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Short Communication

Prevalence, detection and characterisation of fosfomycin-resistant *Escherichia coli* strains carrying *fosA* genes in Community of Madrid, Spain

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ABSTRACT

Objectives: The aim of this this study was to describe the presence of different variants of the *fosA* gene in fosfomycin-resistant *Escherichia coli* strains in Madrid, Spain.

Methods: *fos* genes were searched for in 55 *E. coli* strains collected from seven representative hospitals located in Madrid. A phenotypic screening test was performed following the disk diffusion method with sodium phosphonoformate added as described by Nakamura et al. Additionally, a molecular study based on PCR was used to confirm the screening results. Positive strains for *fos* genes were further subjected to whole-genome sequencing (WGS).

Results: Phenotypic screening was positive in 9/55 strains (16.4%), although genotypic detection was positive in only 3 (*fosA3, fosA4* and *fosA6*). Thus, the prevalence of *fos* genes in Madrid was 5.5% (3/55). WGS data were not available for the *fosA6*-positive strain. One isolate with *fosA3* (ST69) carried a $bla_{\text{CTX-M-55}}$ gene and seven virulence genes (*air, eilA, iha, iss, lpfA, sat* and *senB*). The *fosA4*-positive isolate (ST4038) carried the virulence genes *iss, lpfA, iroN* and *mchF*. Both *fos* genes were located between two IS*26* mobile elements of a plasmid.

Conclusion: We detected the presence of different variants of plasmid-mediated *fosA* genes in fosfomycin-resistant *E. coli* strains in Madrid, Spain. Despite the few reports in Europe, it would be of interest to monitor the spread of these acquired resistance genes.

1. Introduction

Escherichia coli is the most prevalent pathogen in urinary tract infections (UTIs). First-line empirical treatment for uncomplicated cystitis, mainly caused by *E. coli*, is fosfomycin [1], an old antibiotic that has been proposed as a treatment both in monotherapy or in combination for infections caused by multidrug-resistant bacteria [2].

Fosfomycin is a bactericidal antibiotic that acts by interfering with the initiating reaction in the biosynthesis of peptidoglycan, a compound of the bacterial cell wall [2]. Although infrequent, resistance to fosfomycin in *E. coli* is mainly observed in extended-spectrum β-lactamase (ESBL)-producing isolates [3]. In Europe, the main mechanism of resistance to fosfomycin occurs through chromosomal mutations either in GlpT and UhpT transporters or in its target UDP-*N*-acetylglucosamine enolpyruvyl transferase (MurA) [4]. However, in Asian countries, acquired resistance mediated by plasmids carrying *fos* genes is predominant [5].

The *fos* genes encode glutathione-*S*-transferases that inactivate fosfomycin [2]. Those described in Enterobacterales to date are

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Table 1
Screening results for *fosA*-positive isolates

Strain no.	Fosfomycin MIC (μg/mL)	Fosfomycin halo (mm)	Fosfomycin + PPF halo (mm)	Difference between halos (mm)	Molecular detection	ESBL	MLST
1	>256	7	25	18	*fosA4*	No	ST4038
2	>256	6	23	17	Negative	No	N/A
3	>256	10	25	15	Negative	Yes	N/A
4	>256	10	25	15	Negative	Yes	N/A
5	>256	6	19	13	Negative	No	N/A
6	128	6	21	15	Negative	Yes	N/A
7	>256	10	22	12	Negative	No	N/A
8	>256	6	20	14	*fosA3*	Yes	ST69
9	>256	18	27	9	*fosA6*	No	ST345

MIC, minimum inhibitory concentration; PPF, sodium phosphonoformate; ESBL, extended-spectrum β-lactamase; MLST, multilocus sequence typing: ST, sequence type; N/A, not applicable.

fosA1 to *fosA9* and *fosC2* [6–9], with *fosA3* being the most frequent in *E. coli*. Despite the few cases published in Europe [10–12], its prevalence could be increasing due to plasmid dissemination.

In addition to molecular techniques, *fos* genes can also be detected phenotypically by adding sodium phosphonoformate (PPF) to the disk diffusion assay as described by Nakamura et al. [13]. PPF behaves as a competitive inhibitor with fosfomycin and binds to the active site of FosA [14].

In this study, we determined the prevalence of *fos* genes in fosfomycin-resistant *E. coli* isolates in Madrid, Spain, and characterised the *fos*-positive strains.

