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Characterization of FosL1, a Plasmid-Encoded Fosfomycin Resistance Protein Identified in *Escherichia coli*

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ABSTRACT Fosfomycin is gaining renewed interest for treating urinary tract infections. Monitoring fosfomycin resistance is therefore important in order to detect the emergence of novel resistance mechanisms. Here, we used the Rapid Fosfomycin NP test to screen a collection of extended-spectrum-β-lactamase-producing *Escherichia coli* isolates from Switzerland and found a fosfomycin-resistant isolate in which a novel plasmid-mediated fosfomycin resistance gene, named *fosL1*, was identified. The FosL1 protein is a putative glutathione *S*-transferase enzyme conferring high-level resistance to fosfomycin and sharing between 57% to 63% amino acid identity with other FosA-like family members. Genetic analyses showed that the *fosL1* gene was embedded in a mobile insertion cassette and had likely been acquired by transposition through a Tn*7*-related mechanism. *In silico* analysis over GenBank databases identified the FosL1-encoding gene in addition to another variant (*fosL1* and *fosL2*, respectively) in two *Salmonella enterica* isolates from the United States. Our study further highlights the necessity of monitoring fosfomycin resistance in *Enterobacteriaceae* to identify the emergence of novel mechanisms of resistance.

KEYWORDS fosfomycin, plasmid, *Escherichia coli*, FosL1

Fosfomycin is a broad-spectrum phosphonic acid-derived antibiotic that is naturally produced by *Streptomyces fradiae* (1). Fosfomycin acts by inhibiting the UDP-*N*-acetylglucosamine-enolpyruvyl transferase (MurA enzyme) that is involved in a preliminary step of peptidoglycan synthesis by covalently bonding to the latter (1). It is increasingly used as a first-line oral agent for uncomplicated urinary tract infections. Fosfomycin has a large spectrum of activity, good diffusion in the urinary tract, and excellent tolerance among patients. It is considered an alternative treatment option to carbapenems against extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae*. Therefore, its use may significantly contribute to prevent the selection of carbapenem-resistant isolates (2).

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Fosfomycin resistance may occur by three major ways: (i) punctual mutations in the MurA-encoding gene leading to a decrease of affinity between the enzyme and fosfomycin, (ii) decrease of the fosfomycin uptake caused by mutations in the *glpT* and *uhpT* genes encoding a glycerolphosphate permease and a hexose-phosphate system, respectively; and (iii) the production of fosfomycin-modifying enzymes. Those latter enzymes are generally encoded by plasmid-borne genes, and their expression leads to the permanent modification of the fosfomycin molecule by phosphorylation or by addition of a bacillithiol or glutathione group (1).

Glutathione-*S*-transferases are Mn^{2+}- and K^{+}-dependent enzymes predominantly in the FosA family. The *fosA* genes are part of a family of nine variants (*fosA1* to *fosA9*)

TABLE 1 *E. coli* strains used in this study and corresponding resistance genotypes

Strains	MIC (μg/ml)		Resistance genes identified
	FSF[a]	FSF+PPF	
R249	>1,024	64	*aac(3)-IIa*, *aac(6')-Ib-cr*, $bla_{CTX-M-15}$, *catB3*, *sul1*, *dfrA17*, *fosL1*
TC-R249[b]	>1,024	32	*fosL1*, *catB3*
TOP10-p249	>1,024	32	*fosL1*
J53	<4	ND	None
TOP10	<4	ND	None

[a]FSF, fosfomycin.
[b]TC, transconjugant.

conferring high resistance to fosfomycin once expressed (3–10). The natural reservoir of the *fosA* genes has been identified, as members of the *Enterobacteriaceae* family such as *Enterobacter* spp. (*fosA1* and *fosA2*), *Kluyvera* spp. (*fosA3* and *fosA4*), and *Klebsiella* spp. (*fosA3* and *fosA4*) (11, 12). We recently identified a novel *fosA* variant, namely, *fosA8*, for which the progenitor is the enterobacterial species *Leclercia adecarboxylata* (9). The *fosA* genes are mostly located on conjugative plasmids, in particular, in *Escherichia coli*, leading to a high level of resistance to fosfomycin. The *fosC2* gene, identified as a class 1 integron (13), also encodes a glutathione transferase identified in *Enterobacteriaceae*. Other acquired glutathione *S*-transferase genes have been identified in nonenterobacterial species, such as *fosD* in *Staphylococcus rostri*, *fosG* in *Achromobacter denitrificans*, *fosK* in *Acinetobacter soli*, and *fosF* in *Pseudomonas aeruginosa* (14, 15) (see Table S1 in the supplemental material).

