Prevalence of the fosfomycin-resistance determinant, fosB3, in Enterococcus faecium clinical isolates from China

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In order to investigate the prevalence of fosfomycin-resistance (fos) determinants in Enterococcus faecium, clinical strains were collected from hospitals throughout China between January 2008 and December 2009. Antimicrobial susceptibility testing was performed, after which the fos genes in all isolates and van genes in vancomycin-resistant isolates were characterized by PCR and sequencing. Conjugation experiments were carried out with fosB-positive E. faecium, DNA fragments flanking the fosB3 gene were sequenced and the genetic environment of fosB3 was analysed. Fosfomycin-resistant E. faecium (FREF) strains were characterized further by multilocus sequence typing (MLST) and PFGE. Among 145 E. faecium clinical isolates, 10 were resistant to fosfomycin with MICs \( \geq 1024 \) mg l\(^{-1} \) including six vancomycin-resistant strains of which five were vanA-positive and the sixth vanM-positive. All ten FREF strains harboured the fosB3 gene. Fosfomycin resistance and fosB3 could be transferred by conjugation from nine isolates. The fosB3 and tnpA genes were located in a circular DNA intermediate in all FREF strains and reversely inserted into vanA transposon Tn1546 in four vanA-positive FREF isolates. Ten different PFGE types and seven MLST types were found among the ten fosB3-positive isolates, while all strains belonged to the common clonal complex CC17. In conclusion, the transferable fosfomycin-resistance determinant fosB3 plays an important role in E. faecium resistance to fosfomycin in China.

INTRODUCTION

The rapid emergence and spread of vancomycin-resistant enterococci (VRE), together with the relative shortage of new antimicrobial agents, call for a new look at therapeutic options. One of the potential alternative treatments for VRE is fosfomycin (Perri et al., 2002). Fosfomycin is a traditional bactericidal antibiotic that interferes with cell wall synthesis by inhibiting the initial step involving phosphoenolpyruvate synthase (Kahan et al., 1974). It has a broad spectrum of activity against many Gram-positive and Gram-negative bacteria (Michalopoulos et al., 2011).

A significant challenge to the effectiveness of fosfomycin has been the emergence of enzymes that modify the antibiotic. To date, four fosfomycin-modifying enzymes have been described, which act by catalysing the formation of glutathione–fosfomycin (FosA), L-cysteine–fosfomycin (FosB), ATP–fosfomycin (FosC), or water–fosfomycin (FosX) adducts (Michalopoulos et al., 2011). FosB is a divalent-metal-dependent thiol-S-transferase implicated in fosfomycin resistance in Gram-positive pathogens. The fosB gene has

†These authors contributed equally to this work.

Abbreviations: CLSI, Clinical and Laboratory Standards Institute; FREF, fosfomycin-resistant E. faecium; MLST, multilocus sequence typing; ST, sequence type; VRE, vancomycin-resistant enterococci.
been identified both chromosomally and on plasmids in many Gram-positive bacteria including *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecium* and *Bacillus subtilis* (Cao et al., 2001; Lamers et al., 2012; Rigsby et al., 2005; Xu et al., 2013; Zilhao & Courvalin, 1990).

Fosfomycin has been approved for clinical application for decades. In China, the fosfomycin resistance rate in *E. faecium* was as high as 20% in 2007 (Wang et al., 2008) yet molecular studies of fosfomycin resistance in enterococci are rare. Recently, we reported the discovery of *fosB*, a transferable fosfomycin-resistance determinant having high nucleotide identity with the *fosB* gene in *Staphylococcus* spp. and confirmed that this gene was responsible for fosfomycin resistance in three vancomycin- and fosfomycin-resistant *E. faecium* isolates from Shanghai. We found that the *fosB* gene was flanked by a single copy of *tnpA* (ISL3-like transposase-encoding gene) and carried by a transferable extra-chromosomal circular intermediate in *vanM*-positive fosfomycin-resistant *E. faecium* (FREF) strains (Xu et al., 2013). We also found that *fosB* and *tnpA* genes were inserted into the *vanRS–vanH* intergenic region of *vanA* transposon Tn1546 in *vanA*-positive FREF isolates, leading to co-resistance to fosfomycin and vancomycin (Qu et al., 2012).

The purpose of this study was to investigate the prevalence of fosfomycin-resistance genes among FREF clinical isolates in China and to explore the transmission of fosfomycin-resistance determinants in both vancomycin-resistant and vancomycin-susceptible FREF strains. Homology genotyping was also performed to establish the genetic relationship of fos-containing isolates.

