Prevalence of acquired fosfomycin resistance among extended-spectrum β-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* clinical isolates in Korea and IS26-composite transposon surrounding *fosA3*

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**Objectives:** To investigate the prevalence of plasmid-mediated fosfomycin resistance determinants among extended-spectrum β-lactamase (ESBL)-producing *E. coli* and *K. pneumoniae* and their genetic environments.

**Methods:** A total of 347 non-duplicate ESBL-producing *E. coli* (165) and *K. pneumoniae* (182) were collected. The fosfomycin MICs were determined by the agar dilution method according to CLSI guidelines. PCR was used to detect the plasmid-encoded fosfomycin resistance genes (*fosA*, *fosA3*, *fosB* and *fosC*). For isolates harbouring plasmid-encoded fosfomycin resistance genes, sequence types (STs) were determined. The transformation experiment was performed using *E. coli* TOPO10 (Invitrogen, USA) as a recipient strain. With the plasmids from the transformants, plasmid replicon typing was performed and the nucleotide sequences adjacent to *fosA3* were determined.

**Results:** The susceptibility to fosfomycin was 92.9% in *E. coli* and 95.2% in *K. pneumoniae*. Of the 21 isolates non-susceptible to fosfomycin (8 *E. coli* and 13 *K. pneumoniae*), 7 (*E. coli* and 2 *K. pneumoniae*) isolates harboured *fosA3* and all of them co-harboured *blaCTX-M-1* or *blaCTX-M-9* group. The STs of the isolates harbouring *fosA3* were diverse (*E. coli*: ST1, ST1, ST533, ST2 and ST86; *K. pneumoniae*: ST11 and ST101). The plasmid replicon types of transformants co-harbouing *blaCTX-M-1* and *blaCTX-M-9* were IncF and IncN, respectively. By sequence analysis, we found the common feature that the *fosA3* gene, connected to *blaCTX-M* via insertion sequences, was located between two IS26 elements oriented in the opposite direction, composing an IS26-composite transposon.

**Conclusions:** An IS26-composite transposon appears to be the main vehicle for dissemination of *fosA3* in *E. coli* and *K. pneumoniae* of diverse clones.

**Keywords:** IncF, IncN, CTX-M

**Introduction**

The increasing rate of multidrug resistance in bacteria belonging to the family Enterobacteriaceae reduces the number of effective drugs that can be used. Fosfomycin, known for nearly four decades, has a unique mechanism of antimicrobial action that involves the inhibition of UDP-N-acetylg glucosamine enolpyruvyl transferase (MurA), an enzyme that catalyses the first step in bacterial cell wall synthesis.¹ It has a broad spectrum of antimicrobial activity against several Gram-negative and Gram-positive aerobic bacteria.² Although fosfomycin resistance is mostly due to mutation in the chromosomal locus, including *glpT*, plasmid-mediated *fosA3* and *fosC2* were recently reported in CTX-M-producing *Escherichia coli*.³ In Korea, *blaCTX-M* is the
most frequent extended-spectrum β-lactamase (ESBL) in E. coli and the second most frequent in Klebsiella pneumoniae. Therefore we investigated the prevalence of plasmid-mediated fosfomycin resistance determinants among the ESBL-producing E. coli and K. pneumoniae and their genetic environments.

Materials and methods
A total of 347 non-duplicate, ESBL-producing E. coli (165 isolates) and K. pneumoniae (182 isolates) were collected at 25 hospitals in Korea from June to July 2009.

The fosfomycin MICs were determined by the agar dilution method according to the CLSI guideline. The presence of plasmid-encoded fosfomycin resistance genes (fosA, fosB, fosA3 and fosC2) were determined by PCR as described previously, and the PCR products were sequenced on a 3730 DNA analyser (Applied Biosystems, Foster City, CA, USA). For isolates resistant to fosfomycin, but having no plasmid-mediated fosfomycin resistance determinants, we investigated the chromosomal mutation in glpI using primers reported previously for E. coli and K. pneumoniae using KglpT-f, 5′-TAAACCACCCCGCGCATCAA-3′ and KglpT-r, 5′-ATCA TCACCATGAGGCGC-3′. The sequence types (STs) of E. coli and K. pneumoniae isolates harbouring plasmid-encoded fosfomycin resistance genes were determined by analysing the eight housekeeping genes (dnaB, icdA, pabB, polB, putP, trpA, trpB and uidA) and seven housekeeping genes (gaaP, infB, mdh, pgi, phoE, rpoB and tonB), respectively, and they were compared with the multilocus sequence typing (MLST) databases available at http://www.pasteur.fr/recherche/genopole/PF8/mlst/EColi.html and http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html.

