First Report of the Plasmid-Mediated \textit{fosB} Gene in \textit{Enterococcus faecalis} from Pigs

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Abstract: Plasmid-mediated fosfomycin determinants is a global public health concern due to the increasing dissemination of fosfomycin resistance and limited clinical treatment options. Information about the fosfomycin resistant and molecular genetic among \textit{Enterococcus} spp. is still lacking. In this study, we found the first plasmid-mediated \textit{fosB} in \textit{Enterococcus faecalis} from pigs, and all the fosfomycin resistant \textit{Enterococcus} spp. (FRE) isolates were multi-drug resistant. S1-PFGE, Southern blot and conjugation experiments indicated that the \textit{fosB} gene located on ~54.7 kb transferable plasmids. Relative competition assay confirmed that the \textit{fosB}-carrying plasmid impaired fitness in recipient \textit{E. faecalis} JH2-2. Illumina and the MinION sequencing data revealed that both \textit{E. faecalis} ES-1 and ES-2 isolates belonged to novel ST (ST964), and had 71 SNPs difference. WGS showed that the genetic environments of \textit{fosB} were diverse among different species, and the linezolid resistance gene \textit{optrA} was found in the \textit{fosB}-carrying strains. To summarize, for the first time, we reported plasmid-mediated \textit{fosB} in \textit{E. faecalis} from pigs. And, the co-occurrence of \textit{fosB} and \textit{optrA} pose a serious threat to public health.

Keywords: antimicrobial resistance; molecular genetic; fosfomycin resistant; \textit{Enterococcus} spp.

1. Introduction

\textit{Enterococcus} spp. are natural inhabitants of the environment and essential components of the intestinal microbiota of healthy humans and animals [1,2]. The genus \textit{Enterococcus} presently contains over 50 species, among which \textit{Enterococcus faecalis} (\textit{E. faecalis}) and \textit{Enterococcus faecium} (\textit{E. faecium}) are the most predominant isolated species, accounting for more than 80% of isolates [3]. It has been recognized as important major nosocomial pathogens due to quickly acquiring virulence and multidrug resistance determinants via mobile elements [4]. However, the spread of fosfomycin resistant \textit{Enterococcus} spp. (FRE), together with the limited availability of novel antimicrobial agents, has significantly restricted effective therapeutic options [5]. As a broad-spectrum cell wall synthesis inhibitor, fosfomycin interferes with the first step of bacterial cell wall biosynthesis, the formation of the peptidoglycan precursor UDP N-acetylmuramic acid, and then inhibits of enzyme-catalyzed reaction [6]. Therefore, it has considerable bactericidal activity against a range of bacteria by virtue of specific antimicrobial mechanisms [7].

Several mechanisms have been proposed to be related to fosfomycin resistance [8–10]. The mutations in the targeted enzyme MurA could reduce the affinity of enolpyruvyl transferase for fosfomycin [8], and mutations in chromosomal genes encoding fosfomycin transporters are also involved in causing resistance [9,10]. However, a significant challenge to the effectiveness of fosfomycin is the emergence of plasmid encoding enzymes that modify the antibiotic [11,12]. To date, four plasmid-encoded fosfomycin-enzymes (FosA, FosB, FosC and FosX) have been described [13]. Among the above enzymes, FosB, which catalyzes L-cysteine-fosfomycin, was currently the only known plasmid-brone fosfomycin-resistance determinant in \textit{Enterococcus} spp. [14,15]. Reports on \textit{fosB}-plasmid mediated
fosfomycin resistance in *Enterococcus faecium* are available [16]. However, the knowledge of plasmid-brone *fosB* in *E. faecalis* is still unknown. Therefore, further research on the phenotype and genetic molecule of FRE and *fosB*-positive *E. faecalis* is urgently required. The purpose of this study is to investigate the antimicrobial resistance and molecular genetic of FRE from pigs.

