J Antimicrob Chemother 2019; **74**: 843–850
doi:10.1093/jac/dky549 Advance Access publication 10 January 2019

Journal of Antimicrobial Chemotherapy

Characterization of a new transferable MDR plasmid carrying the *pbp5* gene from a clade B commensal *Enterococcus faecium*

Gianluca Morroni [1], Andrea Brenciani[2]*, Alice Litta-Mulondo[3], Carla Vignaroli[3], Gianmarco Mangiaterra[3], Simona Fioriti[2], Barbara Citterio[4], Oscar Cirioni[1], Eleonora Giovanetti[3] and Francesca Biavasco[3]

[1]Infectious Disease Clinic, Department of Biomedical Sciences and Public Health, Polytechnic University of Marche, Ancona, Italy; [2]Unit of Microbiology, Department of Biomedical Sciences and Public Health, Polytechnic University of Marche Medical School, Ancona, Italy; [3]Department of Life and Environmental Sciences, Polytechnic University of Marche, Ancona, Italy; [4]Department of Biomolecular Science, Biotechnology Section, University of Urbino 'Carlo Bo', Urbino, Italy

*Corresponding author. Unit of Microbiology, Department of Biomedical Sciences and Public Health, Polytechnic University of Marche Medical School, Via Tronto 10/A, 60126 Ancona, Italy. Tel: +39 071 220 6299; Fax: +39 071 220 6293; E-mail: a.brenciani@univpm.it

Received 12 July 2018; returned 27 September 2018; revised 16 November 2018; accepted 28 November 2018

Objectives: To evaluate the transferability of antibiotic resistance from an MDR clade B *Enterococcus faecium* and to characterize the genetic elements involved.

Methods: The *erm*(B)-positive strain *E. faecium* 37BA (donor) and strains *E. faecium* 64/3 and *Listeria welshimeri* 11857RF (recipients) were used in mating experiments. Donors and transconjugants were characterized using MIC assays, PFGE, Southern blotting and hybridization, quantitative RT–PCR (RT–qPCR), next-generation sequencing and PCR mapping.

Results: One *E. faecium* and one *L. welshimeri* transconjugant were selected for in-depth investigation. Both acquired an ∼40 kb plasmid carrying *erm*(B). An additional plasmid of ∼200 kb, encoding the full conjugation machinery, was detected in the donor and in the *E. faecium* transconjugant. Next-generation sequencing revealed a new 40 396 bp plasmid that was designated pEf37BA; it contained 10 antibiotic resistance genes, *tet*(M), *tet*(L), *erm*(B), *aadE*, *sat4*, *aphA*, *spw*, *lsa*(E), *lnu*(B) and *pbp5*, resulting from the recombination of pM7M2 of *E. faecium* with an MDR chromosomal region of *Erysipelothrix rhusiopathiae*. A *pbp5*-carrying circular form was also detected. The PBP5 amino acid sequence differed from the C46 variant by two mutations (S39T and D644N). Its expression was documented in both transconjugants. pEf37BA persisted in the absence of selective pressure.

Conclusions: The MDR clade B *E. faecium* plasmid, deriving from the recombination of two different resistance regions, carried a *pbp5* element and was transferable to different bacterial species. This finding further documents the dissemination of ampicillin resistance among community-associated *E. faecium* and the key role of commensal strains in the spread of antibiotic resistance.

Introduction

It is well established that the gut microbiota plays a crucial role in host health.[1] Several factors, including dietary habits and antibiotic use, contribute to shaping the normal gut microbiota in humans.[2] In particular, antibiotic therapy may affect the microbiota composition as well as the long-term persistence of resistant bacteria in the human gut.[3,4] A variety of studies have documented an increase in antibiotic-resistant commensal bacteria, which provide a reservoir of antibiotic resistance (AR) genes that have the ability to spread to other gut bacterial species and genera, either resident or transient, including human pathogens.[4,5] Among human gut commensals, enterococci have a striking ability to colonize healthy carriers and hospitalized patients, and to adapt to adverse environmental conditions, including those created by drug administration, by virtue of their intrinsic resistance to a wide range of antimicrobial agents and of the plasticity of their genome, which favours the acquisition of AR traits.[6,7] After acquiring multiple genetic elements, MDR enterococcal subpopulations easily disseminate from the gastrointestinal tract of patients and healthcare workers to the hospital environment, contributing to hospital-associated infections.[8,9]

In 2016, enterococci ranked second among the bacteria isolated most frequently from bloodstream infections acquired in European ICUs.[10] *Enterococcus faecium* and *Enterococcus faecalis* are the species isolated most frequently from clinical infections. In the past two decades, the prevalence of *E. faecium* has increased worldwide, mainly owing to its greater ability to resist the action of

© The Author(s) 2019. Published by Oxford University Press on behalf of the British Society for Antimicrobial Chemotherapy. All rights reserved. For permissions, please email: journals.permissions@oup.com.

Downloaded from https://academic.oup.com/jac/article-abstract/74/4/843/5288148 by guest on 16 March 2019

antibiotics compared with *E. faecalis*.[11,12] The incidence of *E. faecium* infections in clinical settings has risen since the 1980s along with ampicillin resistance.[12,13] Most hospital-adapted ampicillin-resistant *E. faecium* strains belong to ST17, ST18 and ST78, which are part of clonal complex 17 (CC17).[14,15] More recently, enterococci have been subdivided into two main phylogenetic groups, clade A and clade B: clade A encompasses clinical and animal isolates, whereas clade B mostly includes commensal isolates.[16,17] Clade A enterococci are hospital associated and encompass CC17 isolates, most of which are resistant to ampicillin and aminoglycosides and are either resistant or susceptible to vancomycin. In contrast, clade B enterococci are community associated and are characterized by wide antibiotic susceptibility.[16,18]

E. faecium is intrinsically resistant to low levels of ampicillin through overproduction of the constitutive low-affinity PBP5.[7,19] This enzyme belongs to class B PBPs (monofunctional enzymes, such as PBP2B and PBP2a) and is responsible for peptidoglycan synthesis[20] when all the other PBPs are inactivated.[7,21] The PBP5-encoding gene exists in two allelic forms, whose nucleotide sequences differ by $\sim$5%:[22] *pbp5*-R confers an ampicillin MIC that is usually $\geq$16 mg/L and is harboured by clade A strains, whereas *pbp5*-S confers an ampicillin MIC usually $\leq$2 mg/L and is carried by clade B strains.[16,23]