The transferability of resistance was studied by transformation experiments using E. coli TOP10 (Invitrogen, USA) as a recipient strain. The transformants were selected on LB agar plates supplemented with fosfomycin (40 mg/L) and glucose-6-phosphate (25 mg/L). The conjugation experiments were also performed with azide-resistant E. coli JS3 as a recipient strain by the filter mating method. Transconjugants were selected on LB agar plates supplemented with fosfomycin (40 mg/L) and glucose-6-phosphate (25 mg/L). The conjugation experiments were also performed as described by Carattoli et al. Plasmid profiles were analysed by agarose gel electrophoresis of plasmid DNAs digested with EcoRV and PvuII (New England Biolabs, Beverly, MA, USA) for IncF and IncN, respectively, to demonstrate the relatedness of plasmids. The plasmid multilocus sequence types (pMLSTs) of IncN- and IncF-type plasmids were sequenced as described previously, and the results were compared with the database available at http://pubmlst.org/plasmid/. For plasmids for which pMLSTs were determined, we investigated the genetic environment of the fosA3 gene by primer walking with a set of PCR primers using an ABI 3730 sequencer (Applied Biosystems) and were compared with the GenBank DNA sequence database by using the genomic BLASTN program (available at http://www.ncbi.nlm.nih.gov/BLAST). Antimicrobial susceptibility (to ampicillin, cefalotin, cefuroxime, cefotaxime, cefazidime, cefepime, piperacillin, piperacillin/tazobactam, cefoxitin, imipenem, meropenem, gentamicin, amikacin, trimethoprim/sulfamethoxazole and ciprofloxacin) was determined by disc diffusion for isolates having plasmid-mediated fosfomycin resistance determinants and their transformants.

Table 1. Characteristics of the seven isolates harbouring fosA3 and their transformants

<table>
<thead>
<tr>
<th>Strain Name</th>
<th>Plasmid profile (pMLST)</th>
<th>Replicon type (parental)</th>
<th>CTX-M type</th>
<th>FOS MIC (mg/L)</th>
<th>CTX MIC (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>IncF</td>
<td>FIA, FII</td>
<td>CTX-M-14</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>E. coli</td>
<td>IncN</td>
<td>B, FIA, FII</td>
<td>CTX-M-14</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>E. coli</td>
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<td>E. coli</td>
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<td>CTX-M-14</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>K. pneumonia</td>
<td>IncN</td>
<td>B, FIA, FII</td>
<td>CTX-M-14</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
</tbody>
</table>

There were no detectable transformation sites in the IncN plasmid.
The nucleotide sequence accession numbers of the genetic environments of fosA3 from ECO021TF, ECO096TF and ECO141TF have been deposited in the GenBank database under the numbers JQ343849, JQ343850 and JQ343851, respectively.

Results and discussion

The susceptibility to fosfomycin was 92.9% in E. coli and 95.2% in K. pneumoniae. Of the 21 isolates non-susceptible to fosfomycin (8 E. coli and 13 K. pneumoniae), 7 (5 E. coli and 2 K. pneumoniae) isolates harboured fosA3 and all of them co-harboured ESBLs from the blaCTX-M-1 group or blaCTX-M-9 group. None harboured other plasmid-mediated fosfomycin resistance determinants. By sequencing analysis of the glpT in three E. coli isolates (ECO150, ECO215 and ECO243) resistant to fosfomycin, ECO150 lacked glpT gene, ECO215 was found to harbour two amino acid substitutions (Leu297Phe and Glu448Lys) and ECO243 had one amino acid substitution (Glu448Lys). However, because this study is focused on the acquired fosfomycin resistance mechanism, we did not elucidate if these mutations played a role in developing fosfomycin resistance.

Figure 1. Schematic representation of the fosA3 environment. (a) Genetic environment of the IncN-type plasmid of ECO21TF. (b) Genetic environment of the IncFII:2-type plasmid of ECO96TF. (c) Genetic environment of the IncFII:33-type plasmid of ECO141TF. Arrows indicate open reading frames, grey arrows indicate transposase genes and filled bars indicate inverted repeats of IS26.
fosfomycin-resistant isolates was different between the two species: it was high (62.5%) for *E. coli*, but low (15.4%) for *K. pneumoniae*. Considering that all the isolates harbouring *fosA3* co-harboured *blaCTX-M*, this difference might be derived from the fact that the dominant type of ESBL in *E. coli* and *K. pneumoniae* in Korea is *blaCTX-M* and *blaOXY*, respectively.14,15

The clones of the isolates harbouring *fosA3*, determined by MLST, were diverse: the STs of the five *E. coli* isolates were ST1, ST11, ST533, ST2 and ST86 and those of the two *K. pneumoniae* isolates were ST11 and ST101. Moreover, in the two *E. coli* belonging to the same ST, the plasmid Inc types were different.

