Characterization of Fosfomycin Resistance Gene, *fosB*, in Methicillin-Resistant Staphylococcus aureus Isolates

Zhuyingjie Fu1,2*, Yang Liu1,2*, Chunhui Chen1,2, Yan Guo1,2, Ying Ma1,2, Yang Yang1,2, Fupin Hu1,2, Xiaogang Xu1,2*, Minggui Wang1,2

1 Institute of Antibiotics, Huashan Hospital, Fudan University, Shanghai, China, 2 Key Laboratory of Clinical Pharmacology of Antibiotics, Ministry of Health, Shanghai, China

☯ These authors contributed equally to this work.

* xuxiaogang@fudan.edu.cn

Abstract

To investigate the prevalence, location and genetic environments of fosfomycin-resistance (*fos*) genes in methicillin-resistant *Staphylococcus aureus* (MRSA) clinical strains, 67 fosfomycin-resistant MRSA strains were isolated from the blood and cerebrospinal fluid samples at a teaching hospital in Shanghai. The presence of *fos* genes in these clinical strains was detected by PCR and sequencing. The locations of *fos* genes were determined by Southern blotting and genetic environments were analyzed by primer walking sequencing. Multiple locus sequence typing (MLST) was used to characterize genetic diversity. Conjugation was performed to evaluate the transferability of *fos* genes. Among 67 fosfomycin-resistant MRSA strains, nine high level fosfomycin resistant strains (≥128 μg/ml) were *fosB*-positive. Three new subtypes of *fosB*, designated as *fosB*4, *fosB*5, and *fosB*6, were identified. *fosB*1, *fosB*4 or *fosB*6 genes were located on small plasmids (ca. 2.5 kb) and flanked by an analogous replication gene (*rep*). Differently, the *fosB*5 gene was surrounded by a shorter *rep* gene and two copies of a transposon gene (*tnp*) that shared high identity with the IS257-like transposon. Four MLST types were found among the nine *fosB*-positive strains. Transconjugants with the *fosB* genes were resistant to fosfomycin with MIC 64 or 128 μg/ml. In conclusion, different subtypes and genetic environment of *fosB* genes indicate that gene heterogeneity for fosfomycin resistance in MRSA isolates.

Introduction

Fosfomycin is a bactericidal antibiotic that was first discovered in 1969. By irreversibly interfering with the first committed step of peptidoglycan biosynthesis, fosfomycin can hinder the cell wall synthesis in both Gram-positive and Gram-negative bacteria [1]. Due to its unique mechanism, fosfomycin alone or in combination with other antibiotics is used for the treatment of nosocomial infections due to multidrug-resistant (MDR) Gram-positive and Gram-negative bacteria. [1] But fosfomycin can be inactivated through chemical modification with glutathione, L-cysteine/bacillithiol, phosphate, and H₂O, which can be added to fosfomycin’s epoxide..
ring through the catalyzing of FosA, FosB, FosC, FosX and their subtypes, FosA1-4, FosB1-3, FosC1-2, FosX, FosXcc, respectively [2–7]. Among all plasmid-mediated resistance genes, only the fosB gene has been detected in Gram-positive pathogens [1]. The plasmids harboring fosB sized from 2.4 kb to 4.1 kb that confer resistance to fosfomycin have been found in Staphylococcus spp. [8]. Chromosomal-derived fosB2 has been found in Bacillus anthracis [9] and fosB3 locating on a transferable circular intermediate has been found in Enterococcus faecium [3]. The goal of this study is to characterize fosB gene among 67 fosfomycin-resistant MRSA clinical isolates.

Materials and Methods

Bacterial Strains

Ninety-six MRSA clinical strains isolated from the blood or cerebrospinal fluid were collected from 2004 to 2014 at a teaching hospital [10]. Among them, 67 fosfomycin-resistant MRSA stored frozen at -70°C in L-broth with 40% glycerin for this study. S. aureus strain ATCC25923 (American Type Tissue Culture Collection, Manassas, VA, USA) was used as a recipient in the conjugation assay. S. aureus strain ATCC 29213 was used as a quality control strain in antimicrobial susceptibility testing experiments. E. coli strain V517 was used as a marker in Southern blot.

Antimicrobial Susceptibility Testing

The MIC of fosfomycin against clinical strains and transconjugants was based on the CLSI recommendation to use the agar dilution method [11]. The results were interpreted according to the 2012 EUCAST criteria [12].