The horizontal transfer of the *pbp5* gene, including the possibility of its transfer via a plasmid, has been described in several papers.[18,24–26] A recent study has documented, in *E. faecium* isolates from different clonal lineages, 75 PBP5 variants and the horizontal gene transfer of large chromosomal genetic platforms containing *pbp5* alleles and a number of genes involved in survival in the gastrointestinal tract.[27] However, no *pbp5*-carrying plasmids have yet been described, except in an *Enterococcus hirae* strain; although this plasmid has not been analysed in detail, it has been hypothesized to have been transferred from an *E. faecium* strain.[28]

In a previous study, faecal samples from healthy volunteers eating different diets were screened for AR genes.[29] A collection of intestinal lactic bacteria resistant to erythromycin and tetracycline were thus obtained and analysed. The unusual recovery of an MDR clade B commensal *E. faecium* isolate (strain 37BA) prompted this study, the goals of which were to characterize the transferable plasmid pEf37BA found in *E. faecium* 37BA and to evaluate the transferability of AR traits to different bacterial genera including *Listeria*.

Materials and methods

Bacterial strain

Strain *E. faecium* 37BA was isolated in the framework of a study of the occurrence of AR genes in faecal samples from healthy volunteers eating different diets: omnivorous, ovo-lacto vegetarian and vegan.[29] The strain was resistant to erythromycin (MIC >128 mg/L), ampicillin (MIC 16 mg/L), tetracycline (MIC 128 mg/L), streptomycin (MIC >128 mg/L), kanamycin (MIC >128 mg/L) and gentamicin (MIC 128 mg/L), and susceptible to vancomycin (MIC 1 mg/L) and levofloxacin (MIC 1 mg/L). It carried the resistance genes *erm*(B), *tet*(M), *tet*(L) and *aadE*.

E. faecium 37BA was used as the donor and *E. faecium* 64/3[30] and *Listeria welshimeri* 11857RF,[31] which are resistant to rifampicin and fusidic acid (MIC >128 mg/L) and susceptible to erythromycin (MIC 0.25 mg/L) and ampicillin (MIC 1 and 0.06 mg/L, respectively), were used as recipients in mating experiments. *L. welshimeri* was used as an alternative species to the main pathogen of the genus, *Listeria monocytogenes*, to assess the transferability of pEf37BA to *Listeria* spp.

Susceptibility tests

The MICs of different antibiotics (ampicillin, erythromycin, tetracycline, streptomycin, kanamycin and tigecycline; all purchased from Sigma Chemical Co., St Louis, MO, USA) were obtained by broth microdilution and interpreted according to EUCAST and CLSI clinical breakpoints (version 8.1, www.eucast.org; CLSI 2017 M100-S27). *E. faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 29213 were used for quality control in susceptibility tests.

Mating experiments

Conjugal transfer was performed by filter mating as described previously.[32] Transconjugants were selected on brain–heart infusion agar (BHIA; Oxoid, Basingstoke, UK) plates containing fusidic acid, rifampicin and erythromycin (all at 10 mg/L). The transfer frequency was expressed as the number of transconjugants per recipient.

SmaI- and S1-PFGE, Southern blotting and hybridization assays

SmaI and S1 nuclease digestion followed by PFGE analysis were performed as described previously.[33,34] After S1-PFGE, total DNA was blotted onto positively charged nylon membranes (Ambion-Celbio, Milano, Italy) and hybridized with biotin-labelled *erm*(B) and *pbp5* probes as described elsewhere.[35]

Sequencing and sequence analysis

Plasmid DNA was extracted using a commercial kit (Sigma-Aldrich, St Louis, MO, USA). Next-generation sequencing (NGS) was carried out using Illumina HiSeq (5 Mio read pairs, 2×125 bp) technology (GenProbio srl, Parma, Italy). Contig assembly was performed with SPAdes v 3.11.1 (http://cab.spbu.ru/software/spades/), and ORFs were annotated with the RAST Annotation server (http://rast.nmpdr.org) and ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder). The quality of the final contigs was improved with Burrows–Wheeler Aligner and the SAMtools suite.[36] The gaps between the plasmid contigs were closed by PCR mapping (Table S1, available as Supplementary data at *JAC* Online) using primers targeting unique DNA regions and Sanger sequencing of the resulting amplicons, after purification with a GenElute PCR Cleanup kit (Sigma-Aldrich). The nucleotide sequences were compared with sequences in the GenBank database using BLASTN (http://blast.ncbi.nlm.nih.gov/blast). The ST was determined as previously described.[33]

Amplification experiments

Primer pairs and amplicon size are reported in Table S1 along with the relevant references. The Ex Taq system (TaKaRa Bio, Shiga, Japan) was used in amplification experiments expected to yield PCR products exceeding 3 kb in size. Excision of the *pbp5* genetic context was detected using the outward-directed pair PBP5inv-1 and PBP5inv-2, and the pair PBP5-1 and PBP5-2, which target the flanking regions of the insertion site of the *pbp5* element. The presence of the *pbp5* plasmid ($pbp5_p$) and chromosomal ($pbp5_c$) gene was searched for using two specific primer pairs, (i) $PBP5_p$-FW/$DWPBP5_p$-RV and (ii) $PBP5_c$-FW/$DWPBP5_c$-RV, respectively (Table S1).

