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Identification of FosA8, a Plasmid-Encoded Fosfomycin Resistance Determinant from *Escherichia coli*, and Its Origin in *Leclercia adecarboxylata*

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ABSTRACT A plasmid-located fosfomycin resistance gene, *fosA8*, was identified from a CTX-M-15-producing *Escherichia coli* isolate recovered from urine. Identification of this gene was obtained by whole-genome sequencing. It encoded FosA8, which shares 79% and 78% amino acid identity with the most closely related FosA2 and FosA1 enzymes, respectively. The *fosA8* gene was located on a transferable 50-kb plasmid of IncN type encoding high-level resistance to fosfomycin. *In silico* analysis and cloning experiments identified *fosA8* analogues (99% identity) in the genome of *Leclercia decarboxylata*, which is an enterobacterial species with natural resistance to fosfomycin. This finding adds *L. decarboxylata* to the list of enterobacterial species that are a reservoir of *fosA*-like genes which have been captured from the chromosome of a progenitor and are then acquired by *E. coli*.

KEYWORDS fosfomycin, FOS, *Escherichia coli*, *fosA*

Fosfomycin is a phosphonic acid-derived antibiotic with a broad-spectrum bactericidal activity used as a first-line oral agent for uncomplicated urinary tract infections (1). It is receiving renewed interest worldwide as an antibiotic that contributes to the sparing of carbapenems in the treatment of infections due to extended-spectrum β-lactamase (ESBL)-producing isolates (2, 3). These ESBL producers are increasingly reported worldwide, particularly in *Escherichia coli* (4). Therefore, surveillance studies aiming to evaluate the spread and to identify the nature of the fosfomycin resistance determinants are becoming of growing importance for preserving the efficacy of that antibiotic.

Chromosomally encoded fosfomycin resistance mechanisms in *E. coli* include reduced drug uptake due to mutations in the genes encoding GlpT and UhpT transporters or mutations in the fosfomycin target which is the enzyme catalyzing the first step in peptidoglycan biosynthesis, namely, MurA. Reduced drug uptake remains the most frequent fosfomycin resistance mechanism (1, 5). Transferable and plasmid-encoded resistance mechanisms in *E. coli* are the FosA metalloenzymes, responsible for fosfomycin inactivation by catalyzing the conjugation of glutathione to fosfomycin (1, 5). FosA enzymes are Mn^{2+} and K^{+}-dependent glutathione *S*-transferases that have been shown to inactivate fosfomycin in several Gram-negative bacterial species.

Among the seven known FosA variants, four (FosA3, FosA4, FosA5, and FosA6) have been identified as acquired resistance determinants in *E. coli* isolates (6–15). The FosA3 variant is the most widespread fosfomycin resistance determinant in *E. coli*, reported in human and animal isolates from Asia, Europe, and the United States (6, 7, 16–19). The

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype	Reference or source
Strains		
E. coli		
J53	Recipient strain for conjugation experiment; azide resistant	34
TOP10	Recipient strain for cloning experiments	Invitrogen
376	Clinical isolate resistant to extended-spectrum cephalosporins, fluoroquinolones, kanamycin, tobramycin, tetracycline, and fosfomycin	This study
L. adecarboxylata DSMZ5077	Natural fosfomycin-resistant enterobacterial species	This study
Plasmids		
pACYC184	Cloning plasmid; chloramphenicol and tetracycline resistant	New England Biolabs
p376	Natural ca. 50-kb and IncN plasmid of *E. coli* 376	This study
pFosA8	Recombinant plasmid pACYC184 with a 789-bp PCR fragment (*fosA8*)	This study
pFosA^{LA}	Recombinant plasmid pACYC184 with a 789-bp PCR fragment (*fosA*^{LA})	This study

natural reservoir of some plasmid-mediated *fosA* determinants has been identified. The *fosA3*, *fosA5*, *fosA*^{Tn2921}, and *fosA6* genes originate from *Kluyvera georgiana*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, and *Klebsiella pneumoniae*, respectively (9, 10).

Recently, by performing a screening of fosfomycin resistance determinants among a collection of ESBL-producing *E. coli* isolates from Switzerland, we identified a single isolate harboring a plasmid-mediated fosfomycin resistance determinant that did not correspond to any known *fosA* gene (20). Therefore, the purpose of the study was to characterize this fosfomycin resistant determinant and to identify its origin.