2. Materials and Methods

2.1. Bacterial Isolation and Identification

365 health pig anal swab samples were collected in one commercial pig farms for one time in Jiangsu province, China, 2017. All the samples were screened on the mEI agar plate (Land bridge, Beijing, China) supplemented with 128 mg/L fosfomycin. The identification of bacterial species was performed using MALDI-TOF MS (BruKer Daltonik, Bremen, Germany), and then confirmed by 16S rRNA sequence analysis as described previously [17]. The presence of *fosA*, *fosB*, *fosC* and *fosX* encoding fosfomycin-enzymes were detected by PCR and followed by Sanger sequencing as described previously [10]. Primers used for the detection of fosfomycin resistance genes were listed in Table S1.

2.2. Antimicrobial Susceptibility Testing

The MICs of FRE to penicillin, ampicillin, vancomycin, erythromycin, tetracycline, marbofloxacine, florfenicol, rifampin, linezolid, valnemulin, gentamycin and fosfomycin were determined using the broth microdilution method according to the guidelines of the Clinical and Laboratory Standards Institute (document VET01-A4). The MIC results were interpreted according to CLSI and European Committee on Antimicrobial Susceptibility Testing (EUCAST) (http://www.eucast.org) (6 Decemeber 2020) guidelines.

2.3. S1-PFGE and Southern Blotting

S1 nuclease-PFGE and Southern blotting were performed to locate *fosB* and determine plasmid size in *E. faecalis* as previously described [18]. In brief, strains were embedded in agarose gel plugs and digested by S1 nuclease (TaKaRa, Beijing, China). Specific fosB probe were used for gene localization by Southern blotting using the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Diagnostics). *XbaI* restricted DNA of *S. enterica* serovar Braenderup H9812 was used as DNA marker. This method allowed the detection and estimation of the size of large bacterial plasmids in the presence of genomic DNA using pulsed-filed gel electrophoresis (PFGE).

2.4. Conjugation Assay

The conjugation assay was described in detail in our previous study [19]. Briefly, the horizontal transferability of *fosB* was examined using wild-type strain as donor cell and *E. faecalis* JH2-2 as recipient cell. Transconjugants were confirmed by PCR targeting the *fosB* gene and cooperated with the results of MICs. The transfer frequency was calculated as the number of transconjugants per recipient.

2.5. Fitness Experiment

The biological cost of the acquisition of *fosB*-carrying plasmid was investigated by in vitro competition assays [19]. Competition assay was used to assess the relative fitness of *fosB*-bearing transconjugant JH-ES-1 and JH-ES-2 against *E. faecalis* JH2-2 (the tester strain). The overnight cultures of competitor and the tester were mixed at a rate of 1:1 at 0 h and diluted in LB broth. Subsequently, the mixture was incubated for 24 h, and diluted in 1000-fold into LB broth at 37 °C for 24 h. The mixtures at both startpoint (0 h) and endpoint (24 h) were plated on LB plates without or with 128 mg/L fosfomycin and incubated at 37 °C for 12 h. The relative competitive fitness W was calculated using the formula W = (ln(Rf/Ri)) / (ln(Sf/Si)). Ri and Si indicate transconjugant and recipient cells at 0 h, while Rf and Sf indicate transconjugant and recipient cells at 24 h. Statistical analysis was carried out using GraphPad Prism 5.0.
2.6. Genome Sequence and Analysis

Genomic DNA of the isolates were extracted using the Magen DNA Purification Kit (Magen, Shanghai, China), following the manufacturer’s instructions. The samples were sequenced with two different platforms, comprising Illumina and the Oxford Nanopore Technologies (ONT) MinION platform. Paired-end libraries were constructed and sequenced using the Illumina HiSeq 2500 System (Annoroad, Beijing, China). Sequencing reads were assembled de novo with the SPAdes 3.5 tool. A Rapid Barcoding Sequencing Kit was used to construct the libraries sequenced in a MinION device as previously reported. Guppy basecalling software (v2.2) was used to generate fast5 files harboring the 1D DNA sequence from fast5 files with only raw data in the tmp folder. High-quality complete plasmids were constructed by hybrid de novo assembly of Illumina short reads and nanopore long reads data using the Unicycler v0.3 tool. Reference sequences of antibiotic resistance genes were from database ARG-ANNOT [20]. The sequence type (ST) of *E. faecalis* was performed in silico by multilocus sequence typing (MLST) analysis using the published database [21].