RNA extraction and reverse transcription

Each strain was grown to the exponential phase in triplicate in brain–heart (BH) broth and diluted to OD_{650} = 0.1. Total RNA was extracted from 500 μL of the diluted cultures using an RNeasy Mini kit (Qiagen GmbH, Hilden,

Downloaded from https://academic.oup.com/jac/article-abstract/74/4/843/5288148 by guest on 16 March 2019

Table 1. Conjugal transfer of pEf37BA from the donor *E. faecium* 37BA to recipients *E. faecium* 64/3 and *L. welshimeri* 11857RF

Strain	Genotype	Transfer frequency	MIC (mg/L)					
			ERY	TET	STR	KAN	AMP	TGC
E. faecium 37BA (D)	*tet*(M), *tet*(L), *sat4*, *aadE*, *aphA*, *spw*, *erm*(B), *lsa*(E), *lnu*(B), $pbp5_p$, $pbp5_c$		>128	16	>128	>128	16	0.25
E. faecium 6B2 (T)	*tet*(M), *tet*(L), *sat4*, *aadE*, *aphA*, *spw*, *erm*(B), *lsa*(E), *lnu*(B), $pbp5_p$, $pbp5_c$	5.7×10^{-4}	>128	16	>128	>128	16	0.125
E. faecium 64/3 (R)	$pbp5_c$		0.25	1	2	32	1	0.125
L. welshimeri TW2 (T)	*tet*(M), *tet*(L), *sat4*, *aadE*, *aphA*, *spw*, *erm*(B), *lsa*(E), *lnu*(B), $pbp5_p$	8.5×10^{-8}	>128	4	>128	128	0.125	0.015
L. welshimeri 11857RF (R)			0.125	0.5	8	2	0.06	0.015

D, donor; T, transconjugant; R, recipient; ERY, erythromycin; TET, tetracycline; STR, streptomycin; KAN, kanamycin; AMP, ampicillin; TGC, tigecycline; $pbp5_p$, plasmid *pbp5*; $pbp5_c$, chromosomal *pbp5*.

Germany) according to the manufacturer's instructions. RNA purity and amount were checked with an ND-1000 Nanodrop spectrophotometer (Thermo Scientific, Wilmington, NC, USA). RNA was reverse-transcribed using a QuantiTect Rev. Transcription kit (Qiagen) according to the manufacturer's recommendations.

RT–qPCR assays

Each reaction was performed in technical triplicate in a total volume of 20 μL containing 0.2 μM of each primer, 10 μL of 2×Rotor-Gene SYBR Green PCR master mix (Qiagen) and 2 μL of cDNA. RNase-free water was used as the negative control. Cycling conditions were 95°C for 5 min, followed by 35 cycles of 94°C for 10 s, 30 s of annealing and 72°C for 20 s. A melting curve was obtained by ramping the temperature from 59°C to 95°C (0.5°C/10 s) and analysed with Qiagen's Rotor-Gene Q MDx software. Extracted RNA was tested for the absence of genomic DNA. A delay of 12 cycles between the cDNA and the RNA quantification cycle (Cq) was considered lack of interference. The reference genes *adk* (*E. faecium*) and *scrA* (*L. welshimeri*) were the internal controls. Qiagen's Rotor-Gene Q MDx software was employed for comparative quantification analysis of the expression level of *pbp5* in the donor, the recipient *E. faecium* 64/3 and the two transconjugants using *E. faecium* 37BA as the calibrator.[37] The results are reported as the average of three biological replicates in three RT–qPCR assays ± SD.

Stability of pEf37BA

The stability of the *pbp5*-carrying plasmid was evaluated by serial daily passages on antibiotic-free BHIA. Every week, some colonies were tested by MIC determination for ampicillin susceptibility and by PCR mapping to detect the pEf37BA plasmid.

Nucleotide sequence accession number

The nucleotide sequence of the pEf37BA plasmid has been deposited in GenBank under accession number MG957432.

Results

Conjugation experiments and PCR assays

To confirm the transferability of *erm*(B) and evaluate its possible association with mobile genetic elements, *E. faecium* 37BA was used as a donor in filter mating experiments. *erm*(B) was successfully transferred to *E. faecium* 64/3 and *L. welshimeri* 11857RF at a frequency of 5.7×10^{-4} and 8.5×10^{-8} per recipient cell, respectively. Two transconjugants, *E. faecium* 6B2 and *L. welshimeri* TW2, were selected for further investigation. Their match with the recipient was confirmed by comparing their SmaI-PFGE profiles with those of the donor and the recipient (Figure S1). The erythromycin MIC and PCR amplification assays indicated that both were fully resistant to erythromycin (MIC >128 mg/L) and carried *erm*(B) (Table 1). Moreover, both transconjugants also acquired *tet*(M), *tet*(L) and *aadE* from the donor (Table 1).

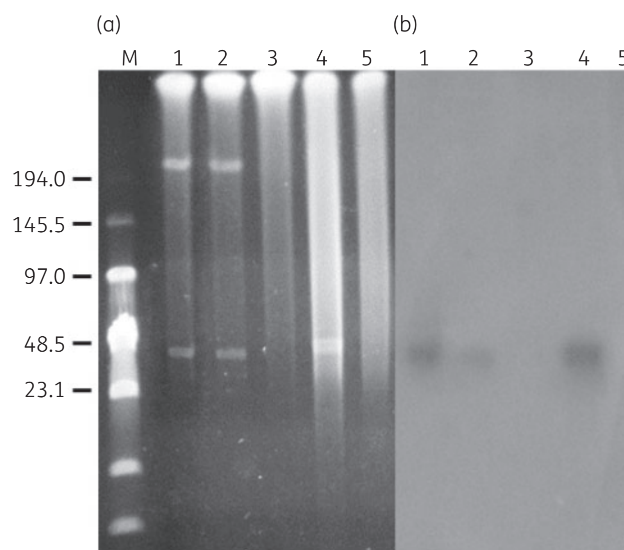

Figure 1. S1-PFGE plasmid profiles of donor, transconjugants and recipients (a), and hybridization with the *erm*(B) probe (b). M, DNA molecular weight low-range PFG marker (New England Biolabs, Ipswich, MA, USA). The size (kb) of fragments is reported on the left; lane 1, *E. faecium* 37BA; lane 2, *E. faecium* 6B2; lane 3, *E. faecium* 64/3; lane 4, *L. welshimeri* TW2; lane 5, *L. welshimeri* 11857RF.

