsequent surveillance from March to June 2011 showed an increase in the proportion of *E. faecalis* to 29 out of 47 VRE (61.7%). Further, ten of these vancomycin-resistant *E. faecalis* strains were found to be resistant to tigecycline. Because resistance to tigecycline in vancomycin-resistant *E. faecalis* is uncommon (Kuch et al., 2012; Cordina et al., 2012), it was important to determine the genetic background and features of these tigecycline-resistant VRE isolates, and to identify mutations as either markers for or contributing to tigecycline resistance.

Prior PFGE analysis revealed that *E. faecalis* VRE57 (Merlo et al., 2015), from 2009, and *E. faecalis* VRE109, from 2011, were the most closely related tigecycline-susceptible isolates to the tigecycline-resistant ST103 strains isolated in 2011 (Fig. 1). Thus, our approach to identify candidate genes or mutations that could contribute to tigecycline resistance was to first compare the genomes of the tigecycline-resistant strains VRE65, VRE69 and VRE80, with the tigecycline-susceptible strain VRE109 and VRE57, and then perform an adaptation experiment *in vitro* with VRE109 in increasing concentrations of tigecycline, following genome analysis of the adapted strains.

2. Materials and methods

2.1. Source of strains

During routine infection control surveillance between March and June 2011 at Risoleta Tolentino Neves, 29 VRE of the species *E. faecalis* were isolated from patients. The hospital sees an average of 280 patients per day in its emergency room, contains 345 beds, and is located in Belo Horizonte, the capital of the state of Minas Gerais and the sixth most populous city in Brazil.

2.2. Antimicrobial susceptibility

Minimum inhibitory concentrations (MICs) for vancomycin, linezolid, daptomycin and tigecycline were determined by broth microdilution following Clinical and Laboratory Standards Institute (CLSI) reccomendations (Clinical and Laboratory Standards Institute, 2018). The tigecycline breakpoint used to define resistance was 0.25 mg/L, as defined by the European Committee on Antimicrobial Susceptibility Testing (European Committee on Antimicrobial Susceptibility Testing, 2018).

2.3. Molecular characterization

PCR was used to detect the presence of *vanA* (*Woodford* et al., *1993*) and to assess strains for virulence genes *elrA*, *cylL$_L$*, *esp* and *gelE*, essentially as described (Camargo et al., 2006; Leavis et al., 2007; Brinster et al., 2007). Extended PCR was used to characterize Tn*1546* (*Woodford* et al., *1997*). Molecular typing of strains was performed by pulsed-field gel electrophoresis (PFGE) after macrorestriction of genomic DNA with *Sma*I (Tenover et al., 1995), and by Multi-Locus Sequence Typing (MLST) (Ruiz-Garbajosa et al., 2006). *E. faecalis* strains from the same hospital, isolated during the earlier 2009 surveillance and described previously by our group (Merlo et al., 2015) were included for comparison. Plasmid *rep* types present in all isolates were determined essentially as described (Jensen et al., 2010).

2.4. In vitro-selection of tigecycline-resistant variants

Tigecycline-susceptible isolate *E. faecalis* VRE109 was selected as the parent strain for attempting to select tigecycline-resistant mutations *in vitro*. The experiment was performed in triplicate as previously described (Dabul et al., 2017), and conducted over 42 days. Briefly, an overnight culture of each of three VRE109 colonies was grown in Brain Heart Infusion (BHI) broth, then diluted to an OD_{600} = 0.1. From this, 30 µL (approximately 3×10^6 CFU) were inoculated into tubes containing 3 mL of BHI and varying concentrations of tigecycline: a) 1/2 MIC (0.03 mg/L); b) 1× MIC (0.06 mg/L); c) 2× MIC (0.125 mg/L); and d) 4× MIC (0.25 mg/L). All tubes were protected from light and grown at 37 °C overnight without shaking. The next day, the tube of each replicate with the highest tigecycline concentration showing visible growth was used to inoculate fresh tubes with that and increasing drug concentrations.

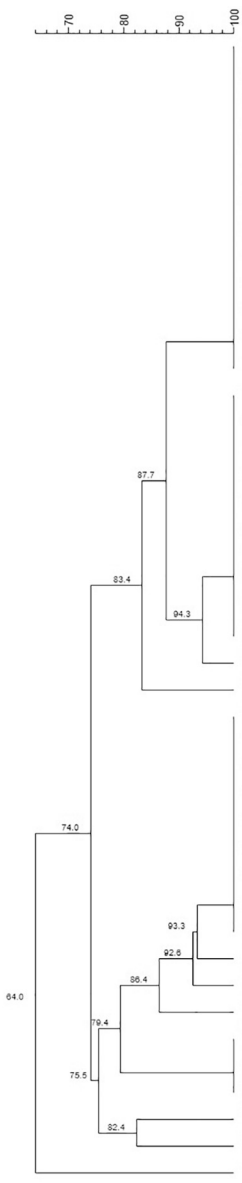
2.5. Genome sequencing, assembly and comparative analysis

Three naturally occurring tigecycline-resistant *E. faecalis* strains, VRE65, VRE69 and VRE80, isolated in March and April 2011 were selected for genome sequencing because they were representative of the first tigecycline-resistant *E. faecalis* to be isolated in the hospital. For comparison, tigecycline susceptible VRE109, and *in vitro*-selected tigecycline resistant variants A1, A11, A20, A42, B1, B21, B42, C1, C6, and C42 were also sequenced (letter designations correspond to the experimental replicate, and numbers correspond to the day of the experiment from which the variant was isolated). Additionally, two tigecycline-susceptible VRE clinical strains, one from 2009 and one from 2011, were selected for sequencing and comparison based on PFGE similarity to the tigecycline-resistant *E. faecalis* strains.

Total genomic DNA was extracted from cultures of each strain using the DNeasy Blood & Tissue kit (QIAGEN, Valencia, USA) following the manufacturer's recommendations for Gram-positive organisms. Sequencing libraries were prepared using the Illumina Nextera XT DNA sample preparation kit (Illumina, San Diego, USA), with recommended modifications for 2 × 250 bp paired-end sequencing. One nanogram of DNA was used for library preparation. Libraries were multiplexed and sequenced on an Illumina MiSeq at the Ocular Genomics Institute in the Massachusetts Eye and Ear Infirmary.