2.7. Nucleotide Accession Numbers

The genome sequences of *E. faecalis* ES-1 determined in this study have been deposited in GenBank under the BioProject number of PRJNA609523.

3. Results

3.1. Antimicrobial Susceptibility Profiles of Fosfomycin Resistant Enterococcus spp.

In total, 54 FRE isolates were obtained from 365 feces samples, of which 28 were *E. faecalis* isolates and the others were *E. faecium* isolates. The isolation rates of fosfomycin resistant *E. faecalis* and *E. faecium* from pigs were 7.67% and 7.12%, respectively, which were higher than 0.3% in *E. faecalis* and 4.9% in *E. faecium*, respectively, from a China teaching hospital [10]. Although epidemiology studies in this field is limited currently, our study suggested that, to some extent, fosfomycin resistant *Enterococcus* spp. in animals maybe more serious than that in human in China [10].

To investigate the antimicrobial susceptibility of FRE, MICs were determined for 11 classes of antimicrobial agents, including 13 kinds of antibiotics. As shown in Figure 1A,B, all those *E. faecalis* and *E. faecium* were resistant to erythromycin, tetracycline and fosfomycin, and all strains were susceptible to vancomycin and valnemulin (Table S2). Additionally, a high frequency of resistance was observed against marbofloxacin (85.71% and 76.92%), florfenicol (96.42% and 88.46%), streptomycin (92.85% and 69.23%) and gentamycin (89.28% and 73.07%), followed by rifampin (46.42% and 42.30%) (Figure 1A,B). Moreover, fosfomycin resistant *E. faecalis* exhibited a low resistance rate to penicillin (3.57%) and ampicillin (3.57%), while fosfomycin resistant *E. faecium* showed a high resistance rate (penicillin, 42.30% and ampicillin, 76.92%). Inversely, fosfomycin resistant *E. faecalis* were highly resistant to linezolid (76.92%), but fosfomycin resistant *E. faecium* presented a low resistance rate (linezolid, 7.69%) (Figure 1A,B). According to the previous study, strains that are resistant to three or more classes of antimicrobial agents are considered as multiresistance [22]. Based on this criterion, all the FRE strains in this study can be determined as multiresistance (Figure 1C,D). More seriously, strains that were resistant to seven classes of antimicrobial agents took the largest proportions, and were up to 50% and 34.6% for *E. faecalis* and *E. faecium*, respectively. Although FRE exhibited multi-resistance, our results showed that they were all susceptible to vancomycin and valnemulin, which indicated that these two drugs may be used as specific medicines for infections caused by FRE strains.
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Figure 1. Antimicrobial resistant profiles of FRE strains. (A, B), the antibiotic-resistance rate of each tested drug; (C, D), the proportion of multiresistance FRE.

To explore the plasmid-mediated determinants in those strains for fosfomycin resistance, PCR was used to detect the plasmid encoding genes (fosA, fosB, fosC and fosX). Results showed that two E. faecalis isolates, named ES-1 and ES-2, were positive for fosB, being confirmed by Sanger sequence. Of note, it was found that most fosfomycin resistance Enterococcus isolates were negative for the known fos genes, indicated that novel fosfomycin resistance determinants or murA mutations may exist among these isolates [10], which requires further analysis.

