**fosI** Is a New Integron-Associated Gene Cassette Encoding Reduced Susceptibility to Fosfomycin

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In this work, we demonstrate that the **fosI** gene encodes a predicted small protein with 134 amino acids and determines reduced susceptibility to fosfomycin. It raised the MIC from 0.125 to 6 μg/ml when the pBRA100 plasmid was introduced into *Escherichia coli* TOP10 and to 16 μg/ml when the gene was cloned into the pBC.SK(–) vector and expressed in *E. coli* TOP10.

Fosfomycin acts as a cell wall biosynthesis inhibitor, blocking the first step in peptidoglycan biosynthesis (1). It has been marketed for decades and has been prescribed mainly for the treatment of urinary tract infections. More recently, it has been used in combination therapy for the treatment of infections caused by multidrug-resistant *Enterobacteriaceae* and *Pseudomonas aeruginosa* with variable success rates (2–7). Consequently, the emergence of plasmid-mediated fosfomycin resistance is a concern. Resistance to this antimicrobial can occur through decreased drug uptake (8), modification of the target site (9), or enzymatic deactivation by fosfomycin glutathione S-transferase enzymes (10). These enzymes are encoded by *fos* genes that are usually plasmid located and frequently found as gene cassettes in integrons (11). To date, nine *fos* genes (fosA, fosB, fosC, fosD, fosE, fosG, fosH, fosK, and fosX) and some variants have been described (12, 13).

The sequencing of a *Mycobacterium abscessus* strain F1725 plasmid detected in São Paulo in 2007 revealed that it was almost identical to plasmid pMAB01 (GenBank accession no. CP003376), which has been reported to have been successfully transferred by conjugation from *M. abscessus* to *Escherichia coli* (14). Both plasmids carry the same integron containing the **aac(6’)-Ib** cassette and a second cassette encoding a hypothetical protein with a glyoxalase-like domain, but to date, the contribution of this gene to antimicrobial resistance has not been elucidated. Because the glyoxalase-like domain is known to be present in *Fos* proteins (11), the aim of this study was to characterize the contribution of this new gene to fosfomycin resistance. *M. abscessus* strain F1725 was the original source of the plasmid containing the **fosI** gene. Plasmid extraction from *M. abscessus* was performed with the QIAfilter Plasmid Midi kit (Qiagen), except that the bacterial suspension was incubated in lysis buffer containing lysozyme (10 mg/ml) overnight at 37°C and the final elution was performed with elution buffer previously heated to 50°C. Chemically competent *E. coli* TOP10 cells were transformed by thermal shock, and the transformants were selected on LB agar containing kanamycin (30 mg/liter). Genomic DNA from *M. abscessus* strain F1725 was extracted as previously described (15) and sequenced with the 454 GS Jr platform (Roche) as recommended by the manufacturer.

The plasmid, herein designated pBRA100 (GenBank accession no. CP003505), was assembled de novo as a single contig with the SeqMan NGen3 software (DNASTAR, Madison, WI), with an average depth of 63. It differs from pMAB01 (14) by 2 bp in the oriV region (nucleotides 43903 and 44166 of the sequence with GenBank accession no. CP003376), but the oriV region of pBRA100 (nucleotides 42145 to 42865 of the sequence with GenBank accession no. CP003505) is 100% identical to the corresponding regions from plasmids pertaining to the IncP-1β incompatibility group. Such plasmids have been isolated on different continents, as is the case for plasmid pFP4, isolated in Australia in 1979 (GenBank accession no. AY365053) (16, 17), and plasmid pAKD18, isolated in Norway in 1999 (GenBank accession no. JN106168) (18).