CLC Genomics Workbench v.7.0.4 (Qiagen, Aarhus, Denmark) was used for genome assembly using default parameters. Genomes were annotated through the NCBI Prokaryotic Annotation Pipeline. Variants were called using CLC Genomics Workbench by comparing the genome sequences of the resistant strains to the annotated sequences of tigecycline-susceptible isolates VRE57 and VRE109. Altered nucleotides and corresponding amino acid changes were further analyzed using BlastN and BlastX from the National Center for Biotechnology Information website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences of genes possessing polymorphisms potentially associated with tigecycline resistance were confirmed by Sanger sequencing in all ten tigecycline-resistant clinical isolates.

All genomes have been deposited at DDBJ/EMBL/GenBank under the accessions: JTEX00000000 (VRE57), JTEY00000000 (VRE65), JTEZ00000000 (VRE69), JTFA00000000 (VRE80), JTFB00000000 (VRE109), LGCB00000000 (A1), LGCA00000000 (A11), LGBZ00000000 (A20), LGBY00000000 (A42), LGBX00000000 (B1), LGBW00000000 (B21), LGBV00000000 (B42), LGBU00000000 (C1), LGBT00000000 (C6), and LGBS00000000 (C42). The versions described in this article are versions JTEX01000000 (VRE57), JTEY01000000 (VRE65), JTEZ01000000 (VRE69), JTFA01000000 (VRE80), JTFB01000000 (VRE109), LGCB01000000 (A1), LGCA01000000 (A11), LGBZ01000000 (A20), LGBY01000000 (A42),



Isolate	PFGE	ST	Patient	Isolation Date	Isolation site	Virulence factors	TGC	VAN	LZD	DAP
VRE2	A1	103	2	07/13/2009	Axillary swab	ElrA/GelE	0.125	>256	2	1
VRE4	A1	103	4	08/24/2009	Perianal swab	ElrA/GelE	0.125	>256	2	1
VRE8	A1	-	8	08/24/2009	Perianal swab	ElrA/GelE	0.125	>256	2	1
VRE12	A1	-	12	08/27/2009	Perianal swab	ElrA/ GelE	0.125	>256	2	1
VRE18	A1	-	8	08/27/2009	Perianal swab	ElrA/GelE	0.25	>256	2	1
VRE21	A1	-	19	09/14/2009	Perianal swab	ElrA/GelE	0.125	>256	2	1
VRE22	A1	-	20	09/24/2009	Foot deep tissue	ElrA/GelE	0.125	>256	2	1
VRE29	A1	103	25	10/08/2009	Perianal swab	ElrA/GelE	0.25	>256	2	1
VRE32	A1	-	28	10/21/2009	Perianal swab	ElrA/GelE	0.125	>256	2	1
VRE38	A1	-	29	11/05/2009	Perianal swab	Cly/ElrA/GelE	0.125	>256	2	1
VRE53	A1	103	6	11/25/2009	Perianal swab	ElrA/GelE	0.125	>256	2	1
VRE56	A1	-	46	12/21/2009	Axillary swab	ElrA/GelE	0.25	>256	2	1
VRE57	A1	103	28	12/21/2009	Perianal swab	ElrA/GelE	0.06	>256	2	1
VRE77	A2	-	54	03/25/2011	Perianal swab	ElrA/GelE	2	>256	2	1
VRE69	A2	103	54	03/11/2011	Perianal swab	ElrA/GelE	1	>256	2	1
VRE65	A2	103	54	03/10/2011	Blood	ElrA/GelE	1	>256	2	0.5
VRE108	A2	-	84	05/09/2011	Surgery wound	ElrA/GelE	2	>256	2	1
VRE104	A2	-	84	05/06/2011	Perianal swab	ElrA/GelE	2	>256	2	1
VRE100	A2	-	81	05/06/2011	Axillary swab	ElrA/GelE	2	>256	4	1
VRE103	A2	103	83	05/06/2011	Perianal swab	ElrA/GelE	2	>256	2	1
VRE105	A2	-	85	05/06/2011	Perianal swab	ElrA/GelE	2	>256	2	1
VRE80	A2	103	66	04/01/2011	Axillary swab	ElrA/GelE	1	>256	2	1
VRE98	A2	-	80	05/05/2011	Perianal swab	ElrA/GelE	2	>256	2	1
VRE109	A3	103	55	05/13/2011	Blood	ElrA/GelE	0.06	>256	1	0.5
VRE15	A4	103	14	09/05/2009	Blood	ElrA/GelE	0.125	>256	2	0.5
VRE110	B1	-	87	06/03/2011	Urine	Cly/Esp/ElrA/GelE	0.125	>256	2	1
VRE67	B1	6	56	03/11/2011	Perianal swab	Cly/Esp/ElrA/GelE	0.06	>256	2	0.5
VRE72	B1	-	60	03/25/2011	Perianal swab	Cly/Esp/ElrA/GelE	0.125	>256	2	0.5
VRE74	B1	-	62	03/25/2011	Perianal swab	Cly/ElrA/GelE	0.06	>256	2	1
VRE75	B1	-	63	03/25/2011	Perianal swab	Cly/Esp/ElrA/GelE	0.125	>256	2	1
VRE87	B1	-	64	04/14/2011	Perianal swab	Cly/Esp/ElrA/GelE	0.125	>256	2	1
VRE88	B1	-	72	04/15/2011	Axillary swab	Cly/Esp/ElrA/GelE	0.125	>256	2	1
VRE95	B1	-	77	05/05/2011	Perianal swab	Cly/Esp/ElrA/GelE	0.125	>256	2	1
VRE96	B1	-	78	05/05/2011	Perianal swab	Cly/Esp/ElrA/GelE	0.125	>256	2	0.5
VRE79	B2	-	65	04/01/2011	Perianal swab	Cly/Esp/ElrA/GelE	0.125	>256	2	1
VRE92	B3	-	76	04/29/2011	Perianal swab	Cly/Esp/ElrA/GelE	0.125	>256	2	1
VRE94	B4	-	72	05/05/2011	Perianal swab	Cly/ElrA/GelE	0.125	>256	2	1
VRE90	B5	-	74	04/29/2011	Perianal swab	Cly/ElrA/GelE	0.125	>256	2	1
VRE99	B5	-	64	05/06/2011	Perianal swab	Cly/ElrA/GelE	0.25	>256	2	1
VRE97	B5	-	79	05/05/2011	Perianal swab	Cyl/ElrA/GelE	0.125	>256	2	1
VRE83	C1	6	69	04/04/2011	Perianal swab	Cly/Esp/ElrA/GelE	0.125	>256	2	2
VRE86	C2	-	55	04/14/2011	Perianal swab	Cly/Esp/ElrA/GelE	0.125	>256	2	1
VRE68	D1	6	57	03/11/2011	Perianal swab	Cly/ElrA/GelE	0.06	>256	2	2