RESULTS

Identification of the *fosA8* gene. *E. coli* isolate 376 was resistant to broad-spectrum cephalosporins, fluoroquinolones, kanamycin, tobramycin, and tetracycline (Table 1). It was also resistant to fosfomycin, according to the result of the rapid fosfomycin NP test (21), and had a fosfomycin MIC value of 1,024 μg/ml. This MIC value was reduced by 16-fold when phosphonoformate (PPF) was added, suggesting the presence of a FosA group glutathione-*S*-transferase activity (22). Using whole-cell DNA from *E. coli* 376 as the template, PCR amplifications were negative for the known *fosA* genes (*fosA1* to *fosA7* genes). Whole-genome sequencing (WGS) of *E. coli* 376 was then performed to possibly identify the mechanism that is responsible for fosfomycin resistance. A gene with a significant homology to *fosA*-like genes was identified. This novel *fosA* gene, which was named *fosA8*, encoded the FosA8 enzyme, which was 141 amino acids long and shared 66% to 79% amino acid identity with the FosA1 to FosA7 enzymes, the most closely related being FosA2. A BLAST search revealed that the same *fosA8* gene was present in two *E. coli* strains isolated in the United States (GenBank accession no. CP041527.1 and CP019910.1).

Phylogenetic analysis performed with FosA8 and the other FosA type enzymes revealed that FosA8 was distantly related to the FosA enzymes, representing a distinct cluster of FosA proteins (Fig. 1). FosA8 possessed conserved residues of FosA proteins that are implicated in the dimer formation of the FosA proteins, of Mn^{2+} and K^{+} binding, and to fosfomycin binding (1) (Fig. 2).

Genetic context of the *fosA8* gene. Analysis of the WGS of *E. coli* 376 showed that the *fosA8* gene was located on a plasmid that also carried a kanamycin resistance marker. Mating-out assays showed that this plasmid was self-conjugative, at a frequency that was estimated to be ca. 10^{-5}. This plasmid did not carry the $bla_{CTX\text{-}M\text{-}15}$ ESBL gene that was identified in *E. coli* 376. PCR-based replicon typing and plasmid analysis following extraction by the Kieser method (23) performed with isolate 376 confirmed the WGS data and identified an IncN natural plasmid 50 kb in size. The *fosA8* gene was inserted into the *sprT* gene and was bracketed by two direct repeat sequences (Fig. 3). The *sprT* gene encoded a hypothetical protein of unknown function (Fig. 3). The occurrence of identical direct repeats, TATTA, on each side of the DNA fragment encompassing the *fosA8* gene strongly suggested that the insertion of the *fosA8* gene onto its plasmid support resulted from a transposition event (Fig. 3).

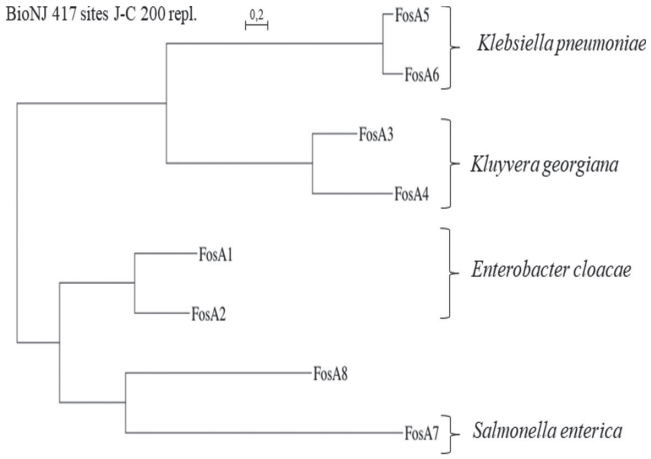

FIG 1 Phylogenetic tree obtained for the FosA proteins identified by distance method using the neighbor-joining algorithm (SeaView version 4 software [33]). Branch lengths are drawn to scale and are proportional to the number of amino acid substitutions with 500 bootstrap replications. The distance along the vertical axis has no significance. The enterobacterial species known as the natural reservoirs of these FosA proteins are shown on the right.