Of the seven isolates harbouring *fosA3*, it was successfully transferred both by transformation and conjugation in six isolates. In one isolate (ECO110), where *fosA3* was not transferred by either transformation or conjugation, the location of *fosA3* was determined to be on the plasmid by S1 nuclease digestion and hybridization with *fosA3* and the 16S rRNA gene. *blaCTX-M* was always co-transferred to the recipient *E. coli* strain, and the replicon types of the transfor-mants co-harbouring the *blaCTX-M* group (*blaCTX-M* or *blaCTX-M*; 53) and *blaCTX-M* group (*blaCTX-M*-14) were IncFII and IncN, respectively (Table 1).

*blaCTX-M*-14 is one of the most dominant plasmid-borne ESBLs in *E. coli* and it shows a worldwide distribution,16 but its presence on an IncN-type plasmid has not been reported. Considering that the IncN-type plasmids are involved in the transmission of various resistance determinants (VIM-1, KPC-2, CTX-M-1 and NDM-1),17 this is a worrisome phenomenon. By plasmid profile analysis, the two FII-type plasmids from *E. coli* and the two IncN-type plasmids from *K. pneumoniae* showed highly similar patterns (Table 1).

As neither IS*ecp1* nor IS*cri1*, the mobile elements for the co-transferred *blaCTX-M* were not detected, we determined the sequences of DNA adjacent to *fosA3*. We found the common feature that the *fosA3* gene, connected to *blaCTX-M* via insertion sequences, was located between two IS26 elements oriented in the opposite direction, composing an IS26-composite transposon. In IncFII-type plasmids, there was another IS26 connecting *blaTEM* (Figure 1).

In the upstream structure of *fosA3* of an IncN-type plasmid, the genetic support of *blaCTX-M*-14 consisted of an upstream truncated IS*ecp1* element and a downstream truncated IS903. Except for the truncation in IS*ecp1* in ECO21TF in this study, this structure is similar to the structure found in IncFII and in other plasmids linked to the spread of CTX-M-14 in China, the UK and Spain.18 A similar structure was also found in Korean isolates of *E. coli* harbouring *blaCTX-M*-14 on an IncF-type plasmid.19

In IncFII-type plasmids, the structure consisted of IS26, truncated IS*ecp1*, *blaCTX-M* and orf*77* upstream of *fosA3*. This structure containing truncated IS*ecp1* instead of an intact copy of IS*ecp1* has been detected in IncN-type and IncI-type plasmids carrying *blaCTX-M*-1 group from German *E. coli* isolates.20 Downstream of *fosA3* there was also a common feature. There was a sequence showing 78% identity to part of the chromosomal nucleotide sequence of *K. pneumoniae* 342 (CP000964). This sequence similarity was also reported in *E. coli* co-harbouring *fosA3* and *blaCTX-M* in Japan.3 As Wachino et al.3 have indicated, considering the fact that the homology region in the chromosome of *K. pneumoniae* is close to the *fosA* gene, our finding supports their speculation that *fosA3* might have originated from *fosA* of *K. pneumoniae*.

We also determined the full susceptibility profiles to a panel of antibiotics in order to obtain an indication of resistance determinants, which may also be encoded on the *fosA3*-encoding plasmids. Resistance to ampicillin, cefalotin, cefuroxime, cefotaxime and piperacillin was found in all six parental strains and their transfor-mants, but resistance to trimethoprim/sulfamethoxazole, ciprofloxacin and gentamicin, which was observed in three, four and two of the six parental strains, respectively, was not observed in transfor-mants. In conclusion, the fosfomycin resistance rate in ESBL-producing *E. coli* and *K. pneumoniae* was low (4.2% and 5.5%, respectively), but up to 62.5% and 15.4% of the fosfomycin-resistant *E. coli* and *K. pneumoniae* isolates, respectively, harboured plasmid-mediated *fosA3*. The *fosA3* genes were always co-harboured with *blaCTX-M* on an IS26-composite transposon in IncN- and IncFII-type plasmids, and were distributed among various clones of *E. coli* and *K. pneumoniae*. Taking into account the emerging importance of IS26 in the spread of *blaESBL* genes, and the wide dissemination of IncN- and IncF-type plasmids, fosfomycin should be used cautiously.

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Transparency declarations

None to declare.

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