Plasmid location of the erm(B) gene

The location of the *erm*(B) gene in the donor *E. faecium* 37BA and in the two transconjugants *E. faecium* 6B2 and *L. welshimeri* TW2 was investigated by S1-PFGE followed by Southern blotting. S1-

Downloaded from https://academic.oup.com/jac/article-abstract/74/4/843/5288148 by guest on 16 March 2019

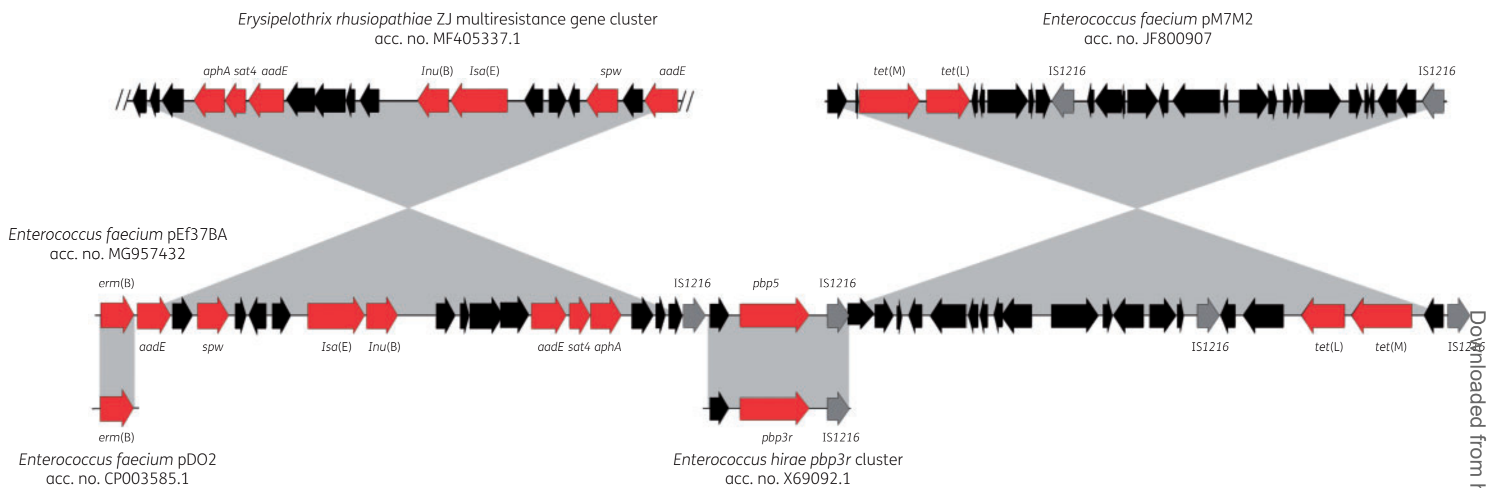

Figure 2. Genetic organization of the *E. faecium* pEf37BA (accession no. MG957432), the *E. faecium* pM7M2 (accession no. JF800907), the *E. rhusiopathiae* multiresistance ZJ region (accession no. MF405337.1) and the *E. hirae pbp3r* (accession no. X69092.1) cluster. Similar ORFs, of different genetic elements are represented by black arrows pointing in the direction of transcription. Antibiotic resistance genes are in red and IS*1216* is in grey. The light grey areas between ORFs denote DNA identities ≥99%.This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

PFGE revealed that the donor and *E. faecium* 6B2 both harboured two plasmids of ∼40 and ∼200 kb, whereas *L. welshimeri* TW2 carried a single plasmid of ∼ 40 kb. Hybridization assays showed that *erm*(B) was located on the ∼40 kb plasmid in all strains (Figure 1).

NGS analysis and characterization of a new mosaic MDR plasmid carrying the pbp5 gene

NGS analysis of the donor *E. faecium* 37BA and the two transconjugants, *E. faecium* 6B2 and *L. welshimeri* TW2, showed that the complete sequence of the *erm*(B)-carrying plasmid, pEf37BA, had a size of 40 396 bp and a G + C content of 36.0% (accession no. MG957432). Sequence analysis identified 44 ORFs encoding proteins ≥50 amino acids. The genetic map of pEf37BA is shown in Figure 2, along with the maps of elements showing high nucleotide similarity and the main ORF features. BLAST analysis revealed a high nucleotide identity with four different AR elements in the following four pEf37BA regions.

(i) Region of erythromycin resistance (1–1351 bp; G + C content, 33%). This *erm*(B) (*orf1*)-carrying region showed 99% nucleotide identity with the *erm*(B)-containing segments found in some enterococcal plasmids [*E. faecium* DO plasmid 2 (accession no. NC_017962.1) and *E. faecalis* V583 plasmid pTEF1 (accession no. NC_004669.1)].

(ii) Region of MDR (1352–15 595 bp; G + C content, 36%). This region carried six AR genes, *aadE* (*orf2* and *orf14*), *spw* (*orf4*), *lsa*(E) (*orf8*), *lnu*(B) (*orf9*), *sat4* (*orf15*) and *aphA* (*orf16*), which confer resistance to streptomycin, spectinomycin, lincosamide/streptogramin/pleuromutilin, lincosamide, streptothricin and kanamycin, respectively. This region exhibited 99% nucleotide identity with a chromosomal multiresistance gene cluster of *Erysipelothrix rhusiopathiae* (accession no. MF405337.1).

(iii) Region of ampicillin resistance (15 595–20 912 bp, G + C content, 38%). This region contained the *pbp5* cluster including the *pbp5* repressor (*orf21*) and the *pbp5* structural gene (*orf22*), which are responsible for β-lactam resistance, and exhibited 100% identity with the *pbp3r* of *E. hirae* (accession no. X69092.1).[28] Remarkably, the *pbp5* gene sequence showed 99% nucleotide identity with the previously described *pbp5* of *E. faecium* BM4107 (C46 variant, accession no. AF364092.1).[38] Alignment of the two amino acid sequences, which showed 100% similarity and 99% identity, demonstrated two mismatches at positions S39T and D644N (Figure S2). The genetic context of *pbp5* was bounded by two identical IS*1216* transposase genes (*orf20* and *orf23*) with the same orientation, which were flanked by 23 bp (GGTTCTGTTGCAAAGTTTTAAAT) inverted repeats. The *pbp5* element was integrated within *orf19*, encoding riboflavin biosynthesis protein RibD (Table S2), leading to loss of 406 bp of the gene. This segment exhibited 99% nucleotide identity with a region of an *E. hirae* plasmid (accession no. X69092.1)[28] and several chromosomal segments of *E. faecium*. PCR and sequencing (Table S2) demonstrated the ability of this region to loop out, resulting in a circular form that contained *pbp5*, the repressor gene and an IS*1216*, and leaving a single IS*1216* copy at the excision site.