Origin of the *fosA8* gene. *In silico* analysis using the GenBank databases confirmed that *fosA*-like genes are present in the chromosomes of several enterobacterial species, such as those of *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Serratia marcescens,* and *Kluyvera ascorbata* (9). FosA8 showed the highest percentage of amino acid identity (98%, differing in 3 amino acids) with a FosA protein, named FosA^{LA}, which is encoded by a gene identified in the chromosome of the enterobacterial species *Leclercia adecarboxylata* strain USDA-ARS-USMARC-60222 (GenBank accession no. CP013990.1). Accordingly, the close sequences located upstream and downstream of the *fosA8* gene shared significant identity at the nucleotide level (99%) with those of *L. adecarboxylata* USDA-ARS-USMARC-60222 (Fig. 3).

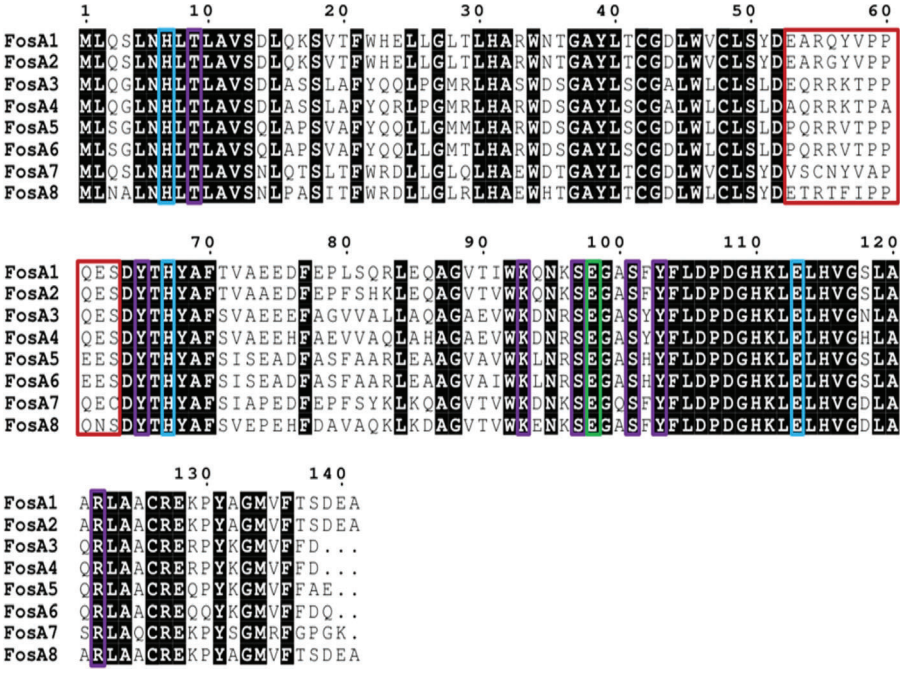

FIG 2 Amino acid alignment of plasmid-mediated FosA proteins. Red, blue, green, and purple boxes bracket amino acids of the dimer interface loop and those implicated in Mn^{2+} coordination, in the K^+ binding loop, and in fosfomycin binding, respectively (11).

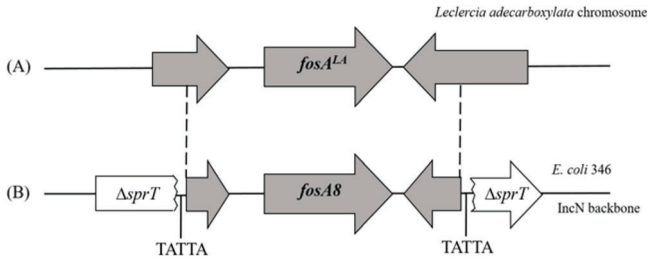

FIG 3 Genetic structures surrounding the *fosA*^{LA} gene on the chromosome of *L. adecarboxylata* (A) and on the *fosA8* gene on plasmid p376 of *E. coli* 376 (B). The grey-shaded sequences indicate sequence identity between plasmid p376 and *L. adecarboxylata* DSMZ5077. TATTA direct repeats bracketing *fosA8* on plasmid p376 are also indicated.

Highly similar *fosA*^{LA}-like genes were identified in six chromosomes of *Leclercia* species available in the genome databases, including two *L. adecarboxylata* isolates (GenBank accession no. CP036199.1 and LR590464.1) and four *Leclercia* species isolates (GenBank accession no. CP026387.1, CP026167.1, CP031104.1 and CP031101.1), sharing from 85% to 97% nucleotide identity with *fosA*^{LA}.

The enterobacterial species *L. adecarboxylata* is rarely identified as a source of human infections and is naturally resistant to fosfomycin (24, 25).

