Published in "Antimicrobial Agents and Chemotherapy doi: 10.1128/AAC.00150-20, 2020" which should be cited to refer to this work.

IS*Ecp1*-Mediated Transposition Leads to Fosfomycin and Broad-Spectrum Cephalosporin Resistance in *Klebsiella pneumoniae*

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ABSTRACT A fosfomycin-resistant and carbapenemase (OXA-48)-producing *Klebsiella pneumoniae* isolate was recovered, and whole-genome sequencing revealed IS*Ecp1*-$bla_{CTX\text{-}M\text{-}14b}$ tandemly inserted upstream of the chromosomally encoded *lysR-fosA* locus. Quantitative evaluation of the expression of *lysR* and *fosA* genes showed that this insertion brought a strong hybrid promoter leading to overexpression of the *fosA* gene, resulting in fosfomycin resistance. This work showed the concomitant acquisition of resistance to broad-spectrum cephalosporins and fosfomycin due to a single genetic event.

KEYWORDS fosfomycin, FOS, IS*Ecp1*, FosA, *Klebsiella pneumoniae*

Acquired resistance to fosfomycin in *Enterobacterales* isolates is often related to the production of Fos-type glutathione *S*-transferases encoded by *fos* genes, among which *fosA*-like genes are the most common and *fosL1* gene was the latest identified (1, 2). It was recently shown that the natural reservoir of *fosA5* and *fosA6* genes is *Klebsiella pneumoniae* (3), whereas that of the latest *fosA8* gene is *Leclercia adecarboxylata* (4). In this species, the *fosA* gene is chromosomally located and is overlapping the end of a putative *lysR* regulator gene (Fig. 1). Scarce information about the role of this regulator in the expression of *fosA* is available. Despite the presence of the *fosA* gene in their chromosome, wild-type *K. pneumoniae* isolates remain susceptible to fosfomycin (<16 μg/ml), suggesting that the chromosomal *fosA* gene is poorly (or not) expressed. In this study, we recovered a carbapenemase-producing *K. pneumoniae* isolate presenting high-level resistance to fosfomycin and submitted it to analysis of its resistance pattern.

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Isolate S54 was recovered from a urine sample of a patient from Switzerland in 2015. It was resistant to all β-lactams, including ceftazidime (MIC, 8 μg/ml), cefepime (MIC, 32 μg/ml), carbapenems (MICs, 128, 8, and 32 μg/ml for ertapenem, imipenem, and meropenem, respectively), chloramphenicol (MIC, >256 μg/ml), ciprofloxacin (MIC, >256 μg/ml), kanamycin (MIC, >256 μg/ml), tobramycin (MIC, 32 μg/ml), and fosfomycin. Isolate S54 also presented reduced susceptibility to amikacin (MIC, 16 μg/ml) and gentamicin (MIC, 1 μg/ml). The isolate remained susceptible only to colistin (MIC, 0.25 μg/ml) and tigecycline (MIC, 0.25 μg/ml).

Fosfomycin resistance of isolate S54 was confirmed by the agar dilution method following CLSI recommendations (5), with an MIC of >1,024 μg/ml. Addition of phosphonoformate in the medium (5 mM) showed a decreased MIC (128 μg/ml), suggesting that the mechanism leading to fosfomycin resistance in that isolate was likely related