(iv) Region of tetracycline resistance (20 103–40 396 bp; G + C content, 35%). This region contained the tetracycline resistance genes *tet*(L) and *tet*(M), which were arranged in tandem (*orf41* and *orf42*), and three genes (*orf31*, *orf32* and *orf33*) that are involved in plasmid replication, including a *repA* belonging to the rep_2 family.[39] This region exhibited 99% DNA identity to the 19 557 bp *E. faecium* plasmid pM7M2 (accession no. JF800907).[40]

NGS analysis also showed the presence of an ∼200 kb mega plasmid (not completely assembled) carrying complete transfer machinery in the donor *E. faecium* 37BA and in the transconjugant *E. faecium* 6B2, in a region showing 99% nucleotide identity (cover, 97%) with the pNB2354_1 plasmid (214 319 bp) of *E. faecium* NRRL B-2354 (accession no. CP004064). This region contained a

Downloaded from https://academic.oup.com/jac/article-abstract/74/4/843/5288148 by guest on 16 March 2019

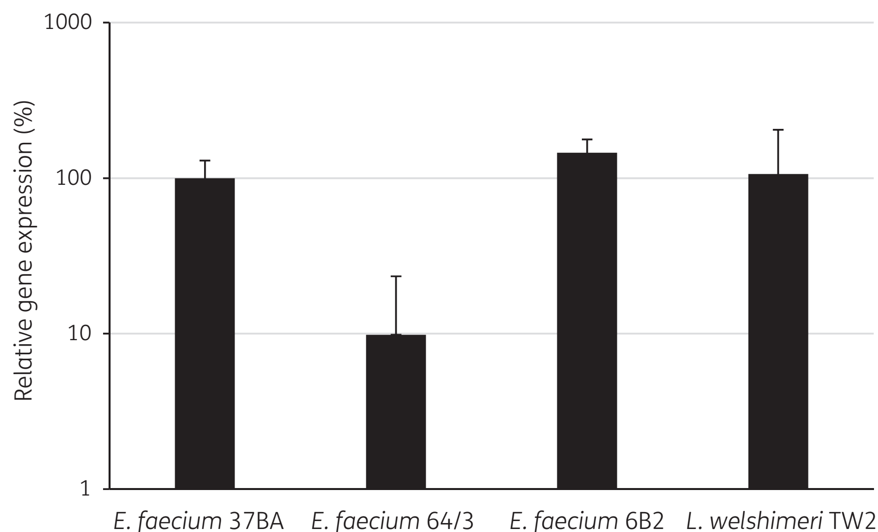

Figure 3. *pbp5* gene expression analysis in the donor *E. faecium* 37BA, recipient *E. faecium* 64/3 and transconjugants *E. faecium* 6B2 and *L. welshimeri* TW2. *E. faecium* 37BA was considered the calibrator (100%). The results are reported as the average of three biological replicates in three RT–qPCR assays ± SD.

pilus assembly and the *traG*, *virB4* and topoisomerase–primase *ltrC*-like genes. The *repA* gene, which was classified as rep20/pLG1, was also detected.[41] Interestingly, β-lactam resistance genes were not detected in the mega plasmid.

Susceptibility assays and MLST

Carriage by pEf37BA of *pbp5* and additional AR genes prompted the analysis of the antibiotic susceptibility patterns of *E. faecium* 37BA, *E. faecium* 6B2 and *L. welshimeri* TW2. All strains were resistant to erythromycin, tetracycline, streptomycin and kanamycin, and fully susceptible to tigecycline; *E. faecium* 37BA and *E. faecium* 6B2 were also resistant to ampicillin (Table 1).

The MLST data showed that *E. faecium* 37BA belonged to ST253 (clade B, which encompasses commensal strains).[42]

pbp5 gene expression and location

To explain the ampicillin susceptibility of the transconjugant *L. welshimeri* TW2, *pbp5* expression was assayed by RT–qPCR in the donor *E. faecium* 37BA, in the recipient *E. faecium* 64/3 and in the two transconjugants *E. faecium* 6B2 and *L. welshimeri* TW2, all of which carried pEf37BA, except for the recipient. Genomic DNA interference was not detected in any of the RNA extracts, since the Cq delay between cDNA and RNA was over the fixed cut-off (12 cycles) and the reference genes were correctly amplified. Interestingly, the donor and the transconjugants exhibited comparable *pbp5* transcription levels, and the recipient strain *E. faecium* 64/3 showed 10-fold lower expression of the same gene (Figure 3).

PCR assays indicated that both the donor, *E. faecium* 37BA, and the transconjugant, *E. faecium* 6B2, harboured two copies of *pbp5*, one plasmid ($pbp5_p$) and one chromosomally ($pbp5_c$) located. This result was confirmed by S1-PFGE, followed by hybridization with a *pbp5* probe (data not shown) and sequencing.

pEf37BA stability assays

E. faecium 37BA, *E. faecium* 6B2 and *L. welshimeri* TW2 were maintained for 30 days in antibiotic-free BHIA. A modification in the ampicillin MIC and the loss of pEf37BA were never recorded.

Discussion

In enterococci, conjugative plasmids are the main elements involved in the horizontal transfer of AR genes.[43,44] Recombination through IS elements allows the exchange of DNA segments between chromosomes and plasmids, enhancing the spread of AR traits.[18,45]

The unusual recovery of a commensal *E. faecium* isolate showing MDR (strain 37BA) prompted its extensive characterization and the investigation of its ability to transfer AR genes. The strain was first used as the donor in intra- and interspecies conjugation assays involving selection for erythromycin resistance. The donor and the two transconjugants selected for analysis, *E. faecium* 6B2 and *L. welshimeri* TW2, harboured an ∼40 kb *erm*(B)-carrying plasmid. Sequencing revealed a new MDR plasmid (which we named pEf37BA) that resulted from the recombination of the *E. faecium* plasmid pM7M2 and an MDR chromosomal region of *E. rhusiopathiae*. *E. rhusiopathiae* causes the disease known as erysipelas, which may affect a wide range of animals. Although it is primarily considered an animal pathogen, the bacterium can also cause erysipeloid, a zoonotic infection affecting humans. Since the genera *Erysipelothrix* and *Enterococcus* belong to different classes, the presence of an *E. rhusiopathiae* DNA region in an *E. faecium* plasmid highlights the ability of the former bacterium to transfer DNA to distantly related bacteria, possibly through IS-mediated recombination. This mechanism thus seems responsible for the construction of the mosaic plasmid pEf37BA, which is characterized by a high potential for AR gene dissemination.