Fig. 1. Dendrogram of vancomycin-resistant *Enterococcus faecalis* isolated from Risoleta Tolentino Neves Hospital in 2009 and 2011. Legend: PFGE – Pulsotype; ST – Sequence Type; TGC – Tigecycline; VAN – Vancomycin; LZD – Linezolid; DAP – Daptomycin. Breakpoints: TGC – S ≤ 0. 25 mg/L/ R > 0.5 mg/L; VAN – S ≤ 4 mg/L/ I = 8–16 mg/L/ R ≥ 32 mg/L; LZD – S ≤ 2 mg/L/ I = 4 mg/L/ R ≥ 8 mg/L; DAP – S ≤ 4 mg/L.

LGBX01000000 (B1), LGBW01000000 (B21), LGBV01000000 (B42), LGBU01000000 (C1), LGBT01000000 (C6), and LGBS01000000 (C42).

2.6. Construction of overexpressing strains

Genes of interest from genome sequence comparisons were amplified using primers designed to add *Bam*HI and *Xba*I restriction sites (Table 1). Genes *rpsJ* (QP83_07505), *tetM* (QP83_12955), a hypothetical protein (QP83_08660), *lepA* (QP83_09250), and the MarR-family transcriptional regulator (QP83_14295) were each amplified from resistant/mutated isolates. Amplicons included the inferred promoter region for each gene, and were digested and ligated into the pAT28 shuttle vector (Trieu-Cuot et al., 1990). Transformants were selected on 100 mg/L spectinomycin for *E. coli*, or 500 mg/L for *E. faecalis*. Since no promoter is predicted immediately adjacent to the gene encoding the multidrug ABC transporter ATP-binding protein (QP83_01175), the reading frame was amplified and inserted into the pMSP3535 expression vector (Bryan et al., 2000). When cloning into pMSP3535, transformants were selected on 150 mg/L erythromycin for *E. coli*, and 10 mg/L for *E. faecalis*.

Following initial propagation and isolation from *E. coli* DH5-α, pAT28-based constructs were transformed into *E. faecalis* VRE80 and VRE109 by electroporation. For the pMSP3535-cloned ABC transporter QP83_01175, following amplification in *E. coli*, it was electroporated into *E. faecalis* OG1RF (selected because VRE80 and VRE109 are resistant to erythromycin).

2.7. Efflux pump inhibitor assays

The effect of efflux pump inhibitors verapamil, reserpine and carbonyl cyanide 3-chlorophenylhydrazone (CCCP) on tigecycline MIC was determined. First, efflux pump inhibitor concentration was titrated to identify the maximum levels usable without themselves inhibiting growth. Pilot experiments identified 800 mg/L for verapamil, 20 mg/L for reserpine, and 1 mg/L for CCCP, as the highest concentrations that did not inhibit *E. faecalis* growth.

Table 1
Primers used for cloning genes of interest into pAT28 or pMSP3535.

Gene	Plasmid	Primers F/R (5′ → 3′)	Amplicon size (bp)
rpsJ	pAT28	F: CGCGGATCCCGCAGAAAATCCTTG R: CGCTCTAGAGAGTACACCTCCATCTTAATT	544
tetM	pAT28	F: CGCGGATCCTTTGATAAAAAATTG R: CGCTCTAGATTTATATAACAACATAAAATACA	2290
QP83_08660	pAT28	F: CGCGGATCCCTGGTTTCCGCCAGCTG R: CGCTCTAGACATTTAAGCATCAATAATCAAGG	839
lepA	pAT28	F: CGCGGATCCTTTTCCATCTCCAATAAATTTA R: CGCTCTAGATTATGATTTTTTCTGATCATCTTCA	2103
QP83_14295	pAT28	F: CGCGGATCCCCTTTTTCTGTGAAAATGGT R: CGCTCTAGATCCTCACTTTTTATTTGTATTCTTGC	672
QP83_01175	pMSP3535	F: CGCGGATCCAAAATTGAAGGGAGTGA R: CGCTCTAGAGGTTATTTAACTCTTTCTATTGTTACG	1853

2.8. Analysis of transposon insertion mutants in genes of interest

Individual, presumed loss-of-function *E. faecalis* OG1RF *mariner* transposon insertion mutants (Dale et al., 2018) in *lepA,* and genes encoding the multidrug ABC transporter ATP-binding protein and the hypothetical protein (kindly provided by Gary Dunny), were tested for their tigecycline sensitivities in a broth microdilution format as follows: Two-fold serial dilutions of tigecycline in Mueller Hinton II were made in a 96-well plate, and wells were inoculated with 100 µL of a 1:5000 dilution of culture normalized to OD_{600} = 0.5, for a total volume of 200 µL and final bacterial dilution of 1:10,000. Plates were incubated at 37 °C for 24 h and OD_{600} measured using a Synergy2 Biotek plate reader (Winooski, VT) with Gen5 software.

2.9. Relative gene expression

To determine whether any of the observed resistance-associated mutations resulted in changes in gene expression, clinical isolates VRE57, VRE65, VRE69, VRE80 and VRE109 were cultured in BHI media overnight at 37 °C with shaking. The next day, all cultures were diluted 1:25 into fresh BHI, and grown to OD_{600} = 0.6–1.0. Cells were collected by centrifugation for 2 min at 14,000 x *g* at 4 °C and lysed by the addition of 3 mg/mL of lysozyme. RNA was then extracted using the SV Total RNA Isolation System (Promega Corporation, Madison, USA), according to manufacturer's recommendations. The concentration of RNA was determined by determining the 260 nm/280 nm absorbance ratio using a NanoDrop 2000c Spectrophotometer (Thermo Scientific, Waltham, USA). All RNA preparations yielded absorbance ratios > 1.8.