3.2. Location and Transferability of fosB-Carrying Plasmid

S1-PFGE and southern blot confirmed that fosB was harboured on a ~54.7 kb plasmid in both ES-1 and ES-2, and there were two plasmids in each of those two isolates (Figure 2A,B). To evaluate the transferability of fosB in E. faecalis ES-1 and ES-2, conjugation assays was performed. Results showed that fosB gene successfully transferred from E. faecalis ES-1 and ES-2 to recipient strains E. faecalis JH2-2. The transfer frequencies of fosB-carrying plasmids were $1.21 \pm 0.96 \times 10^{-5}$ and $3.72 \pm 3.24 \times 10^{-5}$, respectively. Two transconjugants was obtained (named as JH-ES-1 and JH-ES-2), and presented resistance to fosfomycin, gentamicin and erythromycin (Table 1), which indicated that gentamicin and erythromycin resistance determinants co-transferred with fosB, being a potential hazard for public health.
**Figure 2.** The location of dosB in wild-type *E. faecalis* strains ES-1 and ES-2. (A) S1-PFGE of dosB-carrying wild-type *E. faecalis* strains (B) the corresponding Southern hybridization using the dosB-specific probe. Lane M, marker H9812; lanes 1 and 2 represent S1-PFGE patterns of isolates ES-1 and ES-2, lanes 3 and 4 represent southern blot patterns of dosB gene in ES-1 and ES-2. (C) The genetic contents of plasmid pES-dosB. Circular representation of alignments between reference dosB carrying plasmid pKUB3007-3 (accession no. AP018546.1) and the homologous plasmid from *E. faecalis* ES1. The alignments were generated by Blastn and visualized by BLAST Ring Image Generator.

**Table 1.** Characterization of dosB positive strains and their transconjugants.

| Antimicrobial Agents (mg/L)/Other Features | ES-1 | ES-2 | JH2-2 | JH-ES-1 | JH-ES-2 |
|------------------------------------------|------|------|-------|---------|---------|
| Fosfomycin                               | 2048 | 2048 | 64    | 512     | 512     |
| Linezolid                                | 4    | 8    | 0.5   | 0.5     | 0.5     |
| Penicillin                               | 4    | 2    | 1     | 1       | 1       |
| Vancomycin                               | 2    | 4    | 2     | 2       | 2       |
| Gentamicin                               | HLGR | HLGR | -     | -       | -       |
| Streptomycin                             | HSLR | HSLR | -     | HGLR    | HGLR    |
| Florfenicol                              | 64   | 64   | 2     | 2       | 2       |
| Tetracycline                             | 128  | 128  | 2     | 2       | 2       |
| Erythromycin                             | >256 | >256 | 0.5   | 256     | 256     |
| Fusidic acid                             | 4    | 4    | >256  | >256    | >256    |
| Rifampicin ST                            | 2    | 2    |       |         |         |
| **Resistance genes**                     |      |      |       |         |         |
| *aph(3′)-III, dfrG, erm(A), erm(B), fexA, lsa(A), optra, sul*, cat, erm(B), fosB, tet(L), tet(M)* | 964  | 964  |       |         |         |
| *aac(6′)-aph(2′)<sup>″</sup>, erm(B), fosB*    |      |      |       |         |         |

Note: MICs shaded grey represented strains that were resistant to the corresponding antimicrobial agents; Antimicrobial resistance genes located on plasmids were shown in bold. HLGR, high-level gentamicin resistance; HSLR, high-level streptomycin resistance; −, not HLGR/HLSR. MICs of gentamicin and streptomycin > 500 ug/ml and 2000 ug/ml were defined as HLGR and HSLR, respectively, based on the CLSI.
3.3. Fitness Cost of the Transconjugant

fosB-carrying plasmid transferring into the recipient strain may impact the fitness of the host, thus the fitness cost of fosB-carrying plasmid in transconjugant was evaluated by competitive advantage. The in vitro competition assays showed that transconjugants JH-ES-1 and JH-ES-2 had a relative fitness value (W) of 0.78 and 0.73, respectively, when compared with its parental strain JH2-2 (Figure 3). The results further suggested that the acquiring of fosB-carrying plasmid imposed a fitness cost to transconjugant.