PCR Screening

DNA templates were prepared using the Tiangen extraction kit (TIANGEN, Beijing, China) and were screened for the presence of fosA, fosB and fosC genes by primers and PCR conditions as described previously [13]. PCR products were subjected to DNA sequencing for determine subtypes of fos genes.

Genetic Environment Analysis

The plasmid DNA was extracted from fosB positive strains by alkaline lysis using the Plasmid Midi Kit (QIAGEN, Hilden, Germany). Primer walking sequencing was carried out to determine the sequences flanking the fosB genes.

Southern Blot

After gel electrophoresis, plasmid DNA fragments were transferred to a positively charged nylon membrane (Roche, Mannheim, Germany) by a vacuum blotter model 785 (Bio-Rad, Hercules, USA). The fosB PCR product was used as the positive control, while plasmid extracted from Escherichia coli V517 was used as the marker. The membrane was hybridized with fosB probe mixed by fosB1, fosB4, fosB5 and fosB6 probes according to the manufacturer’s instructions for the DIG High Prime DNA Labeling and Detection Starter Kit (Roche, Mannheim, Germany).

Conjugation Assay

Rifampicin-resistant mutants of S. aureus ATCC25923 were generated following overnight incubation in Brain Heart Infusion (BHI) broth containing one-half the MIC of rifampicin, as determined by agar dilution testing. Following overnight incubation at 37°C, bacteria were plated on BHI agar plate containing 10 times the MIC of rifampicin. Each mutant was streaked
onto a BHI agar plate containing 200 μg/ml of rifampicin for 3 generations and a following 3 generation incubation on BHI agar plate containing 400 μg/ml of rifampicin. Then the rifampicin-resistant mutant of S. aureus ATCC25923 was used as recipient, and conjugation assay was carried out as previously described [14]. Putative transconjugants were selected on BHI plates containing fosfomycin (10 μg/ml), Glucose-6-phosphate (25 μg/ml) and additional rifampicin (400 μg/ml). The transconjugants with the same fosB subtype from corresponding donors were confirmed by Multiple Locus Sequence Typing (MLST). The MLST type of real transconjugants were same as the type of S. Aureus ATCC25923, and different from the donor strains.

Multiple Locus Sequence Typing
Isolates were screened using a previously described method [15] to detect the following seven housekeeping genes: arcC, aroE, glp, gmk, pta, tpi, and yqiL. The sequences of the PCR products were compared to the existing sequences available from the MLST website (http://www.mlst.net) for S. aureus [16], and the allelic number was determined for each sequence.

Nucleotide Sequence Accession Numbers
The GenBank/EMBL/DDBJ accession number for the sequences of fosB4, fosB5 and fosB6 genes are KR870311, KT032253 and KR870314, respectively.

Results
Antimicrobial Susceptibility Testing and fos Gene Detection
The MIC of fosfomycin for the 67 MRSA strains ranged from 64 μg/ml to ≥256 μg/ml. Nine isolates with MIC ≥128 μg/ml were positive for fosB (Table 1), and no isolates were positive for fosA or fosC.

Analysis of the fosB Gene and Genetic Environment
The fosB genes found in S. aureus SA0406, SA1280 and SA1278 were different in nucleotide identity and deduced amino acid sequence from fosB1 genes discovered in plasmids from Staphylococcus spp. [17–18], chromosomal-derived fosB2 genes found in Bacillus anthracis [9] and the fosB3 gene from E. faecium [3] (Table 2 and Fig 1). Consequently, the fosB genes from S. aureus SA0406, SA1280 and SA1278 were designated as fosB4, fosB5 and fosB6, respectively. These three fosB genes differing from each other by 2–4 amino acids were all 420 bp in length and encoded a 139-amino acid protein. The strains S. aureus SA0409, S. aureus SA0516, S. aureus SA0849, S. aureus SA1057, and S. aureus SA0406 shared the same fosB4 gene. S. aureus SA0620 carried the same fosB5 gene as S. aureus SA1280. And S. aureus SA1159 carried a fosB1 identical to S. haemolyticus [16]. As it turns out, the fosB4-6 genes shared a high homology (≥97.1%) with fosB1 and fosB3 (Table 2).