Besides *erm*(B), pEf37BA also harboured a large number of AR determinants, including a region coding for PBP5. Differently from earlier work,[27] this region was integrated in an *orf* (*orf 19*) encoding

Downloaded from https://academic.oup.com/jac/article-abstract/74/4/843/5288148 by guest on 16 March 2019

a riboflavin biosynthesis protein and showed high-level identity with several enterococcal chromosomal regions and with a portion of an *E. hirae* plasmid that has not been completely characterized.[28] Although the transferable nature of the *pbp5* determinants in *E. faecium* has been described in several studies,[18,24–26] to the best of our knowledge this is the first report of a *pbp5* element carried by a transferable MDR plasmid of *E. faecium*. This genetic context showed a high level of identity with the chromosomal *pbp5* of *E. faecium* and was inserted into a region flanked by two IS*1216* having the same orientation and capable of looping out from the plasmid by IS*1216*-mediated transposition. These findings suggest the IS*1216*-mediated transposition of a *pbp5*-carrying region from the *E. faecium* chromosome to the plasmid pEf37BA.

To date, 75 PBP5 protein variants have been described in ampicillin-resistant and -susceptible strains. The one detected in our clade B commensal strain was an additional variant differing from the C46 variant of *E. faecium* BM4107, which is commonly found in clade A hospital-adapted isolates, by only two mutations, S39T and D644N.[27]

The transcription level of the *pbp5* gene was comparable in the donor and in the two transconjugants, and was 10 times lower in *E. faecium* 64/3, which only harboured the *pbp5* chromosomal copy. Despite this, the *L. welshimeri* transconjugant was fully susceptible to ampicillin, with an MIC identical to that of the recipient. This behaviour may be ascribed to post-transcriptional regulation or to incorrect protein folding resulting in lack of activity. Moreover, in *E. faecium*, β-lactam resistance can depend on the expression of additional genes. Wall biosynthesis is ensured by co-operation of transpeptidase PBP5 with high-molecular-weight bifunctional class A PBPs, i.e. PBPs exhibiting both transglycosylase and transpeptidase activity.[20,27] Therefore, it cannot be excluded that the resistance phenotype detected in the *E. faecium* strains is due to interactions with species-specific PBPs that are not found in *L. welshimeri*.

The tetracycline resistance region of pEf37BA corresponded to the complete pM7M2 of the dairy strain *E. faecium* M7M2,[40] which contains the tetracycline resistance genes *tet*(M) and *tet*(L) arranged in tandem. The association of the two genes has been described in three tetracycline-resistant *E. faecium* plasmids, pDO1,[46] pLAG[36] and a *tet*(L)/*tet*$(M)_p$ plasmid, the latter conferring high-level resistance to tigecycline in clinical enterococci.[47]

Transferable plasmids can be mobilized by a co-resident conjugative element both in *trans* and in *cis*.[43] We have recently described the ability of a pHTβ-like plasmid to mobilize two co-resident non-conjugative MDR plasmids, $pRUM_{17i48}$ and the newly described pLAG, which lack transfer machinery.[36] The present findings show that pEf37BA can spread not only to other enterococci, but also to different genera such as *Listeria*. Plasmidome analysis demonstrated that the donor *E. faecium* 37BA and the transconjugant *E. faecium* 6B2 harboured two plasmids (∼40–200 kb), whereas the transconjugant *L. welshimeri* TW2 harboured a single ∼40 kb plasmid. Interestingly, sequence analysis demonstrated an ∼200 kb mega plasmid lacking β-lactam resistance genes and containing complete transfer machinery. Since we found no complete conjugation region in pEf37BA and no co-integrate, either in the donor or in *E. faecium* 6B2, it may be hypothesized that pEf37BA underwent trans-mobilization via the transfer apparatus encoded by the co-resident mega plasmid. The presence of a single ∼40 kb plasmid in *L. welshimeri* TW2 can be explained by the transfer of pEf37BA to the transconjugant through the conjugation machinery encoded by the mega plasmid, which may be unable to replicate in *Listeria*.

The 'conjugal' transfer of AR genes in enterococci is a well-known phenomenon.[43] Indirect evidence of *pbp5* transfer between *E. faecium* strains via plasmid intermediaries has been provided by García-Solache *et al.*,[26] who, however, did not identify the genetic element responsible for the transfer. The present data provide the first evidence of the ability of the *pbp5* gene to undergo plasmid-mediated transfer and of the ability of a *pbp5*-carrying plasmid to be transferred and to replicate in different genera such as *Listeria*. Transfer from the non-pathogenic *L. welshimeri* to the main foodborne pathogen *L. monocytogenes* seems likely, as suggested by Katharios-Lanwermeyer *et al.*[48] Although the *pbp5* gene did not confer a resistance phenotype on *L. welshimeri*, the pEf37BA plasmid may contribute to spread the *pbp5* gene among different species, also considering its persistence in the absence of selective pressure.

Altogether, these findings have the potential to advance our understanding of the dissemination of ampicillin resistance among *E. faecium* strains. They show that *pbp5* horizontal gene transfer can be associated not only with the movement of large chromosomal DNA regions,[26,27] but also with plasmid transfer. The identification of a *pbp5*-carrying MDR plasmid in a commensal *E. faecium* strain is cause for concern, since it further documents the spread of antibiotic, including ampicillin, resistance in the commensal clade B *E. faecium* lineage, which can contribute to the spread of ampicillin resistance to the major hospital-adapted *E. faecium* clones and to different bacterial genera that can be found in the gut microhabitat, including the foodborne pathogen *L. monocytogenes*.