Recent studies show that glutathione transferases may be inhibited *in vitro* by the presence of phosphonoformate (PPF), therefore restoring the susceptibility of the isolate to fosfomycin when supplemented (16). This molecule may be used to differentiate resistance to fosfomycin related to the production of glutathione transferases from those that are not.

We recently performed a screening of fosfomycin resistance among a collection of ESBL-producing *E. coli* from Switzerland recovered in 2012 (17). Hence, we identified a fosfomycin-resistant isolate harboring a novel *fos* gene coding for a putative glutathione *S*-transferase located on a conjugative plasmid.

RESULTS AND DISCUSSION

E. coli isolate R249 belonged to the extraintestinal phylogroup B1 and to a novel sequence type (ST) (*adk53*, *fumC40*, *gyrB47*, *icd13*, *mdh36*, *purA28*, and *recA29*). It was resistant to broad-spectrum cephalosporins, fluoroquinolones, tetracycline, sulfonamides, kanamycin, tobramycin, chloramphenicol, fosfomycin, and trimethoprim-sulfamethoxazole. It remained susceptible only to cephamycins (cefoxitin), carbapenems, amikacin, colistin, and tigecycline. PCR screening using specific primers followed by sequencing identified the resistance determinants responsible for the overall resistance phenotype, namely, *aac(3)-IIa*, *aac(6')-Ib-cr*, $bla_{CTX-M-15}$, *catB3*, *sul1*, and *dfrA17* (Table 1).

The MIC of fosfomycin measured by the agar dilution method was 1,024 μg/ml for *E. coli* R249 and decreased to 64 μg/ml after adding PPF (5 mM) in the supplemented medium. The inhibition of the resistance mechanism by PPF suggested that the fosfomycin resistance pattern was likely related to the production of a glutathione *S*-transferase, such as those belonging to the FosA family (16). However, PCR amplification of the known *fosA* and *fosC* (*fosA1-9* and *fosC2*) remained negative.

E. coli J53 transconjugant (TC-249) was successfully recovered by a mating-out assay, showing high-level resistance to fosfomycin but also to chloramphenicol. PCR assays showed that the corresponding conjugative plasmid coharbored the *catB3* gene encoding a chloramphenicol acetyltransferase along with the fosfomycin resistance determinant (Table 1). The conjugation frequency was estimated to be ca. 10^{-2}, therefore quite high but actually corresponding to what was reported for