2. Material and methods

A total of 55 randomly-chosen strains of fosfomycin-resistant *E. coli* [minimum inhibitory concentration (MIC) > 32 mg/L] isolated from urine samples in 2018 from 55 different patients were studied. The strains were collected from seven representative hospitals located in Madrid Autonomous Community (6 662 000 inhabitants).

The fosfomycin MIC was determined by the agar dilution method. To perform the phenotypic screening test, each strain (turbidity adjusted to a 0.5 McFarland standard) was inoculated onto a Mueller–Hinton agar plate previously supplemented with 25 μg/mL glucose-6-phosphate. Two fosfomycin disks (50 μg) were placed on each plate and 20 μL of PPF (50 mg/mL) was added on one of the disks. After 18 h of incubation at 35°C, the diameters of the growth inhibition zones were measured; positive inhibition was considered when the difference between halos was ≥7 mm. Two *E. coli* strains carrying the *fosA3* gene were used as positive controls.

A molecular study was performed to detect *fos* genes. To this end, different PCRs were designed with specific primers for *fos* genes: *fosA* (F, 5′-ATC TGT GGG TCT GCC TGT CGT-3′; R,5′-ATG CCC GCA TAG GGC TTC T-3′); *fosA3* (F, 5′-CCT GGC ATT TTA TCA GCA GT-3′; R, 5′-CGG TTA TCT TTC CAT ACC TCA G-3′); *fosA4* (F, 5′-CTG GCG TTT TAT CAG CGG TT-3′; R, 5′-CTT CGC TGC GGT TGT CTT T-3′); *fosA5* (F, 5′-TAT TAG CGA AGC CGA TTT TGC T-3′; R, 5′-CCC CTT ATA CGG CTG CTC G-3′); *fosA6* (F, 5′-CGA GCG TGG CGT TTT ATC AG-3′; R, 5′-GGC GAA GCT AGC AAA ATC GG-3′); and *fosC2* (F, 5′-TGG AGG CTA CTT GGA TTT G-3′; R, 5′-AGG CTA CCG CTA TGG ATT T-3′). The PCR conditions were 95°C for 5 min; 30 cycles of 95°C for 45 s, 58°C for 45 s and 71°C for 45 s; and 72°C for 10 min.

In those strains that amplified any of the *fos* genes, sequencing was performed and the sequence type (ST) was defined from the multilocus sequence typing (MLST) technique following the Warwick University scheme (https://enterobase.warwick.ac.uk/species/ecoli/allele_st_search). Additionally, these strains together with two control isolates were subjected to whole-genome sequencing (WGS) using an Illumina NextSeq 500 Sequencer System (Illumina Inc., San Diego, CA, USA). A genomic DNA paired-end library was generated using a Nextera XT DNA Sample Preparation Kit (Illumina Inc). Core genome multilocus sequence typing (cgMLST) with 2528 genes provided by SeqSphere+ 3.5.0 (Ridom GmbH, Münster, Germany) was applied to compare isolates from this study with a collection of 114 *E. coli* isolates of the same STs from different geographic regions available in the NCBI database (selected from 842 complete chromosomes of *E. coli*).

To reconstruct the plasmids carrying the *fosA* genes in each genome, an in-house script was used (PlasmidID; https://github.com/BU-ISCIII/plasmidID) as described previously [15].

Antimicrobial resistance genes were analysed using ResFinder (https://cge.cbs.dtu.dk) with an ID threshold of 98% with the exception of β-lactamase variants, which were determined with 100% identity. Virulence genes were identified using VirulenceFinder (https://cge.cbs.dtu.dk) with an ID threshold of 90%.

3. Results

Phenotypic screening with PPF was positive in 9/55 strains (16.4%). The mean difference in the growth inhibition zone with and without PPF was 14.2 mm (range 9–18 mm). The fosfomycin MIC in eight of the nine strains was >256 μg/mL. *fos* genes were detected in three strains (33.3%). Thus, the prevalence of *fos* genes in the strains studied was 5.5% (3/55). The results are summarised in Table 1.

The difference between inhibition halos with and without inhibitor was null or even negative in the 46 remaining strains. All of them were negative for the tested *fos* genes.

Of the 55 strains studied, 27 (49.1%) were ESBL-producers. The prevalence of *fos* genes in fosfomycin-resistant ESBL-producing strains was 1/27 (3.7%).