Primer walking sequencing determined that the fosB genes except fosB5 were located on 2.5 kb-sized plasmids and flanked by an analogous replication (rep) gene. The fosB5 gene located in a unique genetic environment and was surrounded by a shorter rep gene and two copies of a transposon (tnp) gene that shared high identity with the IS257-like transposon (Fig 1).

Southern hybridization analysis verified that the majority of fosB genes were on a small plasmid of about 2.5 kb (Fig 2). Seven of nine strains produced hybridization signal of fosB on one or two bands, and the other two strains (SA0620, SA1280) produced no hybridization signal.

Conjugation Assay
Conjugation experiment verified that the plasmids harboring fosB1, fosB4 or fosB6 separately could confer fosfomycin resistance with the MIC ascending to 64 μg/ml. (Table 1).
Multilocus Sequence Typing

The 9 fosB gene positive MRSA isolates were categorized into 4 ST types and same fosB subtype could be found in different ST strains (Table 1).

Discussion

Nine of 67 strains in this study harbored fosB gene. Etienne et al. reported a 34% fosB-positive rate in 105 fosfomycin-resistant isolates of Staphylococcus spp. (18 fosB-positive strains in 39 S. aureus isolates) [8], which was a higher percentage of fosB positive isolates than we found. This may due to the diversity of strain origin or the larger number of S. aureus isolates examined in our study. Despite of a low detection rate of fosB, we unexpectively found three new subtypes

Table 1. Characteristics of fosB-positive isolates and transconjugants.

| Strains         | fosB subtypes | Fosfomycin MIC (µg/ml) | Source          | Ward             | Fosfomycin exposure* | Plasmid size (kb) | MLST type |
|-----------------|---------------|------------------------|-----------------|-------------------|----------------------|-------------------|-----------|
| SA0406          | fosB4         | >256                   | Blood           | Dermatology       | Existent             | 2.6               | ST5       |
| SA0409          | fosB4         | >256                   | Blood           | Dermatology       | Existent             | 2.3               | ST5       |
| SA0516          | fosB4         | >256                   | Blood           | Dermatology       | Existent             | 2.3               | ST5       |
| SA0849          | fosB4         | >256                   | Blood           | Hematology        | Nonexistent          | 2.3               | ST5       |
| SA1057          | fosB4         | >256                   | Blood           | Infection Department | Nonexistent         | 2.6               | ST764     |
| SA1159          | fosB1         | >256                   | Blood           | Geriatrics         | Nonexistent          | 2.6               | ST2590    |
| SA1278          | fosB6         | >256                   | Spiral fluid    | Neurosurgery       | Nonexistent          | 2.9               | ST5       |
| SA1280          | fosB5         | >256                   | Spiral fluid    | Neurosurgery       | Existent             | Unclear           | ST5       |
| SA0620          | fosB5         | 128                    | Blood           | Neurology          | Existent             | Unclear           | ST239     |
| Transconjugant0406 | fosB4     | 64                     | NA§             | NA§               | NA§                  | 2.6               | NA§       |
| Transconjugant0409 | fosB4     | 64                     | NA§             | NA§               | NA§                  | 2.3               | NA§       |
| Transconjugant0516 | fosB4     | 64                     | NA§             | NA§               | NA§                  | 2.3               | NA§       |
| Transconjugant0849 | fosB4     | 64                     | NA§             | NA§               | NA§                  | 2.3               | NA§       |
| Transconjugant1057 | fosB4     | 64                     | NA§             | NA§               | NA§                  | 2.6               | NA§       |
| Transconjugant1159 | fosB1     | 64                     | NA§             | NA§               | NA§                  | 2.6               | NA§       |
| Transconjugant1278 | fosB6     | 64                     | NA§             | NA§               | NA§                  | 2.9               | NA§       |
| Transconjugant1280 | fosB5     | 128                    | NA§             | NA§               | NA§                  | Unclear           | NA§       |
| Transconjugant0620 | fosB5     | 128                    | NA§             | NA§               | NA§                  | Unclear           | NA§       |
| S. aureus ATCC25923 | NA§   | 2                      | NA§             | NA§               | NA§                  | NA§               | NA§       |

*Exposure to fosfomycin within one month was defined as “Existent” history prior to the positive blood/CSF culture.

Unclear = uncertainty about the location of fosB.