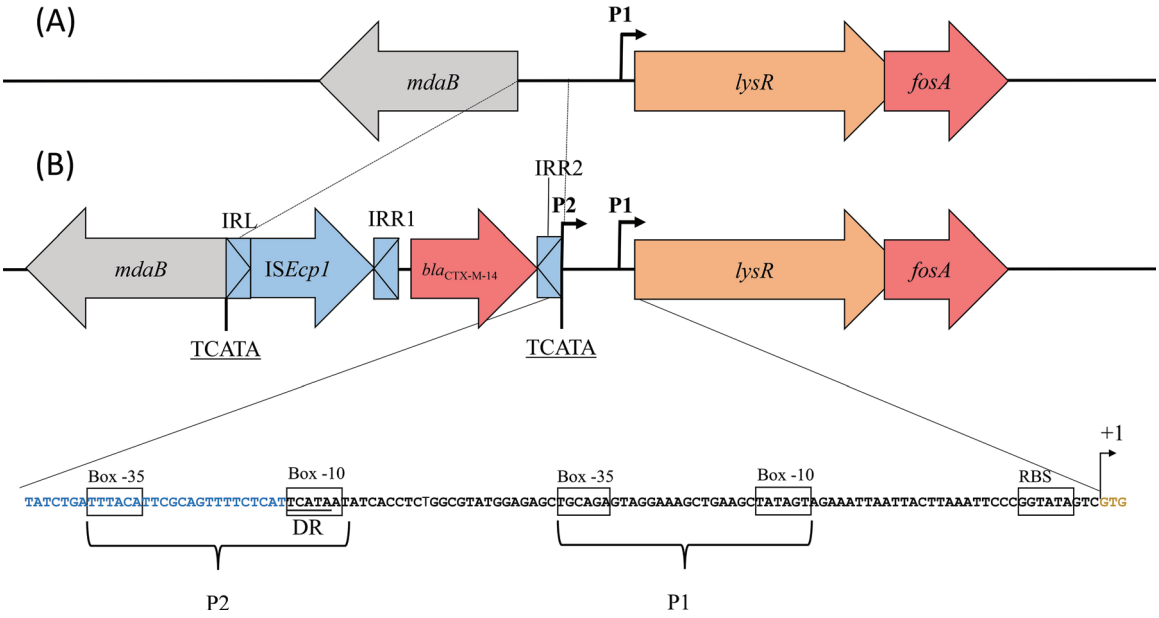

FIG 1 Schematic representation of insertion of the IS*Ecp1*-$bla_{CTX\text{-}M\text{-}14}$ tandem into the *K. pneumoniae* S54 chromosome leading to the formation of a hybrid promoter. (A) Original sequence found in a wild-type *K. pneumoniae* genome. (B) Sequence identified in isolate 54 once the IS*Ecp1*-$bla_{CTX\text{-}M\text{-}14}$ tandem has been acquired by transposition. Arrows show promoters that are described in detail at the bottom. The *lysR* gene encodes a putative LysR regulator; the *fosA* gene encodes the FosA glutathione *S*-transferase; the *mdaB* gene encodes an NAD(P)H-dependent oxidoreductase. Promoter −35 and −10 sequences are boxed. IRL, inverted long repeat; DR, direct repeat; RBS, ribosome binding site; +1, start codon of the *lysR* gene.

to the production of a glutathione transferase (6). PCR amplifications targeting the common plasmid-mediated fosfomycin resistance genes (*fosA1* through *fosA9* and *fosC2*) remained negative except for the *fosA5*- and *fos6*-like genes, being intrinsic to *K. pneumoniae* isolates as mentioned above (3).

Whole-genome sequencing was therefore performed with Illumina technology (Illumina, Inc., San Diego, CA). The genomic DNA was extracted using the Sigma-Aldrich GenElute bacterial genomic DNA kit. Genomic libraries were assessed using the NexteraXT library preparation kit (Illumina, Inc.), and sequencing was performed using the MiniSeq system with 150-bp paired-end reads and 50× coverage. Generated FastQ files were compiled and analyzed using the CLC genomic workbench 7.5.1 (CLC bio, Aarthus, Denmark). The resulting contigs were uploaded to the Center for Genomic Epidemiology server (http://www.genomicepidemiology.org/). Multilocus sequence type and resistome were determined using MLST 2.0 and ResFinder 3.2, respectively (7, 8).

Using those data, the sequence type (ST) of isolate S54 was determined, corresponding to ST101. It carried the $bla_{OXA\text{-}48}$ carbapenemase gene, along with the $bla_{CTX\text{-}M\text{-}14b}$ extended-spectrum β-lactamase (ESBL) gene, $bla_{OXA\text{-}9}$ class D narrow-spectrum β-lactamase gene, and $bla_{TEM\text{-}1D}$ broad-spectrum β-lactamase genes. Additionally, it harbored the *dfrA14*, *sul2*, *aadA1*, and *aac(6′)-Ib-cr* genes, conferring resistance to trimethoprim, sulfonamides, and aminoglycosides, respectively, together with reduced susceptibility to ciprofloxacin. No putative chloramphenicol resistance gene was identified, despite the observed resistance phenotype, suggesting that upregulation of efflux systems might be involved (9).

Detailed *in silico* analysis showed that the $bla_{CTX\text{-}M\text{-}14}$ gene was associated with insertion sequence IS*Ecp1* located upstream of it. IS*Ecp1* is a member of the IS*1380* family known to mobilize adjacent DNA sequences by a so-called one-ended transposition mechanism (10). Furthermore, IS*Ecp1* has been shown to provide a promoter sequence to different antibiotic resistance genes located at its right-hand extremity, such as $bla_{CTX\text{-}M}$ genes, which is the case here with $bla_{CTX\text{-}M\text{-}14}$ (11). Note that the $bla_{CTX\text{-}M\text{-}14}$ ESBL gene was previously reported in animal, environmental, and human isolates from Switzerland (12). In addition, a similar association between IS*Ecp1* and a

bla$_{CTX-M-14}$ gene has already been observed in another *K. pneumoniae* isolate from Switzerland, but it belonged to another ST (ST23) for which the fosfomycin susceptibility status remains unknown (13). The tandem formed by IS*Ecp1* and *bla*$_{CTX-M-14b}$ was located onto the chromosome of isolate S54, where it had inserted upstream of the *lysR-fosA* tandem, at position −2 from the start codon of the proximate *mdaB* gene (Fig. 1).