Expression of *rpsJ*, *tetM*, and the genes encoding multidrug ABC transporter ATP-binding protein, hypothetical protein and MarR-family transcriptional regulator, and reference genes for normalization of expression data (Table 2) was quantified by qPCR. For reverse transcription, a SuperScript™ III First-Strand kit (Invitrogen, Carlsbad, USA), with 50 ng of random hexamers and 1 µg of RNA was used according to manufacturer's recommendations to generate ~ 1 µg of cDNA. The quality of cDNA was assessed by PCR amplification, and verified by gel electrophoresis, the presence of one amplicon of the correct size was observed, the cDNA was considered a good and specific. A cDNA pool, prepared with a mixture of all enterococcal cDNA samples, was used for optimization of annealing temperatures. Additionally, the presence of single peaks in melting curve analyses demonstrated that amplifications by all pairs of primers were target-specific. Then, the cDNA pool was also used to generate a standard curve for primer efficiency, which was used to correct expression values (Bustin et al., 2009), and to evaluate the expression stability of the reference genes *gdh*, *pyrC* and *gyrA* with RefFinder (Xie et al., 2012). All three genes were considered stable for normalization of relative expression.

For qPCR amplification, the fluorescent DNA-binding dye PowerUP™ SYBR® Green PCR Master Mix (Thermo Fisher Scientific, Waltham, USA) was used according to manufacturer's recommendations, with 0.5 ng cDNA added per reaction. A CFX96 Touch™ Real Time PCR Detection System (Bio-Rad, Hercules, USA) was used, with cycling conditions: Initial equilibration at 50 °C for 2 min, followed by denaturation at 95 °C for 2 min, then 40 cycles of 15 s at 95 °C and 1 min at 60 °C. A quantitative melting curve was performed on each product (using a temperature ramp of 0.5 °C per second, from 65 to 95 °C), to verify amplicon identity in each reaction. Each qPCR reaction was performed in triplicate. Relative expression was calculated by normalization to levels of expression for each of the three reference genes, by comparing ΔΔCT values with PCR efficiency correction (Vandesompele et al., 2002; Livak and Schmittgen, 2001).

Table 2
List of genes and primers used for qPCR analysis.

Gene	Gene type	Primers	Tm (°C)	Amplicon size
gdh	Reference	F: 5′- TGTCGGTGAGCTGCCTAAAG −3′ R: 5′- AAAGCGGTTTCAGCTAACGC -3′	60.0 °C 59.8 °C	120 pb
pyrC	Reference	F: 5′- TCGGTAGTGAAACGGCCTTC -3′ R: 5′- ATTTCTGCCGGTTTCACTGC -3′	60.0 °C 59.4 °C	106 pb
gyrA	Reference	F: 5′- AGGTGTTCGTGGAATCCGTC -3′ R: 5′- ACCGCCACGTCCTTTAACTG −3′	60.0 °C 60.6 °C	148 pb
rpsJ	Target	F: 5′- AAGAACTGGAGCTGACGTATC -3′ R: 5′- TATGAGTCGCACGAACAACTG −3′	57.5 °C 58.7 °C	80 pb
tetM	Target	F: 5′- TGTATCACCGCTTCCGTTGG −3′ R: 5′- TCGCAACCATAGCGTATCCC -3′	60.4 °C 60.0 °C	114 pb
QP83_08660	Target	F: 5′- AAAACGGTCAGCAAAGAGGC -3′ R: 5′- GGTACTCCCCGTTTTCTCCG −3′	59.3 °C 60.1 °C	121 pb
QP83_01175	Target	F: 5′- CGTTTCGACGCATGTTTTAC -3′ R: 5′- GCATTGTAGCAGTTTTAGGC -3′	56.4 °C 56.8 °C	126 pb
QP83_14295	Target	F: 5′- CCGTCGGAACACTTACTGTAG-3′ R: 5′- GCCAAGCTTTACTACACGTCG −3′	56.0 °C 59.6 °C	98 pb

Table 3
Sequence changes in the tigecycline-resistant clinical isolates VRE65, VRE69, and VRE80 compared to tigecycline-susceptible isolates VRE57 and VRE109.

Gene[a]	Product	Coding region change	Amino acid change
QP83_07505	S10 protein of the 30S ribosomal subunit	165_176del	Δ56_59HKYK
QP83_01175	Multidrug ABC transporter ATP-binding protein	T735TA	D245E
QP83_12955	Tetracycline resistance protein TetM	T1417A	F473I
QP83_08660	Hypothetical protein	G410A	G137E
QP83_09250	GTP-binding protein LepA	A47T	K16I

[a] Names are from the annotated sequence of the VRE109 isolate.

3. Results and discussion

3.1. Molecular characterization

The emergence of tigecycline resistance among vancomycin-resistant *E. faecalis* was noticed within months of its introduction into use at a Brazilian hospital. It was therefore of considerable interest to understand the nature and basis for this phenotype.

As shown in Fig. 1, 43 VRE *E. faecalis* strains were studied. These included 29 strains collected in 2011 (ten of which were tigecycline-resistant), and 14 additional VRE strains collected in 2009, prior to the spread of tigecycline resistance at this site (Merlo et al., 2015). All isolates contained the *vanA* resistance operon carried by an intact Tn*1546* transposon, and exhibited MICs for vancomycin > 256 mg/L. All isolates were susceptible to daptomycin, and one isolate had linezolid MIC = 4 mg/L, which is the clinical susceptibility breakpoint. A plasmid of the rep_9 family was detected in every 2011 isolate, which was not surprising given the widespread distribution of rep_9-family plasmids among *E. faecalis* (Wardal et al., 2013; Song et al., 2013). All 2009 isolates were positive for rep_1 and seven for rep_2, in addition to rep_9.