In order to characterize the fosfomycin resistance conferred by Fos^{LA}, the *L. adecarboxylata* strain DSMZ5077 was analyzed. It carried a *fos*^{LA} gene identical to that of *L. adecarboxylata* strain USDA-ARS-USMARC-60222. The fosfomycin resistance phenotype observed in *L. adecarboxylata* DSMZ5077 was antagonized by the addition of phosphonoformate (with the fosfomycin MIC value being decreased by 16-fold), indicating the role of FosA^{LA} for conferring fosfomycin resistance in that species. Both *fosA8* and *fosA*^{LA} genes were cloned and expressed in *E. coli* TOP10. They conferred identical levels of resistance to fosfomycin (fosfomycin MIC, 1,024 mg/liter).

DISCUSSION

We report here the identification of a novel plasmid-mediated and transferable fosfomycin resistance determinant, FosA8, in *E. coli*. This enzyme conferred a high level of resistance to fosfomycin. Its <80% amino acid identity with known FosA proteins indicates the diversity of *fosA* genes that may circulate, remaining undetected when screening surveys based on molecular tools are performed. It is noteworthy that analysis of GenBank databases showed that this gene is present in two *E. coli* isolates recovered in the United States, indicating that it is already present on another continent.

The concomitant identification of the natural reservoir of the *fosA8* gene, *L. decarboxylata*, adds to the growing list of enterobacterial species that naturally possess a *fosA*-like gene and from which those genes are translocated onto plasmids in *E. coli* (9, 10). In contrast to other enterobacterial species that possess these *fosA*-like genes, such as *K. pneumoniae* (9), *fosA8*-like genes are likely expressed at a high level in *L. decarboxylata*, since this species is naturally resistant to fosfomycin (25) and the fosfomycin resistance is inhibited by the addition of phosphonormate. The driving forces at the origin of transfer and the spread of *fosA* genes from their natural reservoir to a plasmid location in an enterobacterial species, *E. coli*, which does not possess a *fosA* gene, remain to be investigated.

This novel FosA determinant is reported from an *E. coli* isolate belonging to sequence type 40 (ST40) that is recognized as an emerging extraintestinal *E. coli* clone (26). Of note, the *fosA8* and $bla_{\text{CTX-M-15}}$ genes were not associated on the same plasmid. This is in contrast to the most commonly identified *fosA* gene, *fosA3*, which is always identified on plasmids carrying either ESBL genes, AmpC-type β-lactamase genes, or carbapenemase genes (16–19).

Nonetheless, identification of the FosA8 determinant from a CTX-M-producing

TABLE 2 Oligonucleotide sequences designed for this study[a]

Primer	Sequence
fosA8 BamHI forward	5′-GATGATGGATCCAGCGTGAAATGGAAGTGTGG-3′
fosA8 SalI forward	5′-GATGATGTCGACTAATACTGGTGATCCACGGC-3′
fosA8 intern forward	5′-AACCATCTGACCCTTGCTGT-3′
fosA8 intern reverse	5′-CGAGAAAATAGAACGACGCC-3′

[a]Restriction sites are underlined; intern, internal to the *fosA* gene.

isolate further highlights that ESBL producers are important reservoirs of transferable fosfomycin resistance determinants. This result may be a source of concern, since fosfomycin is becoming a first-line therapy for treating cystitis due to ESBL producers and the prevalence of ESBL producers is still rising worldwide.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* isolate 376 was isolated from urine in the context of screening 1,225 ESBL-producing *E. coli* isolates from a Swiss collection obtained from 2012 and 2013 (20). The list of strains and plasmids used in the study is shown in Table 1.

Fosfomycin resistance determination. Two techniques were used to assess fosfomycin susceptibility. First, the agar dilution method using cation-adjusted Mueller-Hinton agar (MHA-CA, reference 64884; Bio-Rad, Marnes-la-Coquette, France) supplemented with 25 μg/ml glucose-6-phosphate was used to determine fosfomycin MICs, as recommended by Clinical and Laboratory Standards Institute (CLSI) guidelines (27). The breakpoints of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) were used as a reference (28). *E. coli* isolates with MICs of $>$32 μg/ml were categorized as resistant. Second, the rapid fosfomycin/*E. coli* NP test was performed as described by Nordmann et al. (21). The results of this test were read after 1 h 30 min of incubation at 35 $\pm$ 2°C.