3.4. WGS Analysis of fosB-Carrying Isolates

To investigate the molecular genetic characteristic of the two fosB positive strains, whole genome sequencing (WGS) analysis was performed. WGS analysis showed that the two isolates both belonged to novel ST type (ST964), and had 71 SNPs. This indicated that clonal dissemination with novel ST type emerged in fosB-carrying E. faecalis strains. Additionally, one complete chromosome and two plasmids sequences were obtained from each one strain, and fosB gene was located on a 53.121-kb plasmid in both strains, which were correspondence with the S1-PFGE results. The fosB-carrying plasmid pES-fosB had 57% query cover and 97.68% identity with E. faecalis plasmid pKUB3007-3 (accession no. AP018546.1). Both the two plasmids carried fosB, macrolide resistance gene erm(B) and toxin-antitoxin (TA) systems (Figure 2C). Additionally, the pES-fosB plasmid also contained cadmium resistance gene cadA, copper chaperone (CopZ) and IS members of ISEfm, IS1216E and ΔIS1216 (Figure 2C).

Analysis of the genetic environment of fosB-carrying contigs from NCBI showed that fosB in E. faecium was flanked by tnpA. And plasmid pEMA120 (genbank: KX853854) possessed a Tn1546-like element, with tnpA and fosB genes inserted in the vanRS-vanH intergenic region (Figure 4). The locations of the insertion in this region were identical, corresponding to nucleotide 5813 bp of Tn1546 (GenBank M97297). fosB gene also emerged in Staphylococcus haemolyticus and Staphylococcus aureus in small plasmids, and flanked by rep gene at right end (Figure 4). However, fosB was bracketed by hp in E. faecalis, and then surrounded by erm(B) and aac(6′)-aph(2″) (Figure 4). Those analyses indicated that the genetic environments of fosB were diverse.

![Figure 3](image_url)

**Figure 3.** The relative competitive fitness W of the donor JH-ES-1 and JH-ES-2 with the recipient JH2-2 in vitro competition assays. The values were represented as mean ± SD of 5 independent parallels.
The fosB gene and genetic environment of fosB in different species or genera. fosB genes were indicated by red arrows, and regions of 100% nucleotide sequence identity were shaded in grey.

The second plasmid in ES-1 and ES-2 named pES-erm(B), which has a size of 85.096-kb, and carried erm(B), chloramphenicol resistance gene cat, tetracycline resistance genes tet(L) and tet(M), TA systems, IS1216E and A1IS1297, copper ATPases (CopA and CopB), and copper transport repressor (CopY) (Figure S1).

Further analysis of WGS showed that one copy optrA gene, which conferred resistance to oxazolidinones and phenicols, was in chromosome. Macrolide resistance gene erm(A) and florfenicol resistance gene fexA were flanked on the left of optrA, and the 11.739 kb (tnpB-erm(A)-fexA-optrA-hp) segment has 100% query cover and 99.81% identity with E. faecalis 743142 (accession no. MF443377.1), which located on plasmid. Partial segment containing fexA-optrA in E. faecalis ES1 also emerged in homo E. faecalis plasmid from China and Ireland with accession no. MH018572.1 and MN831417.1, respectively, as well as homo E. faecalis chromosome (accession no. MH225421.1) (Figure S2). Additionally, chromosome sequence also contained aminoglycoside-modify enzyme gene aph(3′)-III, lincosamide and streptogram A resistance gene lsa(A), sulphonamide resistance gene dfrG and macrolide-lincosamide-streptogram B resistance gene erm(B). The emergence of multidrug-resistant genes will render antibiotic treatment ineffective.

4. Discussion

In this study, fosfomycin resistance in clinical Enterococcus spp from health pigs in Jiangsu Province, China, was evaluated. As previously reported, fosfomycin has good activity for the treatment of complicated Enterococcus spp infection [15]. However, in this study, all the FRE strains were multi-resistance, and the fosfomycin resistance rate was higher than that in the previous report [15]. According to our results, strains resistance to seven classes antimicrobial agents accounted for the largest proportion. Those indicated that fosfomycin should not usually be suggested in the treatment of Enterococcus.
spp infection at least in some commercial pig farms, and it is necessary to monitor the antimicrobial resistance rate in FRE. Additionally, our results showed that all the FRE in this study were susceptible to vancomycin and valnemulin, which means that these two drugs could inhibit FRE growth at a low concentration, therefore, they have the potential to be used as specific medicines for infections caused by FRE strains.