PFGE analysis revealed that most 2009 isolates clustered in a single pulsotype (designated pulsotype A), as did 11 out of 29 isolates from 2011, with 87.7% DNA fragmentation similarity (Fig. 1). The virulence factor profile for the pulsotype A strains was $elrA^+gelE^+$. All subtype A2 samples ($n = 10$) were resistant to tigecycline, and one of them also showed intermediate resistance to linezolid (VRE100). These isolates were collected from seven different patients, indicating dissemination of this resistant clone in the hospital during the 2011 sampling period. VRE109 was the only isolate of subtype A3, and it was susceptible to tigecycline. The 18 remaining vancomycin-resistant *E. faecalis* strains from 2011 belonged to pulsotypes B-D and were all susceptible to tigecycline. Three representative isolates from these pulsotypes were typed by MLST and identified as ST6. Their virulence factor profile was either $cyl^+esp^+elrA^+gelE^+$ or $cyl^+elrA^+gelE^+$ (Fig. 1).

Six isolates from pulsotype A (including one from subtype A2) were typed by MLST and found to belong to ST103 (Fig. 1), a sequence type previously detected in this hospital (Merlo et al., 2015), but distinct from a tigecycline-resistant ST6 *E. faecalis* lineage encountered in Germany (Werner et al., 2008). Tigecycline resistance among *E. faecalis* isolated in Brazil is a rare event, as highlighted by data from the Tigecycline Evaluation and Surveillance Trial between 2004 and 2015, which showed that only 14.3% of vancomycin-resistant *E. faecalis* in Brazil were resistant to tigecycline (Vega and Dowzicky, 2017), and also by results from the SENTRY Antimicrobial Surveillance Program, which revealed 100% susceptibility to tigecycline among *E. faecalis* isolated in Latin America between 2011 and 2014 (Sader et al., 2016), as did a study of isolates from 2016 (Pfaller et al., 2018).

3.2. Analysis of the genome sequence of the ST103 tigecycline-resistant lineage

Virulence determinants and resistant genes are frequently carried by plasmids and disseminated among bacterial cells by conjugation. The fact that no other plasmid family and no other virulence genes were introduced in the tigecycline-resistant isolates could suggest the accessory genome does not play a role in tigecycline resistance, so our deeper analysis focused in the core genome.

PFGE analysis revealed that *E. faecalis* VRE57 (isolated in December 2009) (Merlo et al., 2015) and *E. faecalis* VRE109 (isolated in May 2011) were the most closely related tigecycline-susceptible isolates to the tigecycline-resistant ST103 strains isolated in 2011 (Fig. 1). In order to identify candidate genes or mutations that could contribute to tigecycline resistance, we first compared the genomes of the tigecycline-resistant strains VRE65, VRE69 and VRE80, with the tigecycline-susceptible strain VRE109. Results of that comparison were then filtered to remove polymorphisms that were not shared upon comparison with VRE57, the tigecycline-susceptible isolate from 2009. From this analysis, five genetic changes were found to be common to all three tigecycline-resistant clinical isolates, and absent from the two tigecycline-susceptible comparators (Table 3). Further, these five differences were confirmed to occur in the remaining tigecycline-resistant strains VRE77, VRE98, VRE100, VRE103, VRE104, VRE105 and VRE108. These mutations do not occur in publicly accessible sequences in the NCBI database, suggesting that they are unique to this lineage. The wild type alleles for each gene, however, were identified in the database. The nature of the changes in each of the five genes is discussed in greater detail below.

3.2.1. rpsJ (QP83_07505)

A 12 bp deletion was identified in the *rpsJ* gene (*QP79_02985*), encoding the S10 protein of the 30S ribosomal subunit, in each of the tigecycline-resistant isolates. The deletion begins at position 165 of the gene, and results in removal of amino acids HKYK from the protein at positions 56–59 (Table 3). In *Enterococcus faecium*, an Asp60Tyr substitution in the same gene was found to be responsible for the reduced susceptibility to tigecycline in three mutant strains generated *in vitro* (Cattoir et al., 2015). Additionally, the same study characterized a clinical isolate of *E. faecium* with reduced tigecycline susceptibility and found that the isolate had a Lys57Glu mutation in *rpsJ* (*Cattoir et al., 2015*). Codon 57 is located at the vertex of a well conserved loop in close proximity to the tigecycline target site in the 30S ribosomal subunit (Villa et al., 2014). Mutations in this region have also been observed in other tigecycline-resistant *E. faecium* clinical isolates, as well as in *K. pneumoniae*(Villa et al., 2014; Niebel et al., 2015). Of particular interest, Beabout et al. experimentally proved that a modification (R53Q-Δ54-57ATHK) that occurred in tigecycline-resistant *E. faecalis* strain selected *in vitro* resulted in a 4-fold increase in tigecycline resistance (Beabout et al., 2015). Here, we observed a 12 bp deletion at the same region of the S10 protein in clinical *E. faecalis* isolates, highlighting the relevance of alteration in this specific region of the *rpsJ* gene for tigecycline resistance in *E. faecalis*. We attempted to express the mutant *rpsJ* allele *in trans* in the tigecycline-susceptible VRE109 strain, but we did not observe a change in tigecycline MIC. This likely stems from the fact that the wild type gene is highly expressed in *E. faecalis* (Table 4) on a polycistronic message, and the ribosome is assembled co-translationally (Shieh et al., 2015). Allelic replacement at this locus in enterococci has not yet been successful. (Beabout et al., 2015; Cattoir et al., 2015) The polymorphism in *rpsJ* occurring in tigecycline-resistant strains VRE65, VRE69, and VRE80 was not

Table 4
Tigecycline Minimal Inhibitory Concentrations (MICs) in the absence and presence of efflux pump inhibitors.