Acknowledgements

We are grateful to Professor F. Clementi and the microbiological research group of the Department of Agricultural, Food and Environmental Sciences of the Polytechnic University of Marche for providing the *E. faecium* 37BA strain, and to Dr Laura Di Sante for technical assistance.

Funding

This study was supported by internal funding.

Transparency declarations

None to declare.

Supplementary data

Tables S1 and S2 and Figures S1 and S2 are available as Supplementary data at *JAC* Online.

References

1 Sekirov I, Russell SL, Antunes LC *et al.* Gut microbiota in health and disease. *Physiol Rev* 2010; **90**: 859–904.

2 Jandhyala SM, Talukdar R, Subramanyam C *et al.* Role of the normal gut microbiota. *World J Gastroenterol* 2015; **21**: 8787–803.

Downloaded from https://academic.oup.com/jac/article-abstract/74/4/843/5288148 by guest on 16 March 2019

3 Jakobsson HE, Jernberg C, Andersson *et al*. Short-term antibiotic treatment has differing long-term impacts on the human throat and gut microbiome. *PLoS One* 2010; **5**: e9836.

4 Rolain JM. Food and human gut as reservoirs of transferable antibiotic resistance encoding genes. *Front Microbiol* 2013; **4**: 173.

5 Broaders E, Gahan CGM, Marchesi JR. Mobile genetic elements of the human gastrointestinal tract: potential for spread of antibiotic resistance genes. *Gut Microbes* 2013; **4**: 271–80.

6 Cattoir V, Leclercq R. Twenty-five years of shared life with vancomycin-resistant enterococci: is it time to divorce? *J Antimicrob Chemother* 2013; **68**: 731–42.

7 Miller WR, Munita JM, Arias CA. Mechanisms of antibiotic resistance in enterococci. *Expert Rev Anti Infect Ther* 2014; **12**: 1221–36.

8 Gilmore MS, Lebreton F, van Schaik W. Genomic transition of enterococci from gut commensals to leading causes of multidrug-resistant hospital infection in the antibiotic era. *Curr Opin Microbiol* 2013; **16**: 10–6.

9 Guzman Prieto AM, van Schaik AM, Rogers W *et al*. Global emergence and dissemination of enterococci as nosocomial pathogens: attack of the clones? *Front Microbiol* 2016; **7**: 788.

10 European Centre for Disease Prevention and Control. *Annual Epidemiological Report for 2016: Healthcare-associated Infections Acquired in Intensive Care Units*. Stockholm, Sweden: ECDC, 2018.

11 Arias CA, Murray BE. The rise of the *Enterococcus*: beyond vancomycin resistance. *Nat Rev Microbiol* 2012; **10**: 266–78.

12 Cattoir V, Giard JC. Antibiotic resistance in *Enterococcus faecium* clinical isolates. *Expert Rev Anti Infect Ther* 2014; **12**: 239–48.

13 Galloway-Peña JR, Nallapareddy SR, Arias CA *et al*. Analysis of clonality and antibiotic resistance among early clinical isolates of *Enterococcus faecium* in the United States. *J Infect Dis* 2009; **200**: 1566–73.

14 Willems RJ, Top J, van Santen M *et al*. Global spread of vancomycin resistant *Enterococcus faecium* from distinct nosocomial genetic complex. *Emerg Infect Dis* 2005; **11**: 821–8.

15 Top J, Willems R, Bonten M. Emergence of CC17 *Enterococcus faecium*: from commensal to hospital-adapted pathogen. *FEMS Immunol Med Microbiol* 2008; **52**: 297–308.

16 Lebreton F, van Schaik W, McGuire AM *et al*. Emergence of epidemic multidrug-resistant *Enterococcus faecium* from animal and commensal strains. *mBio* 2013; **4**: e00534–13.

17 Raven KE, Reuter S, Reynolds R *et al*. A decade of genomic history for healthcare-associated *Enterococcus faecium* in the United Kingdom and Ireland. *Genome Res* 2016; **26**: 1388–96.

18 Palmer KL, Godfrey P, Griggs A *et al*. Comparative genomics of enterococci: variation in *Enterococcus faecalis,* clade structure in *E. faecium*, and defining characteristics of *E. gallinarum* and *E. casseliflavus*. *mBio* 2012; **3**: e00318–1.

19 Zorzi W, Zhou XY, Dardenne O *et al*. Structure of the low-affinity penicillin-binding protein 5 PBP5fm in wild-type and highly penicillin-resistant strains of *Enterococcus faecium*. *J Bacteriol* 1996; **178**: 4948–57.

20 Rice LB, Carias LL, Rudin S *et al*. Role of class A penicillin-binding proteins in the expression of β-lactam resistance in *Enterococcus faecium*. *J Bacteriol* 2009; **191**: 3649–56.

21 Kristich CJ, Rice LB, Arias CA. Enterococcal infection—treatment and antibiotic resistance. In: MS Gilmore, DB Clewell, Y Ike, N Shankar, eds. *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection*. Boston, MA, USA: Massachusetts Eye and Ear Infirmary, 2014; 89–134.

22 Galloway-Peña JR, Rice LB, Murray BE. Analysis of PBP5 of early U.S. isolates of *Enterococcus faecium*: sequence variation alone does not explain increasing ampicillin resistance over time. *Antimicrob Agents Chemother* 2011; **55**: 3272–7.

23 Galloway-Peña J, Roh JH, Latorre M *et al*. Genomic and SNP analyses demonstrate a distant separation of the hospital and community-associated clades of *Enterococcus faecium*. *PLoS One* 2012; **7**: e30187.

24 Carias LL, Rudin SD, Donskey CJ *et al*. Genetic linkage and cotransfer of a novel, *vanB*-containing transposon (Tn*5382*) and a low-affinity penicillin-binding protein 5 gene in a clinical vancomycin-resistant *Enterococcus faecium* isolate. *J Bacteriol* 1998; **180**: 4426–34.

25 Rice LB, Carias LL, Rudin S *et al*. *Enterococcus faecium* low-affinity *pbp5* is a transferable determinant. *Antimicrob Agents Chemother* 2005; **49**: 5007–12.