FosA-like role in fosfomycin resistance. The contribution of a potential FosA-like enzyme in fosfomycin resistance was assessed first by using the disk diffusion method as described by Nakamura et al. (22). Briefly, the fosfomycin-resistant *E. coli* 376 and the *L. decarboxylata* DBZ isolate at an ~0.5 McFarland standard were inoculated onto Mueller-Hinton agar plates supplemented with 25 μg/ml of glucose-6-phosphate. Two disks containing 200 μg of fosfomycin, with and without 0.5 mg of the FosA inhibitor sodium phosphonoformate (PPF) (Sigma-Aldrich), were added to the plates, which were incubated at 35 $\pm$ 2°C overnight. Actually, PPF selectively inhibits the FosA-like proteins, which allows differentiation between plasmid-mediated FosA and other mechanisms of resistance to fosfomycin. An increase in the diameter of the growth inhibition zone by $\geq$5 mm in the presence of PPF is interpreted as FosA-related resistance.

Molecular analyses. DNA was extracted from *E. coli* strain 376 with the QIAamp DNA minikit and the QIAcube workstation (Qiagen, Courtaboeuf, France), according to the manufacturer's instructions. PCR amplification (Microsynth, Balgach, Switzerland) was performed to detect any known plasmid-mediated *fosA* genes (*fosA1* to *fosA7*), as previously described (21). PCR conditions were 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 52°C for 30 s and 72°C for 30 s, with a final extension of 72°C for 5 min. Sequences were analyzed with CloneManager Professional (Sci-Ed Software, Denver, CO, USA) (29). Whole-genome DNA of the *fosA8*-positive *E. coli* isolate and its transconjugant was extracted using a Sigma-Aldrich GenElute bacterial genomic DNA kit. Genomic libraries were assessed using a Nextera XT library preparation kit (Illumina Inc., San Diego, CA), and sequencing was performed using an Illumina MiniSeq system with 300-bp paired-end reads and a coverage of 50 times. The generated FastQ data were compiled and analyzed using the CLC Genomic Workbench (version 7.5.1; CLC Bio, Aarhus, Denmark). Reads were *de novo* assembled with automatic bubble and word size, and contigs with a minimum contig length of 800 nucleotides were generated using the mapping mode map reads back to contigs. The resulting contigs were uploaded into the Center for Genomic Epidemiology server (http://www.genomicepidemiology.org/). The plasmid replicon type and multilocus sequence type according to the Warwick typing scheme were determined using the PlasmidFinder (version 1.3) and MLST (version 1.8) programs, respectively (26, 30, 31). Sequence alignments was performed with ESPript 3.0 software, and construction of phylogenetic trees was performed with the SeaView alignment tool (version 4; Prabi, La Doua, Lyon, France) (32).

The *fosA8* gene from *E. coli* 376 and the fos^{LA} gene from *L. adecarboxylata* were cloned into the pACYC184 vector by using primers listed in Table 2 and expressed in *E. coli* TOP10.

Plasmid analysis and conjugation. Plasmid analysis was performed using the Kieser extraction method (23), followed by gel electrophoresis, in order to further confirm the size of the plasmid containing the *fosA8* gene using *E. coli* strain 50192 harboring four plasmids of 154, 66, 48, and 7 kb as plasmid size markers (33). The determination of the plasmid incompatibility group was confirmed by PCR-based replicon typing (PBRT) (30). Conjugation experiments were performed using *E. coli* strain 376 and azide-resistant strain *E. coli* J53 as donor and recipient strains, respectively, as described previously (20). Both donor and recipient strains were cultured in exponential phase and then mixed on solid Luria Broth agar using filters at a 1:10 donor/recipient ratio. After 5 h of incubation, the filters were resuspended in 0.85% NaCl and the bacterial mixture was plated onto agar plates supplemented with fosfomycin (25 μg/ml) and sodium azide (100 μg/ml). The susceptibility of all *E. coli* transconjugants to

fosfomycin was assessed by disk diffusion with azide- and fosfomycin-containing disks and by the PPF test (22).

Accession number(s). The sequence data corresponding to the *fosA8* gene reported in this paper was submitted to the GenBank nucleotide database under accession no. MN150127. In addition, the contigs corresponding to the whole genome of isolate 376 have been deposited as a BioProject under accession no. PRJNA558667.

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