Strain	TGC	TGC + CCCP	TGC + RES	TGC + VER
	(mg/L)			
VRE57	0.060	0.060	0.060	0.030
VRE65	1.000	1.000	1.000	0.250
VRE69	1.000	1.000	1.000	0.250
VRE80	1.000	1.000	1.000	0.250
VRE109	0.060	0.030	0.060	0.030
A1	0.060	0.030	0.060	0.030
A11	0.125	0.125	0.125	0.030
A20	0.500	0.500	0.500	0.060
A42	1.000	1.000	1.000	0.125
B1	0.060	0.060	0.060	0.030
B21	0.250	0.250	0.250	0.030
B42	0.500	0.500	0.500	0.125
C1	0.060	0.030	0.060	0.030
C6	0.125	0.125	0.125	0.06
C42	1.000	1.000	0.500	0.125
VRE109(pAT28)	0.060	N/A	N/A	N/A
VRE109(pAT28-*hyp*^{VRE80})	0.125	N/A	N/A	N/A
VRE109(pAT28-*rpsJ*^{VRE80})	0.060	N/A	N/A	N/A
OG1RF(pMSP3535)	0.060	N/A	N/A	N/A
OG1RF(pMSP3535-*abc*^{VRE80})	0.060	N/A	N/A	N/A
VRE109(pAT28-*marR*^{VRE109})	0.060	N/A	N/A	N/A
VRE109(pAT28-*marR*^{B42})	0.125	N/A	N/A	N/A
B42(pAT28)	1.000	N/A	N/A	N/A
B42(pAT28-*marR*^{VRE109})	1.000	N/A	N/A	N/A
B42(pAT28-*marR*^{B42})	2.000	N/A	N/A	N/A

TGC: tigecycline; CCCP: carbonyl cyanide 3-chlorophenylhydrazone; RES: reserpine; VER: verapamil; N/A: not applicable.

associated with significantly altered levels of mRNA abundance in these strains (Fig. 2). Nevertheless, the data collectively support the deduction that the deletion in *rpsJ* observed in these tigecycline-resistant clinical isolates of *E. faecalis* contributes to the resistant phenotype.

3.2.2. tetM (QP83_12955)

Comparison of genomes identified a Phe473Ile substitution in the gene encoding the tetracycline resistance protein TetM. However this polymorphism was located far away from codons for amino acids responsible for excluding tetracycline from the ribosome by altering nucleotide conformation of the 16S rRNA (Donhofer et al., 2012). Linkevicius et al. (2016) reported several *tetM* mutations involved with increased tigecycline resistance in *E. coli*, and the one with the greatest capacity to increase tigecycline MIC was a L505 deletion, resulting in a shortened III loop in TetM (Linkevicius et al., 2016). TetM-mediated tigecycline resistance has also been attributed to an overlap of the 9-t-butylglycylamido moiety of tigecycline and the domain IV loop of TetM, but it seems unlikely that the Phe473Ile substitution we identified would alter this overlap, since amino acid 473 does not interact directly with the tigecycline molecule (Jenner et al., 2013). Recently, Fiedler et al. (2016) reported that high-level expression of *tetM* due to its presence on a high copy-number plasmid was able to confer tigecycline resistance in enterococcal clinical isolates (Fiedler et al., 2016). We therefore examined *tetM* expression in our tigecycline-susceptible and resistant strains. We found that the abundance of *tetM* message was in fact higher in VRE65, VRE69 and VRE80 compared to VRE57 and VRE109 strains (Fig. 2). We currently do not know whether the polymorphism we observed in the tigecycline-resistant strains might stabilize the mRNA resulting in greater abundance, or whether increased abundance is due to higher levels of transcription of this gene in these strains. No polymorphisms were detected in the region upstream of the *tetM* ORF, which is known to regulate expression *via* transcriptional attenuation (Su et al., 1992).

3.2.3. Multidrug ABC transporter ATP-binding protein (QP83_01175)

Another polymorphism that was common to tigecycline-resistant strains resided in a predicted multidrug ABC transporter ATP-binding protein. The base change causes an Asp245Glu substitution in the amino acid sequence of the protein. This protein has a primary sequence similar to the Gram-negative lipid A export permease/ATP-binding protein MsbA (Doerrler and Raetz, 2002), and to the ABC-type bacteriocin and lantibiotic exporters belonging to the SunT superfamily (Paik et al., 1998). Because this protein appears to be involved in transmembrane transport, we hypothesized that it could play a role in tigecycline efflux. We found that the efflux pump inhibitor verapamil was able to produce a 4-fold decrease in the tigecycline MIC for VRE65, VRE69 and VRE80, but only a 2-fold decrease in the tigecycline MIC for VRE57 and VRE109 (Table 4). On the other hand, reserpine and CCCP were not able to produce any changes in the tigecycline MIC. According to DeMarco et al. (2007), an efflux pump inhibitor-associated 4-fold decrease in MIC is indicative of efflux (Demarco et al., 2007). The observed synergy with verapamil suggests that efflux may contribute to the resistance phenotypes of VRE65, VRE69 and VRE80.

To test whether this multidrug ABC transporter ATP-binding protein could efflux tigecycline, we tested a presumed loss of function transposon insertion mutant that was generated in *E. faecalis* strain OG1RF. In this strain, a *mariner* insertion occurs at base 418 of the gene and would be predicted to permit only the first 139 amino acids of 594 in the full-length protein to be made, resulting in truncation to only ~20% of the full-length protein and notably removing the ATP-binding domain. However, no difference in tigecycline MIC was observed between wild type OG1RF and the transposon insertion mutant (Table 4). We also observed no change in tigecycline MIC when the mutant allele was overexpressed in the VRE109 background (Table 4). Finally, no differences in mRNA abundance for this gene were observed when comparing the sensitive and resistant strains (Fig. 2). Thus, it seems that the polymorphism that occurs in the multidrug ABC transporter ATP-binding protein is not involved in the resistance of *E. faecalis* to tigecycline.

3.2.4. Hypothetical protein (QP83_08660)

We identified a Gly137Glu substitution in the sequence of a gene encoding a hypothetical protein that could be involved in translation, based on the occurrence of conserved domains. Because tigecycline inhibits protein translation by blocking the entry of aminoacyl-tRNA into the A site of the ribosome (Jenner et al., 2013), we could not rule out the possibility that mutating this hypothetical protein was somehow important for tigecycline resistance. Expression of the mutant allele of the hypothetical protein *in trans* in the VRE109 background did not increase the tigecycline MIC, and a transposon mutant that truncates 96% of the protein did not affect the tigecycline MIC of OG1RF (Table 4). No differences in mRNA abundance for this hypothetical protein were observed when comparing the sensitive and resistant strains to one another (Fig. 2). Thus, it seems that hypothetical protein (QP83_08660) is not involved in tigecycline resistance in these strains.