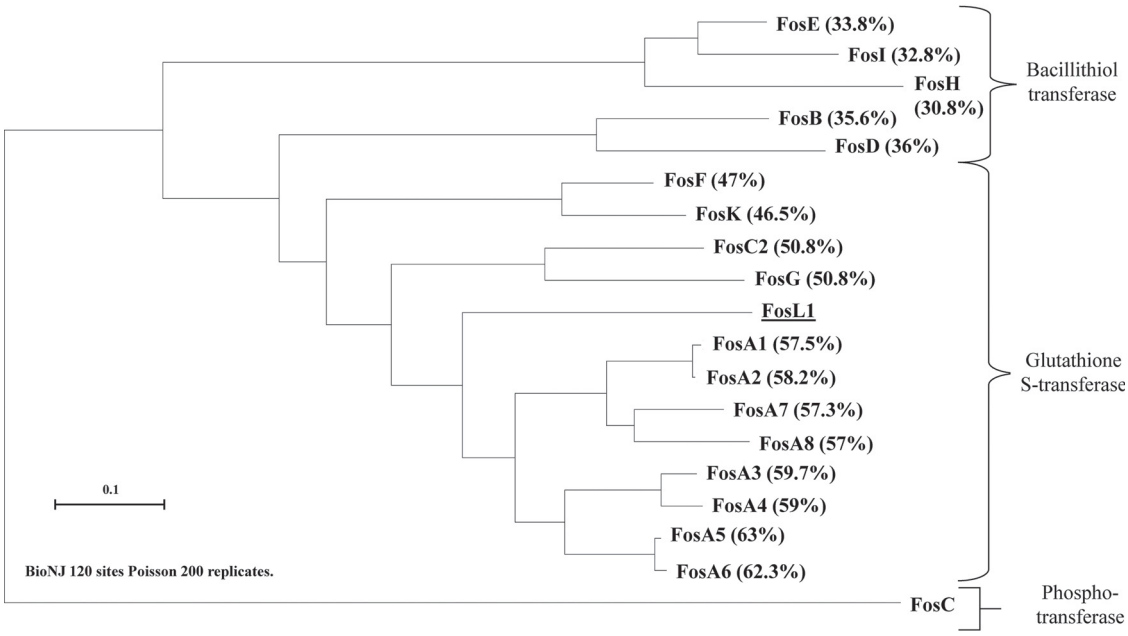

FIG 1 Phylogenetic tree obtained for all the identified Fos enzymes, including the bacillithiol and glutathione transferases by distance method using a neighbor-joining algorithm (SeaView version 4 software). Branch lengths are drawn to scale and are proportional to the number of amino acid substitutions with 200 bootstrap replications. The distance along the vertical axis has no significance. Percentages of amino acid identity shared between the FosL1 enzyme and the other Fos enzymes are indicated in brackets.

IncX-type plasmids (18). PCR-based replicon typing (PBRT) analysis and Kieser extraction revealed that this plasmid was ca. 40 kb in size and belonged to the IncX1 incompatibility group.

Shotgun cloning using whole-cell DNA of *E. coli* TC-249 gave recombinant *E. coli* TOP10 strains resistant to fosfomycin, a resistance also antagonized by the addition of PPF. Restriction profiling using the HindIII enzyme revealed that a ca. 2-kb insert was cloned into the pACYC184 recombinant vector. Sequencing of the DNA insert identified a novel fosfomycin resistance gene, which was named *fosL1* (GenBank accession number MN464149) to follow the current nomenclature with the latest *fos* gene, identified as *fosK* (15). This gene was 411 bp long coding for a putative protein of 136 amino acids. The FosL1 protein shared 57% and 63% of identity with the FosA1 to FosA8 enzymes and 50% of identity with the FosC2 enzyme (Fig. 1). Alignment of the amino acid sequences of the main Fos proteins showed that FosL1 possessed the conserved amino acid residues involved in the binding of the Mn^{2+} and K^{+} cations, a feature shared with other Fos-like glutathione transferases (Fig. 2). To gain insight into the backbone of the *fosL1*-bearing plasmid, whole-genome sequencing of the *E. coli* transconjugant DNA but also the *E. coli* clinical isolate was performed. DNA extraction was performed using a Sigma-Aldrich GenElute bacterial genomic DNA kit. Then, genomic libraries and sequencing were performed using a strategy adapted to the Illumina MiniSeq system as reported previously (9) 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), with reads assembled as described previously (9); resulting contigs were uploaded into the Center for Genomic Epidemiology server (http://www.genomicepidemiology.org/). Even though the total assembly process could not close the sequence of the natural plasmid because of repeated sequences, it did confirm that the *fosL1* plasmid was indeed the IncX1, with no other plasmid backbone identified from the transconjugant. Corresponding sequences have been deposited under BioProject accession number PRJNA596098, and the whole genome of isolate 249 has been deposited in GenBank under accession number WTUN00000000 (BioSample SAMN13612190).