NA = not applicable

Table 2. Percent identity in nucleotide and deduced amino acid sequence for fosB subtypes.

| New subtype | fosB1 (%) | fosB2 (%) | fosB3 (%) | fosB1 (%) | fosB2 (%) | fosB3 (%) |
|-------------|-----------|-----------|-----------|-----------|-----------|-----------|
| fosB4      | 99.5%     | 62.2%     | 99.3%     | 99.3%     | 59.0%     | 98.6%     |
| fosB5      | 99.8%     | 62.2%     | 99.5%     | 99.3%     | 59.0%     | 98.6%     |
| fosB6      | 99.3%     | 60.5%     | 99.0%     | 97.8%     | 58.3%     | 97.1%     |

Table 2. Percent identity in nucleotide and deduced amino acid sequence for fosB subtypes.
Fig 1. Comparison of the sequences of fosB genes. Diversity of amino acids among fosB gene subtypes (A). Diversity of deduced amino acid sequences of fosB and genetic environment (B). Amino acid
of fosB gene, fosB4, fosB5 and fosB6. The low homology between fosB and other fos genes were responsible for the different bacterial origins (Fig 1). On the other hand, the high homology between fosB3 and other fosB subtypes implied a possible transfer between Enterococcus faecium and Staphylococcus spp. (Fig 1, Table 2).

The results of Southern hybridization analysis and conjugation assay show that the majority of fosB genes were on a small plasmid of about 2.5 kb (Fig 2). Some strains (SA1057, SA1159) with two fosB positive bands may be attributable to variations in the structure of the same plasmid (Fig 2). Two strains (SA0620, SA1280) produced no hybridization signal of fosB. This negative result might be attributed to a low copy number plasmid. By primer walking, we obtained two identical sequences adjacent to the fosB5 genes from SA1280 and SA0620 and conjugation result suggested that they are more likely located on a larger plasmid. Though whether fosB5 gene is located on the plasmid or chromosome is not yet known clearly, we gain genetic environment of fosB5 (Table 1 and Fig 2). Unlike the sequences flanking fosB4 and fosB6 which were similar to those found in Staphylococcus spp. [17–18], the sequences adjacent to fosB5 gene has never been reported (Fig 1B). In addition to rep genes,
there were two copies of the \textit{tnp} gene with 99.4\% nucleotide identity to IS257 found in \textit{S. aureus} \[19\]. The 17-bp sequence GGTCTTGGCAAGTT of the terminal inverted repeat sequence (IR) exists at both ends of two copies of the IS257-like structure. Moreover, these IS257s share high identity in both their nucleotide and deduced amino acid sequences with the IS15 family and ISS1 found in Gram-negative bacteria and \textit{Streptococcus lactis}, respectively \[20–22\]. Plasmids harboring multiple copies of IS257 may provide several sites for the excision or insertion of resistance genes through homologous recombination of an IS257-containing plasmid conferring erythromycin resistance (pOX7-IS) into the IS257s of pJ3356, as observed previously \[19,23\], implying that IS257 is an active mobile genetic element conferring fosfomycin resistance.

Meanwhile, MLST profiles indicated that \textit{fosB} genes were spreading in MRSA clinical strains. The result of conjugation also show that the \textit{fosB} genes can be transferred and confer fosfomycin resistance to \textit{S. aureus} ATCC25923. However, although conjugants became resistant to fosfomycin after transformation with \textit{fosB} genes, their MIC values were only 64 or 128 \(\mu\text{g/ml}\), 2 to 3 times lower than observed in the donor strains. These results imply that other potential mechanisms contribute to fosfomycin resistance in MRSA.

In conclusion, we report three new \textit{fosB} subtype genes that play a role in fosfomycin resistance in MRSA. Despite the diversity of these three \textit{fosB} subtype genes in deduced amino acid and genetic environment, the strains bearing them could confer fosfomycin resistance by the plasmid or maybe through an active mobile genetic element. The different subtypes and genetic environment of \textit{fosB} genes indicate that gene heterogeneity for fosfomycin resistance in MRSA isolates. Due to the complicated resistance and transmission mechanisms in fosfomycin-resistant MRSA, more research is warranted.

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\textbf{Author Contributions}

Conceived and designed the experiments: XX MW. Performed the experiments: ZF YL CC YG. Analyzed the data: YM FH. Contributed reagents/materials/analysis tools: YY. Wrote the paper: ZF CC XX.

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