3.2.5. lepA (QP83_09250)

Another polymorphism that plausibly could be related to tigecycline resistance in *E. faecalis* occurred in *lepA*, which encodes a GTP-binding protein (Gibbs et al., 2017). A K16I substitution in tigecycline-resistant isolates distinguished them from sensitive isolates and others in the NCBI database. An OG1RF transposon insertion mutant missing ~70% if the LepA protein did not exhibit an altered tigecycline MIC (Table 4). Thus, we conclude that *lepA* is unlikely to be involved in the observed tigecycline resistance in *E. faecalis*.

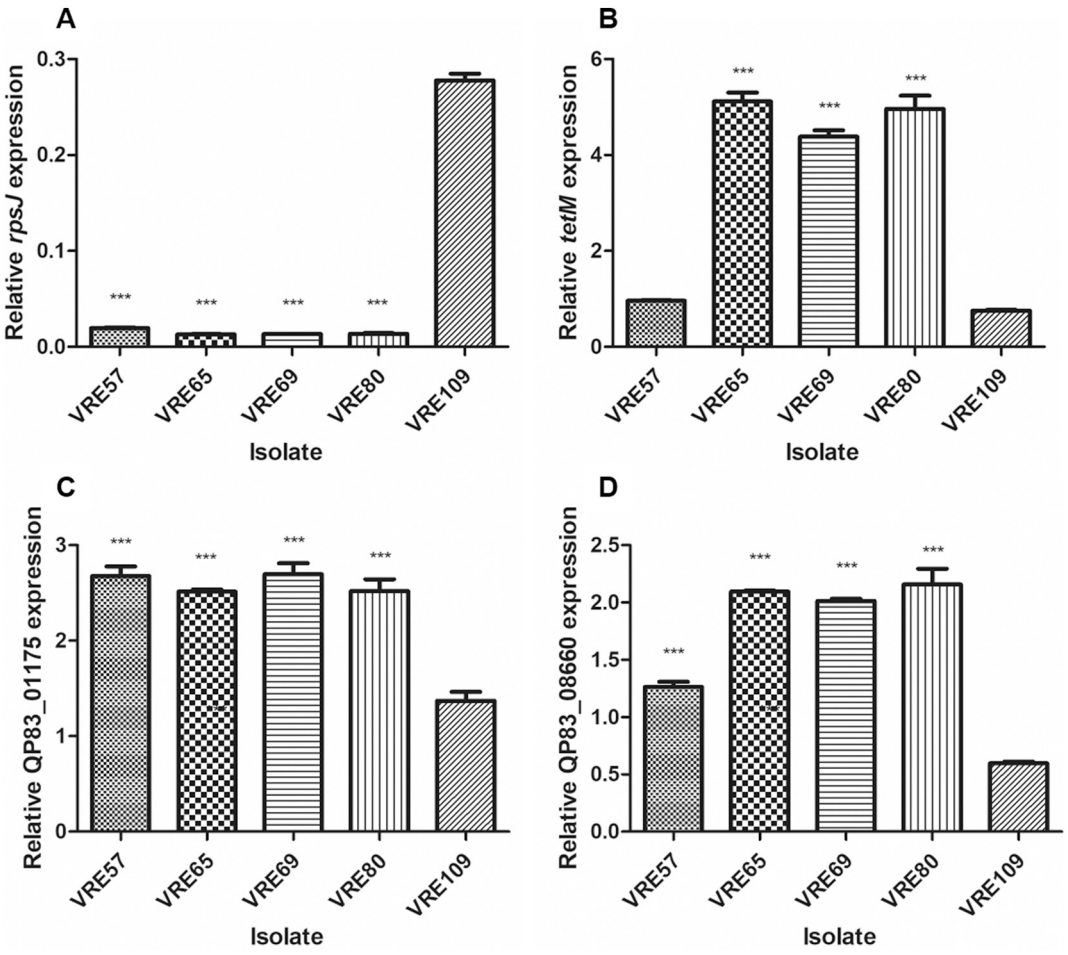


Fig. 2. Relative mRNA abundance levels in tigecycline-susceptible and resistant *E. faecalis* clinical isolates. Mean levels of *rpsJ*, *tetM*, Multidrug ABC transporter ATP-binding protein QP83_01175 and Hypothetical protein QP83_08660 mRNA in tigecycline-susceptible VRE57 and VRE109, and tigecycline-resistant VRE65, VRE69, and VRE80 strains normalized to reference genes *gdh*, *pyrC* and *gyrA* ($p > .05$, as determined by ANOVA). Error bars represent standard deviations of at least three experimental replicates, and statistically significant differences are marked with ***.

3.3. Polymorphisms in tigecycline-resistant variants of VRE109 selected in vitro

To explore evolutionary trajectories leading to tigecycline resistance in the ST103 lineage, and to attempt to recapitulate some the most relevant polymorphisms, we conducted independent *in vitro* resistance evolution experiments in triplicate using VRE109 as the tigecycline-sensitive parent strain. Comparison of genome sequences of resistant variants selected *in vitro* to the VRE109 parent revealed no consistent differences occurring exclusively in reading frames. However, examining of non-coding sequences revealed multiple changes upstream of a gene coding for a MarR-family transcriptional regulator. Mutations occurred in all three evolution experiments in either its predicted promoter region, or within the coding sequence. Tigecycline resistant mutants A11, A20 and A42 had an A → G substitution at position −41 (relative to the *marR* start codon); mutants B21 and B42 had a G400A transversion in the *marR* coding sequence resulting in an Ala to Thr change (Ala134Thr), and mutant C42 had a C → T substitution at position −10.

We wondered if the effect of the upstream mutations was to alter *marR* mRNA abundance in the tigecycline-resistant strains. Counterintuitively, we found a modest but significant increase in *marR* expression in strains B21 and B42 only (Fig. 3). We constructed several *marR* trans complementation strains (Table 4), but none affected tigecycline MIC. This mutation may have been selected for simply for enhanced ability to rapidly divide *in vitro* over the extended course of the experiment.

Other resistance-associated mutations that we identified occurred uniquely in each of the triplicate experiments, suggesting that multiple evolutionary trajectories lead to tigecycline resistance (Table 5). Variants in the S10 protein-encoding gene *rpsJ* as well as *tetM* arose in resistant strains from experiments A and C, respectively, recapitulating the importance of these genes in the clinical isolates, although we did not observe any relationship between the *rpsJ* mutations and expression levels (Fig. 3). Additionally, variants in several other ribosomal proteins arose in experiments A and B.