**METHODS**

**Bacterial strains.** A total of 145 *E. faecium* clinical strains were obtained from patients at 12 hospitals in different areas of China between January 2008 and December 2009: eastern China (74 strains), south-west China (19), northern China (17), southern China (14), north-west China (14) and central China (7).

**Antimicrobial susceptibility testing.** The MICs of ten antimicrobial agents were measured by agar dilution susceptibility testing. High-level gentamicin resistance was determined by the Kirby Bauer method. Results were interpreted according to recommendations of the Clinical and Laboratory Standards Institute (CLSI). Due to the lack of acknowledged fosfomycin breakpoints for *E. faecium*, we used the fosfomycin breakpoints for *E. faecalis* proposed by the CLSI (CLSI, 2010). *E. faecalis* ATCC 29212 was used as a quality control for MIC determination.

**Detection of fosfomycin- and vancomycin-resistance determinants.** All isolates were tested for the presence of *fosA*, *fosB* and *fosC* genes by PCR amplification with the primers listed in Table 1, followed by sequencing of the PCR product. Vancomycin-resistance (*van*) genes were detected in FREF strains by PCR as previously described (Xu et al., 2010). The PCR products were sequenced to determine the *van* genotype.

**PFGE.** PFGE analysis was performed using a CHEF mapper system (Bio-Rad). Agarose plugs were prepared with proteinase K (Merck) at 1000 μg ml⁻¹ and digested with S1 nuclease or Smal (Takara), and the digested DNA was subjected to electrophoresis at 6 V cm⁻¹, 14 °C, in a 1.0% agarose gel with pulse times of 5 to 30 s for 22 h. Banding patterns were analysed with BioNumerics software version 5.0 (Applied Maths). Isolates were classified into the same PFGE group if their Dice similarity coefficient was over 80% (Carriço et al., 2005).

**Multilocus sequence typing (MLST).** MLST was performed as described previously (Homan et al., 2002). Sequence types (STs) were assigned using http://faecium.mlst.net/. Clusters of related sequence types (STs) were grouped into clonal complexes using the eBURST program v3 (http://eburst.mlst.net/) (Feil et al., 2004).

**Conjugation.** Filter matings were performed using the fosfomycin-resistant clinical strains as donors and *E. faecium* BM4105RF (resistant to rifampicin and fusidic acid) as the recipient strain as described by Franke & Clewell (1981). Transconjugants were selected on BHI agar (Oxoid) plates containing fosfomycin (1024 μg ml⁻¹) with glucose 6-phosphate (25 μg ml⁻¹), fusidic acid (10 μg ml⁻¹) and rifampicin (100 μg ml⁻¹).

**DNA sequence analysis.** Inverse PCR and nucleotide sequencing were performed to determine the flanking region of the *fosB* gene using primers *fosBrF* and *fosBrR* listed in Table 1 (Xu et al., 2013). Overlap PCR and nucleotide sequencing were carried out to further determine the genomic environments of the *fosB* gene as previously described (Qu et al., 2012).

**RESULTS**

**Antimicrobial susceptibility of *E. faecium* clinical isolates**

Of the 145 *E. faecium* clinical isolates, ten were resistant to fosfomycin (MIC>1024 mg l⁻¹), all of which were isolated in eastern China. Twenty-eight isolates were intermediate to fosfomycin (MIC=128 mg l⁻¹) and 107 strains were susceptible to fosfomycin (MIC≤64 mg l⁻¹). Six FREF isolates were resistant to vancomycin (MIC≥128 mg l⁻¹), and four of these were resistant to teicoplanin (MIC 32–64 mg ml⁻¹). The resistance rates of FREF to ampicillin, levofloxacin, erythromycin and high-level gentamicin were 100%, 90%, 80% and 60%, respectively. All ten FREF isolates were susceptible to linezolid (Table 2).

**Screening of fosfomycin-resistant determinants and *van* genes**

The fosfomycin-resistance gene *fosB* was present in all ten FREF isolates. Sequencing established that it was subtype *fosB*3. No *fosB* gene was detected in the other 135 strains, including those that showed intermediate resistance to fosfomycin. *fosA* and *fosC* genes were not found in any of the isolates tested. Among the six vancomycin-resistant FREF isolates, five strains were *vanA*-positive and one was *vanM*-positive (Fig. 1).

**Molecular typing of *fosB*3-positive strains**

MLST analysis of the *fosB*3-positive *E. faecium* strains revealed seven different STs. ST18, ST78 and ST203 were found in two isolates each. ST34, ST323, ST389 and ST559
were found in one strain each. All strains belonged to the clonal complex CC17. PFGE profiles of SmaI-digested chromosomal DNA divided the FREF strains into ten different PFGE types (Fig. 1).