The *fosA3*-producing strain belonged to clone ST69, produced an ESBL and was resistant to trimethoprim/sulfamethoxazole (SXT). Strains carrying *fosA4* and *fosA6* genes belonged to ST4038 and ST345, respectively, and were susceptible to all of the antibiotics tested except fosfomycin.

WGS data were not available for the *fosA6*-positive strain, therefore only the *fosA3*- and *fosA4*-positive isolates were analysed. The number of differences between the sequenced isolates in this study was 2.360 alleles (Fig. 1). Additionally, sequenced isolates in this study were not clonally related to other *E. coli* strains of the same STs available in the NCBI database (Fig. 1).

The number of acquired resistance genes in both sequenced isolates was six. The isolate with *fosA3* (ST69) carried a $bla_{\text{CTX-M-55}}$ gene and seven virulence genes (*air, eilA, iha, iss, lpfA, sat* and *senB*), some of them commonly described in uropathogenic *E. coli*. The *fosA3* gene was carried by an IncFII plasmid of ~64 kb, almost identical to plasmid pCREC-591_2 (GenBank accession no. **NZ_CP024823.1**; average identity, >95%; average coverage percent-

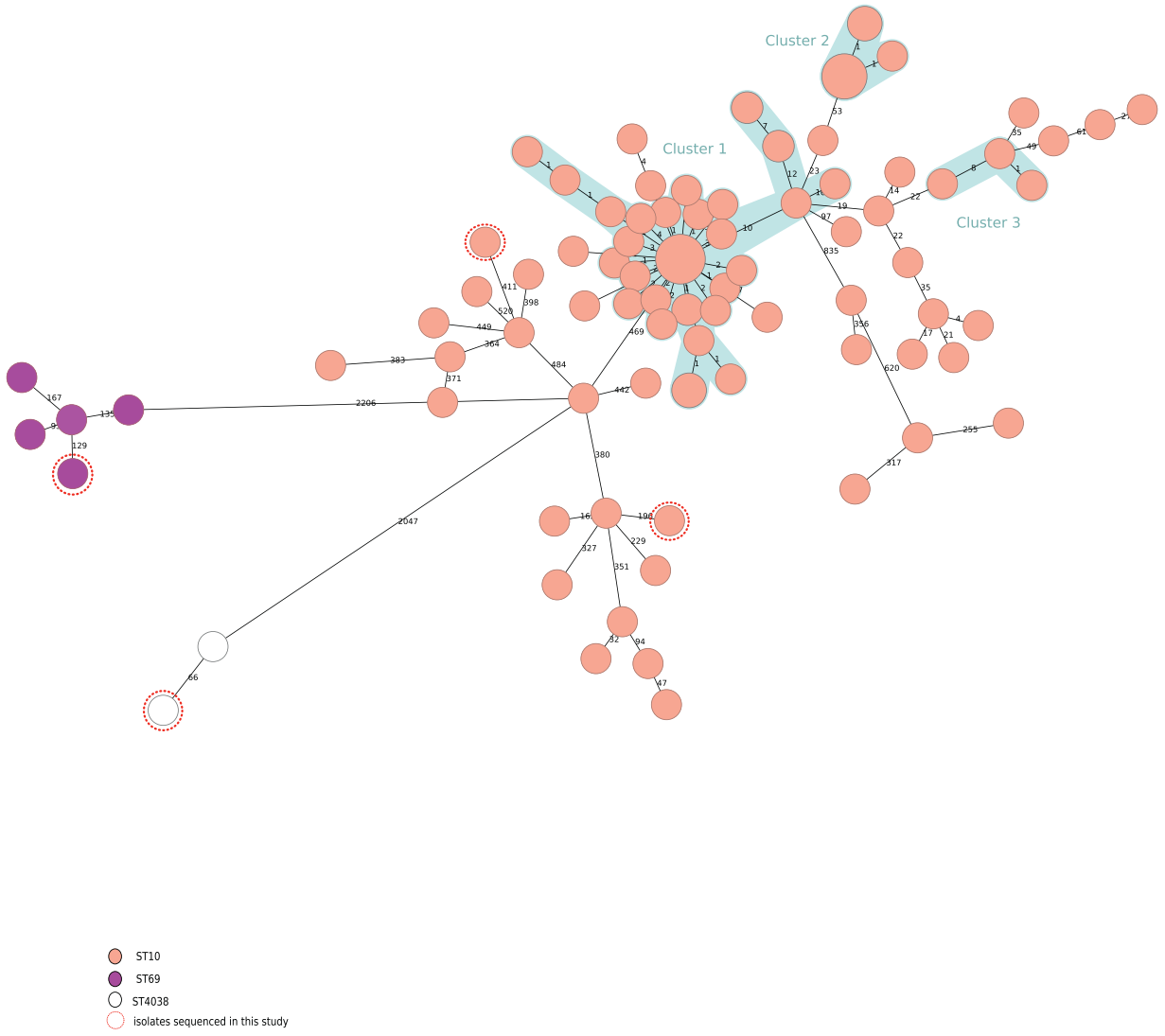

Fig. 1. Minimum spanning tree of two *fosA*-positive *Escherichia coli* isolates from this study compared with two control isolates harbouring the *fosA*3 gene and 114 *E. coli* isolates from the NCBI database belonging to the same MLST sequence types (STs). Distance is based on a 2528-gene schema provided by SeqSphere+ 3.5.0 (Ridom, Münster, Germany). Isolates from this study are represented with red dashed circles coloured according to ST. MLST, multilocus sequencing typing.

age, 99.14%); in this plasmid the mobile element IS*26* associated both with *fosA3* and *bla*$_{\text{CTX-M-55}}$ was detected (Fig. 2).

The *fosA4*-positive isolate (ST4038) carried the virulence genes *iss, lpfA, iroN* and *mchF*. The *fosA4* gene was located in an IncI plasmid of ~118 kb, similar to pCREC-591_1 (GenBank accession no. **NZ_CP024822.1**; average identity, >95%; average coverage percentage, 80.39%) that harbours only this resistance gene located between two IS*26* elements (Fig. 2).