Quantitative PCR showed that *tetM* expression increased proportionally to changes in tigecycline MIC in each individual experiment (Fig. 3). TetM is a ribosomal protection protein able to bind to the ribosome and chase tetracyclines from their binding sites (Arenz et al., 2015). Interestingly, the tigecycline-resistant strain C42 possesses a 125 bp deletion upstream of *tetM*, which includes the region that encodes its leader peptide (Roberts and Mullany, 2009), as well as a polymorphism in the *tetM* structural gene itself. This variant also exhibited the greatest levels of *tetM* mRNA as well as the highest tigecycline MIC. Since *tetM* transcription is regulated *via* attenuation (Su

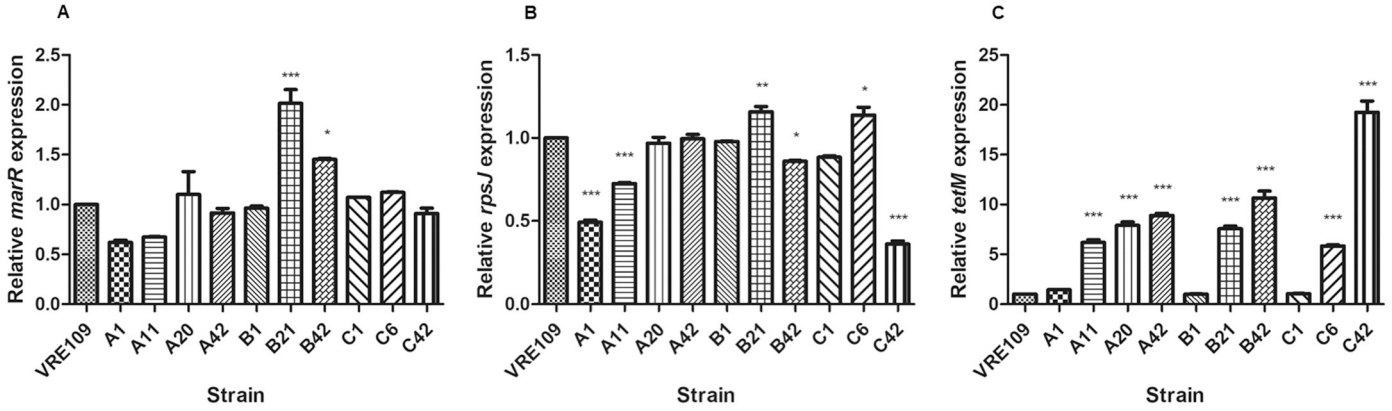


Fig. 3. Relative mRNA levels for MarR-family transcriptional regulator QP83_14295 (A), *rpsJ* (B) and *tetM* (C) for *E. faecalis* VRE109 and tigecycline-resistant variants selected *in vitro*. Mean expression is plotted relative to the reference genes *gdh*, *pyrC* and *gyrA*. Relative QP83_14295, *rpsJ* and *tetM* expression were normalized to VRE109 ($p > .05$, as determined by ANOVA). Error bars represent the standard deviations, and statistically significant differences are marked with *, ** or *** indicating the intensity of the difference, being *** the more intense.

Table 5
Mutations in coding regions detected among *in vitro* tigecycline-resistant strains.

Strain	Tigecycline MIC (mg/L)	Product	Coding region change	Amino acid change
A1	0.06	–	–	–
A11	0.125	–	–	–
A20	0.5	iron ABC transporter substrate-binding protein	G943 T	G315*
		30S ribosomal protein S10	C166T	H56Y
A42	1.0	iron ABC transporter substrate-binding protein	G943 T	G315*
		30S ribosomal protein S10	C166T	H56Y
		translation initiation factor IF-2	G1514A	G505D
		50S ribosomal protein S3	493_494insGTTCAG	S164E165insGS
B1	0.06	–	–	–
B21	0.25	MarR-family transcriptional regulator	G400A	A134T
B42	0.5	MarR-family transcriptional regulator	G400A	A134T
		PhoB-family transcriptional regulator	A320G	E107G
		50S ribosomal protein L11	C76T	P26Ser
		Integrase	T707A	F239 L
		50S ribosomal protein L6	G196A	G66Arg
		Membrane protein	A1029T	L343F
C1	0.06	–	–	–
C6	0.125	–	–	–
C42	1.0	TetM	G1826 T	G609 V

et al., 1992), this could explain why *tetM* mRNA levels are so much higher in C42 compared to other strains. Similar mutations upstream of *tetM* were not observed in tigecycline-resistant variants with elevated mRNA levels deriving from the other two *in vitro* selection experiments. Whether other factors affect *tetM* regulation in those strains, or mRNA turnover, remains unknown.

4. Conclusions

The introduction of tigecycline into the formulary at Risoleta Tolentino Neves Hospital in early 2011 appears to have selected for the emergence and proliferation of an endemic, highly transmissible ST103 VRE *E. faecalis* lineage. The Hospital Infection Control Committee was notified so that measures could be taken to limit its spread. Our results suggest that resistance in the ST103 lineage stems from polymorphisms in *rpsJ*, which encodes the S10 protein of the 30S ribosomal subunit, and polymorphisms leading to increased *tetM* expression. Selecting for tigecycline-resistant variants *in vitro* generated similar types of mutations, with the most consistently observed effect being the emergence of strains with altered ribosomal proteins and elevated levels of *tetM* expression. In one case, this could be directly attributed to loss of the transcription attenuator that limits *tetM* expression. The fact that resistance to tigecycline can arise from endogenous genetic traits already present in many strains of *E. faecalis* indicates that to maximize its utility, it should be reserved for treating *E. faecalis* infections where other options have been exhausted, or where the course of therapy is likely to be short.

Our results suggest that *rpsJ* and *tetM* play a major role in the development of resistance to tigecycline in *E. faecalis*. When it comes to the *in vitro* settings, the tigecycline resistance phenotype of *E. faecalis* ST103 strains seems to be a multifactorial event, with contributions of many ribosomal proteins, but increased expression of TetM is a common theme.

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Transparency declarations

We declare no conflicts of interest.

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