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                    10                                                  50
FosL1     MLSGMNHLTL AVQSLERSVS FYKDTLGFRL AARWKNGAYL ELGDIWLCLS LDDKRSAQP/ /WPEYTHYAF SALEQDFSSF
FosL2     ---------- ---------- ---------- ---------- -V-N------ ---------/ /-S------- -V--------
FosA1     --QSL----- --SD-QK--T -WHEL--LT- H---NT---- TC----V--- Y-EA-QYV-P QESD------ TVA-E--EPL
FosA3     --Q-L----- --SD-AS-LA --QQLP-M-- H-S-DS---- SC-A------ --EQ-RKT-P QESD------ -VA-EE-AGV
FosA6     ----L----- --SQ-AP--A --QQL--MT- H---DS---- SC-------- --PQ-RVT-P EESD------ -IS-A--A--
FosA7     --QSL----- --SN-QT-LT -WR-L--LQ- H-E-DT---- TC----V--- Y-VSCNYVAP QECD------ -IAPE--EP-
FosC2     --R-L--I-I --SD-----E --TRL--MKA HV--DS---- S-EAT-I--- C-EVHPS/// //QD-C-I-- DVS-EN-EPV
FosK      -IT-I--I-F S-RD-SS-IE --R-L--M-- HVT-EA---F TA--T-V--- VGEPKPAN// ///D-T-V-- -VG-RELVEL
                                 100                                 130
FosL1     VAKLIQKGVV EWKKNRSEGN SFYFLDPDGH KLEVHVGNLE SRLKQCRVHP YTDMEIFD
FosL2     ---------- ---------- ---------- ---------- ---------- -A------
FosA1     SQR-E-A--T I--Q-K---A ---------- ---L---S-A A--AA--EK- -AG-VFTSDE A
FosA3     --L-A-A-AE V--D------A -Y-------- ---L-----A Q--AA--ER- -KG-VF--
FosA6     A-R-EAA--A I--L------A -H-------- ---L---S-A Q--AA--EQQ -KG-VF--Q
FosA7     SY--K-A--T V--D-K---Q ---------- ---L---D-A ---AQ--EK- -SG-RFGPGK
FosC2     TK--REAH-- ---Q------L -L-L------ ---I-S-S-Q ---ESLKSK- -QGLVWL
FosK      H-R-KEA--E ---Q-I----- -V-L---N-- RI-L-C-T-A T--AELEKS- -KRLVWC
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FIG 2 Amino acid sequence comparison between FosL1 and different members of glutathione transferases. Boxes bracket amino acids implicated in Mn^{2+}, K^{+}, and fosfomycin bond. Hyphens represent the conserved amino acids with the FosL1 sequence and dashes represent the gaps in the amino acid sequence.

Analysis of the sequences surrounding the *fosL1* gene showed that it was part of a 1,597-bp-long cassette along with a putative *N*-acetyl-transferase (Fig. 3A). Noteworthy, 5-bp direct repeat sequences (CCCGG) were identified at each extremity of this cassette, suggesting that the acquisition of that latter had occurred through a transposition mechanism, although no transposase gene was identified in this cassette. *In silico* analysis using the BLAST alignment tool showed that the sequences directly adjacent to the direct repeats and therefore corresponding to the cassette boundaries were highly similar to those found at the extremities of Tn*7*-like transposons, namely, the Tn*7L/R* sequences. The latter sequences have been shown to play a major role in the mobilization of Tn*7*-like elements (19, 20). Further analysis of adjacent sequences flanking this *fosL1*-containing cassette identified sequences of an IS*91*-like element on both extremities. In fact, it may be speculated that this IS*91*-like element had been truncated by the *fosL1* cassette, probably by a Tn*7*-like-mediated transposition mechanism with an undefined transposase activity likely acting in *trans*.