Transfer of resistance

Fosfomycin resistance was transferred by filter mating at frequencies of $10^{-2}$–$10^{-7}$ per donor c.f.u. from nine of the ten donor strains. All transconjugants exhibited fosfomycin resistance of $>1024$ mg l$^{-1}$ and were fosB3-positive. Vancomycin resistance and van genes (vanA and vanM) were co-transferred with fosfomycin resistance by conjugation from three of the six co-resistant strains at rates of $10^{-2}$–$10^{-7}$ per donor c.f.u. (Table 2).

Genetic environment of fosB3

In all FREF isolates the fosB gene was flanked by a single copy of tnpA. According to inverse PCR and sequencing

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primers</th>
<th>Sequences (5’ to 3’)</th>
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<td>GCTGCAGCCCGGCTGGAATA</td>
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</tr>
<tr>
<td></td>
<td>fosAR</td>
<td>GACGCCCCCTCAGTTTGT</td>
<td>This study</td>
</tr>
<tr>
<td>fosB</td>
<td>fosBF</td>
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<td></td>
<td>fosBR</td>
<td>CTCAGTCATTCCTAAGCTCTG</td>
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<td>fosC</td>
<td>fosCF</td>
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<td></td>
<td>fosCR</td>
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<td>This study</td>
</tr>
<tr>
<td>van genes</td>
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<td>GTTTGGGGGTTGCTCAGAGG</td>
<td>Xu et al., (2010)</td>
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<tr>
<td></td>
<td>NvanR</td>
<td>TCACCCCTTTAACGCTAATACGATC</td>
<td>Xu et al., (2013)</td>
</tr>
</tbody>
</table>

Table 2. Antimicrobial susceptibility of fosB-positive E. faecium clinical strains and their transconjugants

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Antibiotic susceptibility (mg l$^{-1}$)*</th>
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<tbody>
<tr>
<td></td>
<td>FOS  VAN  TEC  LZD  CHL  LEV  DOX  ERY  RIF  AMP  GM†</td>
</tr>
<tr>
<td>Clinical isolates</td>
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<td>SH08118</td>
<td>$&gt;1024$ 1 0.25 1 4 64 $\leq 0.06$ $&gt;128$ 8 128 6</td>
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<td>SH08154</td>
<td>$&gt;1024$ $&gt;128$ 8 1 4 128 16 $&gt;128$ 8 128 6</td>
</tr>
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<td>HZ0948</td>
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</tr>
<tr>
<td>HZ0952</td>
<td>$&gt;1024$ $&gt;128$ 64 1 16 64 32 $&gt;128$ 4 128 10</td>
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</tr>
<tr>
<td>HZ0963</td>
<td>$&gt;1024$ 128 32 1 4 32 0.125 $&gt;128$ 8 128 6</td>
</tr>
<tr>
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<td>$&gt;1024$ $&gt;128$ 64 2 4 128 $\leq 0.06$ 1 4 128 21</td>
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<tr>
<td>HZ0966</td>
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</tr>
<tr>
<td>HZ0967</td>
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</tr>
<tr>
<td>Transconjugants</td>
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</tr>
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<tr>
<td>Recipient</td>
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<td>BM4105RF</td>
<td>32 0.5 0.25 1 2 2 $\leq 0.06$ 0.125 $&gt;128$ 0.5 23</td>
</tr>
</tbody>
</table>

*FOS, fosfomycin; VAN, vancomycin; TEC, teicoplanin; LZD, linezolid; CHL, chloramphenicol; LEV, levofloxacin; DOX, doxycycline; ERY, erythromycin; RIF, rifampicin; AMP, ampicillin; GM, gentamicin.

†High-level gentamicin MIC was determined by Kirby Bauer method (mm).
results, *fosB* and *tnpA* genes formed a circular DNA (ISL3-like family) in all FREF isolates and their transconjugants (Fig. 2a). In the five *vanA*-positive FREF isolates and two of their transconjugants (BM64 and BM66), *fosB* and *tnpA* genes reversibly inserted into the *vanA* transposon Tn1546 in the *vanRS–vanH* intergenic region (Fig. 2b).

**DISCUSSION**

Fosfomycin has good activity against *E. faecium* and has been used therapeutically across the globe. The reported fosfomycin resistance rate in *E. faecium* was 0% in Turkey (Butcu et al., 2011) and 1% in South India (Sahni et al., 2013). Some studies have also shown a high *in vitro* susceptibility of VRE to fosfomycin (Allerberger & Klare, 1999; Perri MB et al., 2002). However, in this study, we observed 6.9% fosfomycin resistance rate in *E. faecium* clinical strains, which may relate to more common use of fosfomycin as an empirical treatment for VRE infection in Chinese hospitals.