Plasmid pBRA100 is 56,265 bp long and has a mosaic structure with the mating pair formation genes *trbA* to *trbP* separated from the conjugative transfer genes *traC* to *traO* by a class 1 integron designated In781 according to INTEGRALL (19). This class 1 integron displays a PcWTGN-10 variant of the Pc promoter, the usual 5’ and 3’ conserved segments (*qacE1α, sulI*, and *orf5* genes) of class 1 integrons, and two gene cassettes. The second one is **aac(6’)-Ib**, while the first one, here designated **fosI**, is a novel gene cassette. This new **fosI** gene cassette is 462 bp long and has a 60-bp **attC** region that displays the L’-L” and R’-R” regions required for functional **attC** sites. **attC** from In781 has two pairs of imperfect repeats, as each pair differs by a single base pair. The L’ and L” repeats are separated by a 20-bp spacer (Fig. 1A). The **fosI** gene encodes a predicted 134-amino-acid protein (GenBank protein ID no. AFJ38137). Alignment with other Fos proteins highlighted the conservation of key amino acids/motifs, such as the metal binding histidine residues at alignment positions 8 and 75 and the conservation of key amino acids/motifs, such as the metal binding histidine residues at alignment positions 8 and 75 and the conservation of key amino acids/motifs, such as the metal binding histidine residues at alignment positions 8 and 75 and the conservation of key amino acids/motifs, such as the metal binding histidine residues at alignment positions 8 and 75 and the conservation of key amino acids/motifs, such as the metal binding histidine residues at alignment positions 8 and 75 and the conservation of key amino acids/motifs, such as the metal binding histidine residues at alignment positions 8 and 75 and the conservation of key amino acids/motifs, such as the metal binding histidine residues at alignment positions 8 and 75 and the conservation of key amino acids/motifs, such as the metal binding histidine residues at alignment positions 8 and 75 and the conservation of key amino acids/motifs, such as the metal binding histidine residues at alignment positions 8 and 75 and the conservation of key amino acids/motifs, such as the metal binding histidine residues at alignment positions 8 and 75 and the conservation of key amino acids/motifs, such as the metal binding histidine residues at alignment positions 8 and 75 and the
glutamic acid residue at alignment position 124 (Fig. 1B, arrows).

When the amino acid sequence of FosI was compared to the sequences available in the GenBank and RAC databases (20), the highest similarity observed (81%) was that to FosE (GenBank accession no. AY029772).

Primers fosI-EcoRI-16906-26 (5'-AAAAGAATTCCAGTGTGACGGAATCGTTGCT) and fosI-PInt-EcoRI-17804-785 (5'-AAAAGAATTCATGCCTGACGATGCGTGGAG) were designed to amplify an 898-bp fragment including the integron promoter and the fosI gene. After EcoRI digestion, the fragment was cloned into the pBC_SK(H11002) vector (Agilent). The resulting construct was used to transform electrocompetent E. coli TOP10 (Thermo) before selection on LB medium containing chloramphenicol (100 mg/liter) and 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-Gal; 100 mg/liter). Amplification and sequencing with primers M13-20F and M13-R confirmed the absence of mutations in the construct.

Fosfomycin MICs were determined in triplicate with the Etest (bioMérieux) and Muller-Hinton agar (bioMérieux) as recommended by the manufacturer. The MICs for E. coli TOP10 containing the pBC_SK(−) plasmid and for E. coli TOP10 were 16, 6, 0.125, and 0.125 μg/ml, respectively. There was a 128-fold increase when the E. coli strain containing the pBC_SK(−) plasmid was compared with the strain containing the same plasmid with the fosI gene under the control of the In781 integron PcWTGN-10 promoter. Therefore, the new fosI gene seems to confer a significant level of resistance to fosfomycin on E. coli, but the MICs for the transformants were below the fosfomycin resistance breakpoint (64 μg/ml) recommended by the European Committee on Antimicrobial Susceptibility Testing (21).

M. abscessus strain F1725 had a fosfomycin MIC of 1,024 μg/ml. This very high MIC is most likely due to the presence of an aspartic acid residue at position 117 of the MurA protein, as deduced from the sequence of the murA gene of M. abscessus F1725, which was used in this study (GenBank accession no. KT446471). The presence of this substitution in the MurA protein is known to confer high-level resistance to fosfomycin on Mycobacterium (22).

In a recent publication, the in vitro activity of fosfomycin in combination with tobramycin and the mutant prevention concentration for multidrug-resistant P. aeruginosa strains was evaluated (23). The authors showed the occurrence of mutations in fosfomycin resistance determinants in P. aeruginosa.
the permease gene glpT in all of the mutants with high fosfomycin MICs recovered during the study. If we consider that fosI encodes reduced susceptibility to fosfomycin, it is possible that FosI could facilitate mutant selection during fosfomycin exposure, but the prevalence of fosI among fosfomycin-susceptible and fosfomycin-resistant strains of both \textit{P. aeruginosa} and \textit{Enterobacteriaceae} remains to be determined.

In summary, we describe a new integron-associated \textit{fosI} gene cassette encoding reduced susceptibility to fosfomycin.

**Nucleotide sequence accession numbers.** The complete nucleotide sequences of the pBRA100 plasmid and the \textit{murA} gene have been deposited in GenBank under accession numbers CP003505 and KT446471, respectively.

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