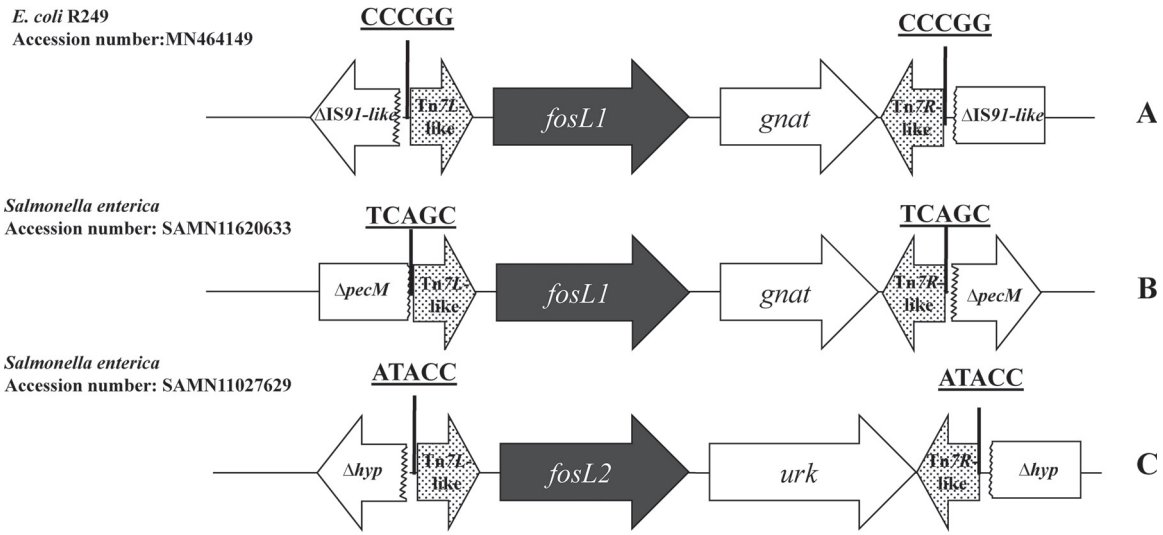

FIG 3 Schematic structures of the different mobile insertion cassettes harboring the *fosL* gene. (A) Genetic structure identified in R249 (this study). (B and C) Genetic structures identified *in silico* in *Salmonella* species isolates. Tn*7L/R*, sequences showing high nucleotide identity with the Tn*7* extremities recognized by the transposases of the transposon Tn*7*; *gna*, gene encoding a putative acetyltransferase; IS*91*-like, insertion sequence truncated by the insertion of the *fosL1* cassette between nucleotides 150 and 151; *pecM*, gene encoding a histone acetyltransferase HPA2 and related acetyltransferases (protein ID BBB37423.1); *urk*, gene encoding a putative uridine kinase; *hyp*, hypothetical gene.

Altogether, these data showed that the *fosL1* gene is located in a mobile insertion cassette (mic). Those genetic structures were first described in a *Bacillus cereus* isolate (21). They are small genetic elements containing one or several open reading frames that present imperfect inverted repeats (IRs) in their extremities. They are not capable of mobility by themselves, since they lack a transposase gene but they can be effectively mobilized in *trans* by a transposase that is able to recognize its extremities. Those mics were previously described containing resistance genes such as the plasmid-mediated quinolone resistance *qnrS2* or more recently the colistin resistance gene *mcr-5* (22–24).

In silico analysis using the NCBI database identified the exact same *fosL1* gene in a *Salmonella enterica* isolate recovered from the United States (accession number EAW2818841.1) (Fig. 3B). This isolate was sequenced by the National Subtyping Network for Foodborne Disease Surveillance established by the CDC (25). In that case, the *fosL1* gene was also part of a mic-type structure sharing 99% nucleotide identity, differing by only three nucleotide substitutions in the Tn*7L*-like sequence. That element was also bracketed by 5-bp-long direct repeats (TCAGC) (Fig. 3B). Furthermore, another gene encoding a FosL1-like enzyme was also identified *in silico* in the genome of another *S. enterica* from the United States (GenBank accession number SAMN11027629). This enzyme that we named FosL2 shares 96% amino acid identity (only five amino acid substitutions, namely, L41V, D43N, P61S, A70V, and T130A) with FosL1. The *fosL2* gene was also located in a mic element; however, it was different from the one bearing the *fosL1* gene, being 1,491 bp long and bracketed by 5-bp-long direct repeats (Fig. 3C). Using the PlasmidFinder tool (26), the *fosL1* and *fosL2* genes identified in the *Salmonella* sp. isolates were found to be located on IncQ1 and IncP-like plasmids, respectively.

These data highlight that FosL-like resistance determinants are already disseminated among enterobacterial isolates in different parts of the world, and their presence on various plasmid supports may be the consequence of a functional mobility of the mic-*fosL* elements.

Interestingly, a previous study described a mobile insertion cassette containing a *fosD*-like gene coding for bacillithiol transferase acquired in the chromosome of a *Bacillus cereus* isolate. The extremities of that mic structure showed significant homology with the inverted repeats of insertion sequence IS*231* (IS*4* family) (27).