4. Discussion

Since 2004, fosfomycin resistance in ESBL-producing *E. coli* has increased in Spain [16]. Although the prevalence of *fos* genes in Europe is not yet established, our results show a very low rate (5.5%) compared with the high prevalence observed in Asia [5]. However, recent data suggest that *fos* genes could be spreading in Europe [8,10–12].

The *fos* genes are found in plasmids that usually carry other resistance genes (mainly *bla*$_{\text{CTX-M}}$) [6]. However, unlike other published studies [17,18], two of the three *fos*-carrying strains did not present other phenotypes of resistance to any tested antibiotic, both being susceptible to amoxicillin/clavulanic acid, piperacillin/tazobactam, fluoroquinolones, gentamicin, SXT and carbapenems. The third strain was an ESBL-producing strain and showed resistance to SXT. Finally, the presence of an IS*26* composite transposon both in *fosA3* and *fosA4* plasmids is in accordance with the importance of this transposon as the main vehicle for dissemination of *fosA3* genes, as previously described in the literature [17].

Regarding the phylogeny of the strains, no clonality was found among the three isolates. Strains belonging to the ST69 lineage are quite frequently isolated in UTIs both in the community and the hospital environment [19] and they are usually associated with ESBL production, which is in agreement with the profile of our strain. The clonal groups ST345 and ST4038, although much less frequent, are also characterised as causing human infections and by carrying resistance genes such as those encoding β-lactamases of the CTX-M-14 type [20].

In a recent study, Nakamura et al. [13] performed the screening assay with PPF in *E. coli* and observed that two positive screened strains were negative when specific *fosA1–fosA7* PCR was performed; in contrast, a *fosA3*-positive *E. coli* was negative when per-