The complete sequences of two fosB-carrying E. faecalis was obtained by WGS. The two strains shared the same novel ST964 with 71 SNPs difference. It was noted that clonal dissemination emerged in fosB-carrying E. faecalis strains. Additionally, competitive assay showed that fosB-carrying plasmid brought a fitness cost to the recipient strain. This finding partially explained why fosB is unfrequently detected in E. faecalis. The comparison of pES-fosB and related E. faecalis strains (genebank: AP018546.1) revealed that their plasmid backbones were relatively conserved. The sequence surrounding the fosB gene was diverse among different species. For example, the rep genes were adjacent to fosB in staphylococcal plasmid, while a tnpA gene existed in the downstream of fosB and was included in an IS3-like transposon in E. faecium plasmid, [23]. However, hp genes located up- and downstream of fosB in E. faecalis strains, and we speculated that recombination of the fosB gene may have occurred when it was transferred to E. faecalis. Previous study demonstrated that fosB and vanRS co-located on the same plasmid [22]. And in our study, fosB, erm(B) and aac(6′)-aph(2′) emerged on one single plasmid, this may be the results of cross-use antimicrobial agents. Since the fosB-carrying plasmid pKUB3007-3 hasn’t been reported until now, this is the first report of transferable fosB-carrying plasmids in E. faecalis.

The presence of two copies of erm(B) on pES erm(B) plasmid was detected, and one copy erm(B) located on pES-fosB plasmid. The complicated existence of erm(B) could explain why ES-1 and ES-2 had high MIC values of erythromycin. Additionally, the coexistence of fosB and optrA in one single E. faecalis strain was firstly found, which was consistent with the bacterial drug resistance phenotype. As we know, essential trace elements such as chromium (Cr), zinc (Zn), and copper (Cu) participate in a number of biological processes and are important constituents of several key enzymes being involved in several oxidation-reduction reactions [24,25]. However, it would become harmful if excessive feeding was applied. In this study, cadmium resistance gene cadA, copper transport related protein CopZ/B/Y were incorporated into plasmid pES erm(B) and pES-fosB, which hinted that these genes may be spread to divergent bacteria by horizontal transfer [26]. Additionally, trace elements related genes emerged in transferable plasmid, which may be induced by feed additive in livestock diets. These findings indicated that excessive feeding could be harmful in animal production through inducing the spread of trace elements.

In conclusion, we firstly reported the emergence of plasmid-mediated fosfomycin resistant gene fosB in E. faecalis strains with novel ST964 from pigs, and also studied the antimicrobial resistance and molecular genetic of fosfomycin resistant Enterococcus spp.. The fosB-carrying plasmid could transfer between different strains, which lead to decreased sensitivity to multiple antibiotics in the recipient bacteria. fosB-carrying plasmid brought fitness cost in recipient E. faecalis JH2-2, which partially explained the low detection rate of fosB in E. faecalis. The genetic environments of fosB were diverse in different species, and fosB could coexist with optrA, which has a potential threat to public health. Given that fosfomycin and linezolid are the last-resort antibiotic for treating infections caused by VRE, the co-occurrence of fosB and optrA in one single E. faecalis may seriously compromise the effectiveness of clinical therapy. This finding, taking a “One Health” perspective, provided an importance theory to the increasing fosfomycin resistance. Continuous monitoring will be necessary to prevent further dissemination of these resistant elements in Enterococcus spp. isolates from humans and food producing animals.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/genes12111684/s1, Table S1: primers used in this study; Table S2: Minimum inhibitory concentrations (MIC, μg/mL) of tested antimicrobial agents for the studied bacterial isolates, Figure S1: The genetic contents of plasmid pES erm(B). Circular representation of alignments between reference erm(B) carrying plasmid pL15 (accession no. CP042214.1) and the homologous plasmid.
from E. faecalis ES1. The alignments were generated by Blastn and visualized by BLAST Ring Image Generator, Figure S2: Genetic environment of optrA. Schematic representation and comparison of the genetic environment of the optrA-flanking region from this study and previous reported. Arrows indicate the direction of transcription of each of the genes, and different genes are shown in different colors. Regions of ≥70.0% nucleotide sequence identity are shaded in grey

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