Very recently, we identified another novel *fosA* variant, namely, *fosA8* (9), identified from an *E. coli* isolate. The progenitor of the *fosA8* gene was identified, being the enterobacterial species *Leclercia adecarboxylata*. In the present study, we did not find any putative progenitor species for *fosL* when searching the GenBank databases. Our finding constitutes further evidence that a variety of Fos-like determinants are currently circulating in *Enterobacteriaceae*, and particularly, in *E. coli*, being transferable sources of acquired resistance among clinical isolates. Their identification may not simply rely on PCR experiments using primers designed from the previously described *fosA-fosC* genes, since sequences of those two recently identified genes are too divergent compared to the former. Since fosfomycin is currently considered a critical antimicrobial molecule (used as first-line therapy in many situations but also as a last-resort option when dealing with infections caused by some carbapenem-resistant isolates), strategies should be implemented to identify those resistance determinants and limit their spread when it is still possible.

MATERIALS AND METHODS

Bacterial strains and plasmids. Isolate R249 was recovered in 2012 from a patient's urine sample in Switzerland (17). The azide-resistant *E. coli* J53 strain was used as recipient for the conjugation experiments. The plasmid-free *E. coli* TOP10 strain (Thermo Fisher, Zug, Switzerland) was used as recipient of the genomic bank generated by the shotgun cloning experiment.

Antimicrobial susceptibility testing and determination of fosfomycin resistance. Antimicrobial susceptibility testing was performed according to the standard disc diffusion methods using Muller-Hinton (MH) agar plates (Bio-Rad, Cressier, Switzerland) according to the CLSI recommendations (28). Fosfomycin resistance determination was performed using the Rapid Fosfomycin NP test (29) followed by MIC determination by agar dilution using MH agar plates supplemented with glucose-6-phosphate

(G6P) at 25 μg/ml and with concentrations of fosfomycin (Sigma-Aldrich, Switzerland) ranging from 4 to 2,048 μg/ml.

Molecular analyses. Screening of resistance determinants was performed by PCR using specific primers. Multilocus sequence type was obtained using the Center for Genomic Epidemiology server (MLST 1.8) (30). Phylogroup determination was performed using the PCR-based Clermont method (31).

Plasmid analysis. Transferability of the fosfomycin resistance determinant was assessed by a mating-out assay. Briefly, isolate R249 and *E. coli* strain J53 were separately inoculated overnight in LB broth. The samples were then mixed at a ratio of 10:1 (recipient/donor) for 5 h and plated on LB agar plates supplemented with azide (100 μg/ml), G6P (25 μg/ml), and fosfomycin (50 μg/ml). Antimicrobial susceptibility testing was performed on the *E. coli* transconjugant in order to identify putative coresistance associated. Kieser extraction (32) followed by gel electrophoresis analysis were performed for the resulting *E. coli* transconjugant strain (TC-249) in order to estimate the size of the transferred plasmid. *E. coli* strain 50192 carrying four plasmids with known sizes (7 kb, 48 kb, 66 kb, and 154 kb) was used as molecular marker.

PCR-based replicon typing (PBRT) was performed to identify specific replicase genes eventually differentiating plasmid incompatibility groups (33). Replicon typing of *in silico*-identified plasmid was performed using the web-based tool PlasmidFinder version 2.1 (https://cge.cbs.dtu.dk/services/PlasmidFinder/) (26).

Shotgun cloning. Total DNA of *E. coli* TC-249 was extracted using the GenElute bacterial genomic DNA kit (Sigma-Aldrich, Switzerland). Then, DNA from both isolate R249 and the recombinant plasmid pACYC184 were digested by the restriction enzyme HindIII. Generated fragments were ligated using a T4 DNA ligase (Thermo Fischer, Switzerland) and transferred into the *E. coli* TOP10 strain by heat shock. Putative transformants containing the fosfomycin resistance determinant were selected on LB agar supplemented with G6P (25 μg/ml) and fosfomycin (50 μg/ml). Recombinant clones were analyzed by PCR followed by Sanger sequencing using specific primers at each extremity of the cloning site of plasmid pACYC184.

Data availability. DNA sequencing has been deposited in GenBank under accession number MN464149. Corresponding sequences have been deposited as BioProject accession PRJNA596098; the whole genome of isolate 249 has been deposited in GenBank under accession number WTUN00000000 and BioSample SAMN13612190.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.05 MB.

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

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