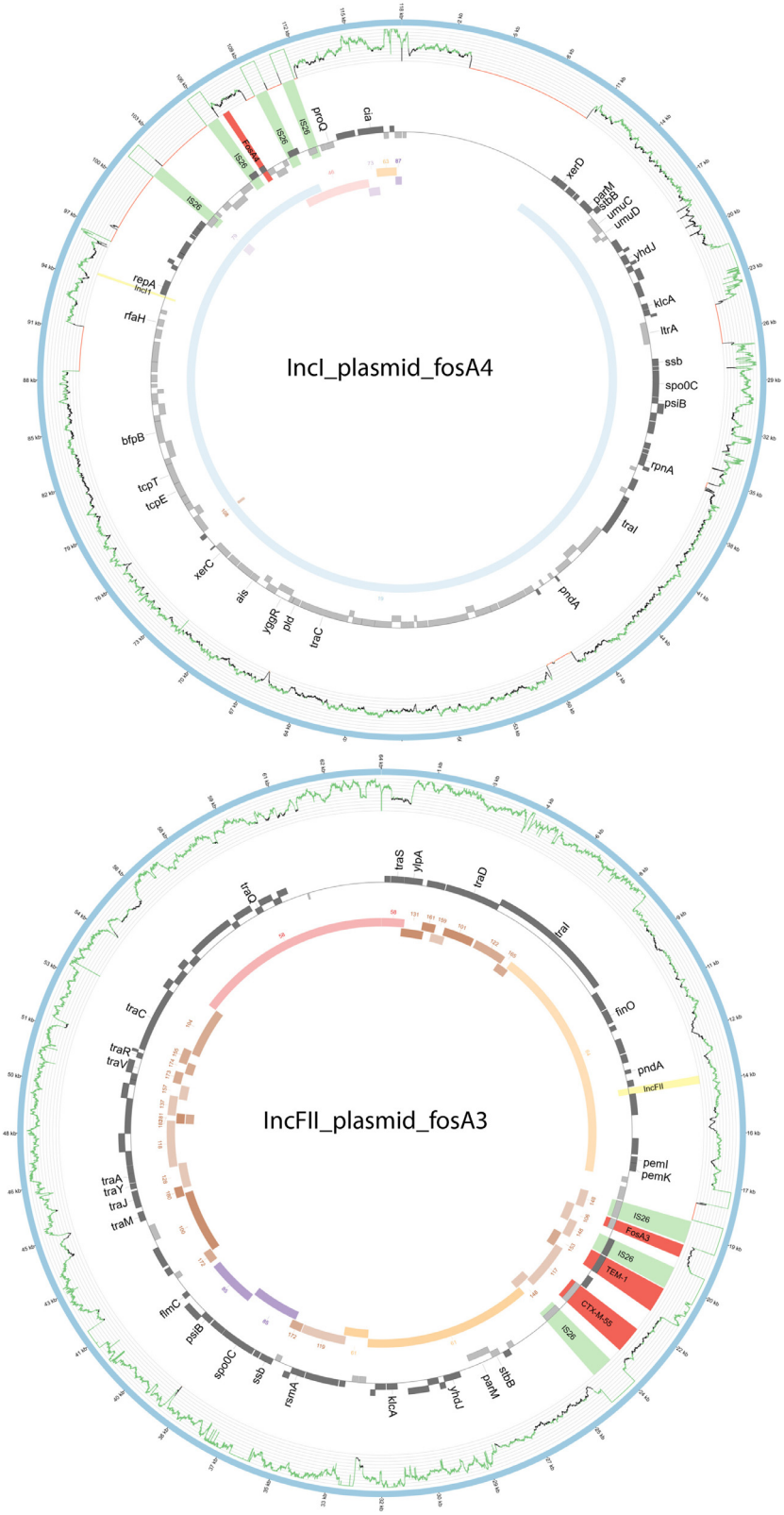

Fig. 2. Overview of plasmids harbouring *fosA* genes detected in *Escherichia coli* in this study. The figure represents the plasmid according to the homology with a highly similar plasmid from the GenBank database (blue outer ring). The graph represents the reads mapped against this reference sequence with a depth of coverage ranging from 0 (red) to 500, with orange indicating values of 1–20 reads and green indicating values >200 reads. Grey boxes represent the coding sequence from automatic annotation, with dark and light colours being used when they were found on the forward or reverse strand, respectively. Coloured stripes represent a more detailed annotation that includes antibiotic resistance genes in red, insertion sequence (IS) elements in blue and *rep* genes in yellow. Homology between the reference plasmid and the assembled contigs is represented in the inner ring, with each contig coloured according to its number.

forming the PPF assay. In our study, most of the strains with positive phenotypic screening did not amplify any *fos* genes tested. This event suggests either the presence of different *fos* genes not sought in this study or another non-*fos* resistance mechanism inhibited by PPF.

In conclusion, in this study we detected the presence of different variants of plasmid-mediated *fosA* genes in fosfomycin-resistant *E. coli* strains in Madrid, Spain. As far as we know, this is the first description of *fosA4* and *fosA6* genes in Spain. Regarding the screening method, in our experience, a poor concordance with the molecular detection of *fos* plasmid genes is observed. Despite this, it seems to be a very sensitive method and thus it could be used as a laboratory screening test given its simplicity, although genotypic confirmation of positive results is necessary.

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