26 García-Solache M, Lebreton F, McLaughlin RE *et al*. Homologous recombination within large chromosomal regions facilitates acquisition of β-lactam and vancomycin resistance in *Enterococcus faecium*. *Antimicrob Agents Chemother* 2016; **60**: 5777–86.

27 Novais C, Tedim AP, Lanza VF *et al*. Co-diversification of *Enterococcus faecium* core genomes and PBP5: evidences of *pbp5* horizontal transfer. *Front Microbiol* 2016; **7**: 1581.

28 Raze D, Dardenne O, Hallut S *et al*. The gene encoding the low-affinity penicillin-binding protein 3r in *Enterococcus hirae* S185R is borne on a plasmid carrying other antibiotic resistance determinants. *Antimicrob Agents Chemother* 1998; **42**: 534–9.

29 Milanovic V, Osimani A, Aquilanti L *et al*. Occurrence of antibiotic resistance genes in the fecal DNA of healthy omnivores, ovo-lacto vegetarians and vegans. *Mol Nutr Food Res* 2017; **61**: 1–13.

30 Werner G, Klare I, Witte W. Arrangement of the *vanA* gene cluster in enterococci of different ecological origin. *FEMS Microbiol Lett* 1997; **155**: 55–61.

31 Biavasco F, Giovanetti E, Miele A *et al*. In vitro conjugative transfer of VanA vancomycin resistance between enterococci and listeriae of different species. *Eur J Clin Microbiol Infect Dis* 1996; **15**: 50–9.

32 Morroni G, Di Cesare A, Di Sante L *et al*. *Enterococcus faecium* ST17 from coastal marine sediment carrying transferable multidrug resistance plasmids. *Microb Drug Res* 2016; **22**: 523–30.

33 Vignaroli C, Luna GM, Pasquaroli S *et al*. Epidemic *Escherichia coli* ST131 and *Enterococcus faecium* ST17 in coastal marine sediments from an Italian beach. *Environ Sci Technol* 2013; **47**: 13772–80.

34 Barton BM, Harding GP, Zuccarelli AJ. A general method for detecting and sizing large plasmids. *Anal Biochem* 1995; **226**: 235–40.

35 Vignaroli C, Zandri G, Aquilanti L *et al*. Multidrug-resistant enterococci in animal meat and faeces and co-transfer of resistance from an *Enterococcus durans* to a human *Enterococcus faecium*. *Curr Microbiol* 2011; **62**: 1438–47.

36 Di Sante L, Morroni G, Brenciani A *et al*. pHTβ-promoted mobilization of non-conjugative resistance plasmids from *Enterococcus faecium* to *Enterococcus faecalis*. *J Antimicrob Chemother* 2017; **72**: 2447–53.

37 McCurdy RD, McGrath JJ, MacKay-Sim A. Validation of the comparative quantification method of real-time PCR analysis and a cautionary tale of housekeeping gene selection. *Gene Ther Mol Biol* 2008; **12**: 15–24.

38 Sifaoui F, Arthur M, Rice L *et al*. Role of penicillin-binding protein 5 in expression of ampicillin resistance and peptidoglycan structure in *Enterococcus faecium*. *Antimicrob Agents Chemother* 2001; **45**: 2594–7.

39 Jensen LB, Garcia-Migura L, Valenzuela AJ *et al*. A classification system for plasmids from enterococci and other Gram-positive bacteria. *J Microbiol Methods* 2010; **80**: 25–43.

40 Li X, Alvarez V, Harper WJ *et al*. Persistent, toxin–antitoxin system-independent, tetracycline resistance-encoding plasmid from a dairy *Enterococcus faecium* isolate. *Appl Environ Microbiol* 2011; **77**: 7096–103.

41 Mikalsen T, Pedersen T, Willems R *et al*. Investigating the mobilome in clinically important lineages of *Enterococcus faecium* and *Enterococcus faecalis*. *BMC Genomics* 2015; **16**: 282.

Downloaded from https://academic.oup.com/jac/article-abstract/74/4/843/5288148 by guest on 16 March 2019

42 Lasch P, Fleige C, Stämmler M *et al*. Insufficient discriminatory power of MALDI-TOF mass spectrometry for typing of *Enterococcus faecium* and *Staphylococcus aureus* isolates. *J Microbiol Methods* 2014; **100**: 58–69.

43 Clewell DB, Weaver KE, Dunny GM *et al*. Extrachromosomal and mobile elements in enterococci: transmission, maintenance, and epidemiology. In: MS Gilmore, DB Clewell, Y Ike, N Shankar, eds. *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection*. Boston, MA, USA: Massachusetts Eye and Ear Infirmary, 2014; 225–303.

44 Laverde Gomez JA, Hendrickx APA, Willems RJ *et al*. Intra- and interspecies genomic transfer of the *Enterococcus faecalis* pathogenicity island. *PLoS One* 2011; **6**: e16720.

45 Manson JM, Hancock LE, Gilmore MS. Mechanism of chromosomal transfer of *Enterococcus faecalis* pathogenicity island, capsule, antimicrobial resistance, and other traits. *Proc Natl Acad Sci USA* 2010; **107**: 12269–74.

46 Qin X, Galloway-Peña JR, Sillanpaa J *et al*. Complete genome sequence of *Enterococcus faecium* strain TX16 and comparative genomic analysis of *Enterococcus faecium* genomes. *BMC Microbiol* 2012; **12**: 135.

47 Fiedler S, Bender JK, Klare I *et al*. Tigecycline resistance in clinical isolates of *Enterococcus faecium* is mediated by an upregulation of plasmid-encoded tetracycline determinants *tet*(L) and *tet*(M). *J Antimicrob Chemother* 2016; **71**: 871–81.

48 Katharios-Lanwermeyer S, Rakic-Martinez M, Elhanafi D *et al*. Coselection of cadmium and benzalkonium chloride resistance in conjugative transfers from nonpathogenic *Listeria* spp. to other Listeriae. *Appl Environ Microbiol* 2012; **78**: 7549–56.

Downloaded from https://academic.oup.com/jac/article-abstract/74/4/843/5288148 by guest on 16 March 2019