Given the long distance separating IS*Ecp1* from the *lysR-fosA* tandem, it was unlikely that the promoter sequence provided by IS*Ecp1* could play a significant role in *fosA* expression. Therefore, *in silico* promoter identification was performed using BPROM software (http://www.softberry.com/berry.phtml?topic=bprom) (14), identifying two putative promoters named P1 and P2. Promoter P1 was located upstream from the *lysR-fosA* tandem and was therefore hypothesized to be the intrinsic promoter of these genes. Promoter P2 was identified as a putative hybrid promoter constituted by a −35 box located at the end of the IS*Ecp1-bla*$_{CTX-M-14b}$ tandem combined with a −10 box intrinsic to the *K. pneumoniae* sequence. This −10 box actually corresponded to the entire 5-bp direct repeat flanking the IS*Ecp1-bla*$_{CTX-M-14b}$ tandem, together with an additional nucleotide (Fig. 1). The presence of this putative hybrid promoter P2 was therefore hypothesized to be responsible for overexpression of the *fosA* gene and the source of acquired resistance to fosfomycin in isolate S54.

To confirm the impact of both promoters, two plasmid constructs were generated in the pBAD$_b$ vector carrying an L-arabinose-inducible promoter and expressed in *Escherichia coli* (15). These two recombinant plasmids, namely, pBAD-P1 and pBAD-P2, respectively, encompassed (i) the *lysR-fosA* tandem together with promoter P1 and (ii) the same tandem with both P1 and P2 promoters. Both plasmids were transformed into *E. coli* strain TOP10 (ThermoFisher Scientific), selection being performed with ampicillin 50 μg/ml (resistance marker of plasmid pBAD). Of note, *E. coli* is a bacterial species that does not possess any naturally occurring *fosA* gene. The recombinant *E. coli* strains carrying the pBAD-P1 and pBAD-P2 plasmids showed fosfomycin MICs of 128 and 2,048 μg/ml, respectively, whereas that of *E. coli* TOP10 was <4 μg/ml. This confirmed the significant role of P2 as an efficient promoter of *fosA* expression.

Experiments were performed to evaluate the expression of *lysR* and *fosA* genes in the two recombinant strains. Briefly, total RNAs of the two recombinant strains were extracted using the Quick-RNA MiniPrep kit (Zymo Research, CA). Then a total of 200 ng of each RNA sample was reverse transcribed using the qScript cDNA SuperMix (Quantabio, Beverly, MA) according to the recommendation of the manufacturer. Quantitative real-time PCR was performed using RotorGene Q (Qiagen, Hilden, Germany). Reactions were set up in a total volume of 25 μl with PerfeCTa SYBR green FastMix (Quantabio). The selected genes in this assay were 16S rRNA (internal control), the putative regulator gene *lysR*, and *fosA*. Three independent replicates were performed, with three different RNA extractions, cDNA amplifications, and measurements.

Results showed that the recombinant strain carrying both P1 and P2 promoters had an overexpression of *lysR* and *fosA* genes of 380- and 340-fold, respectively, in *E. coli* isolates compared with those measured from the recombinant strain harboring promoter P1 only.

Altogether, these results showed that P2 constitutes an additional promoter responsible for overexpression of the *fosA* gene leading to the observed fosfomycin resistance phenotype in isolate S54. It is well known that IS elements may provide promoter sequences to resistance genes, such as for *bla*$_{TEM-6}$ or *bla*$_{SHV-2a}$ genes (16, 17). However, the identified structure here was singular. First, the promoter was chimeric with a −35 box belonging to the sequence bracketing the IS*Ecp1-bla*$_{CTX-M-14b}$ tandem (with this sequence actually corresponding to the one recognized by IS*Ecp1* as an imperfect right inverted repeat) and a −10 box sequence being the target-site duplication generated on transposition by IS*Ecp1*. Second, insertion of the IS*Ecp1-bla*$_{CTX-M-14b}$ tandem led to overexpression of another resistance gene, namely, *fosA*, providing a coresistance phenotype. Hence, coacquisition of fosfomycin and broad-spectrum cephalosporin occurred through a single genetic event. A similar phenomenon has been reported

with the insertion of an IS*Ecp1*-$bla_{CTX\text{-}M\text{-}15}$ tandem into the chromosomal *mgrB* gene of a *K. pneumoniae* isolate, leading to coresistance to broad-spectrum cephalosporins and colistin. However, in the latter case, coresistance related to IS*Ecp1* insertion was associated with expression of a resistance gene ($bla_{CTX\text{-}M\text{-}15}$) and truncation of a regulatory gene (*mgrB*) involved in the lipopolysaccharide biosynthesis pathway (18).

Finally, our finding further highlights how the genetic plasticity involving an IS element may play a pivotal role in acquired resistance to antibiotics by creation of mosaic structures (19). In the present report, a single IS-mediated event compromised the efficacy of two important classes of antibiotics, i.e., β-lactams and fosfomycin, in a significant nosocomial pathogen, i.e., *K. pneumoniae*.

Data availability. The whole genome of isolate S54 has been deposited in GenBank under accession number JAAIKF000000000.

ACKNOWLEDGMENTS

This work was funded by the University of Fribourg, the Swiss National Science Foundation (project FNS-310030_188801), and the Laboratoire Européen Associé INSERM Emerging Antibiotic Resistance in Gram-Negative Bacteria.

L.P. and P.N. designed the study and provided the material. N.K., L.M., and S.M. performed the experiments. N.K., L.P., and P.N. wrote the manuscript.

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