In this study the *fosB3* gene was detected in all of the 10 FREF isolates and, similar to our previous results with three different FREF strains (Xu et al., 2013), we found that *fosB3* could be transferred by conjugation from nine strains tested. Differing from our previous study, however, the *fosB3* gene was detected not only in six VanA- or VanM-type vancomycin-resistant isolates, but also in four vancomycin-sensitive FREF strains.

Circular DNA (ISL3-like family) was found in all FREF isolates and their transconjugants. The *vanA*-positive strains possessed a previously unidentified variation in a Tn1546-like element (containing the *van* operon), whereby the *fosB3* and *tnpA* (ISL3-like transposase) genes were inserted in the *vanRS–vanH* intergenic region. These findings suggest that the *fosB3* gene can be transferred via an extra-chromosomal circular intermediate, or via the Tn1546-like element, resulting in co-resistance to fosfomycin and vancomycin.

The 10 FREF clinical isolates demonstrated high diversity by PFGE typing with each having a different fragment pattern. Similar findings from earlier studies also demonstrated a high genetic diversity amongst *E. faecium* clinical strains isolated from the same and/or different hospitals (d’Azevedo et al., 2006; Xu et al., 2013). PFGE has been considered as the ‘gold standard’ for enterococci typing because of its high

**Fig. 1.** Strain particulars and dendrogram of patterns generated by PFGE of *fosB3*-positive *E. faecium*. CC: clonal complex. FPH: Shanghai First People’s Hospital, Shanghai, China. HSH: Huashan Hospital, Fudan University, Shanghai, China. RJD: Renji Hospital of Shanghai Jiaotong University, Shanghai, China. ZFH: First Affiliated Hospital of Zhejiang University, Hangzhou, China.

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Hospital</th>
<th>Year</th>
<th>MLST</th>
<th>CC type</th>
<th><em>van</em></th>
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<td>HZ09064</td>
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<td>2009</td>
<td>ST389</td>
<td>CC17</td>
<td>vanA</td>
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<td>ZFH</td>
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<td>ST559</td>
<td>CC17</td>
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<tr>
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<td>2008</td>
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<td>CC17</td>
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<td>2009</td>
<td>ST203</td>
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<td>2009</td>
<td>ST78</td>
<td>CC17</td>
<td>vanA</td>
</tr>
</tbody>
</table>

**Fig. 2.** The genetic environment of the *fosB3* gene in fosfomycin-resistant *E. faecium* isolates. (a) Circular DNA containing *fosB3* and *tnpA* genes; (b) Tn1546-like element with *tnpA* and *fosB3* genes inserted between *vanRS* and *vanH*. 
degree of isolate discrimination (Barbier et al., 1996). However, MLST has emerged as an important tool to study long-term epidemiology, microbial population biology and patterns of evolutionary descent (Feil et al., 2004).

MLST analysis of the FREF isolates revealed seven different STs: ST18, ST78, ST203, ST389, ST323, ST34 and ST559. All of these STs belong to clonal complex CC17. CC17, which is characterized by its resistance to ampicillin and quinolones, is a major genetic lineage of E. faecium that has spread worldwide and is associated with hospital outbreaks (Willems et al., 2005). It has also been reported that CC17 strains are the main epidemic lineage responsible for E. faecium nosocomial infections in China (Qu et al., 2012; Sun et al., 2012; Xu et al., 2011). In our study, the 10 CC17 FREF isolates exhibited high level resistance to vancomycin, teicoplanin, ampicillin, levofloxacin, erythromycin and gentamicin, although all of them were susceptible to linezolid. The multidrug resistance suggests the hypothesis that CC17 E. faecium are better able than other types to exploit the clinical environment through successive acquisition of multiple adaptive mechanisms, including the acquisition of resistance genes (such as van genes and fosB), to gain a selective advantage (Willems et al., 2005).

Our results indicate that spread of CC17 strains may play an important role in the increase of resistance to fosfomycin and co-resistance to vancomycin in E. faecium. Therefore, it will be important to monitor the emergence of CC17 strains to limit their spread in hospitals. As ampicillin resistance is a specific genetic marker of CC17, increasing isolation rates of ampicillin-resistant enterococci may represent the first sign of the emergence of CC17 in hospitals (Willems et al., 2005).

In conclusion, fosB is responsible for fosfomycin resistance in E. faecium and could be transferred between different strains. CC17 is the dominant clonal complex in clinical FREF isolates. A transposon or extrachromosomal circular intermediate is associated with the transmission of fosfomycin resistance in E. faecium. Therefore, continuous monitoring will be necessary to prevent further dissemination of fosfomycin-resistance genes, together with prudent use of fosfomycin in clinical settings.

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