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MECHANISMS OF RESISTANCE

Proposing *Kluyvera georgiana* as the Origin of the Plasmid-Mediated Resistance Gene *fosA4*

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ABSTRACT A putative *fosA* gene in *Kluyvera georgiana* 14751 showed 99% nucleotide identity with plasmid-encoded *fosA4*. Due to a single-nucleotide insertion translating to a truncated protein, *K. georgiana* 14751 *fosA* does not confer fosfomycin resistance. However, analysis of another genome deposit (*Kluyvera ascorbata* WCH1410) that could be recategorized as *K. georgiana* after phylogenetic analysis revealed a *fosA* gene 100% identical to the plasmid-borne *fosA4* gene. We suggest that *Kluyvera georgiana* represents the most probable origin of *fosA4*.

KEYWORDS FosA, *Kluyvera ascorbata*, *Kluyvera georgiana*, whole-genome sequencing, fosfomycin resistance

Fosfomycin is an old, broad-spectrum antibiotic that inhibits cell wall biosynthesis by inactivating UDP-*N*-acetylglucosamine-3-enolpyruvyltransferase (MurA), acting as a phosphoenolpyruvate analogue (1). It has regained attention in the past few years due to its activity against multidrug-resistant and extremely drug-resistant microorganisms, typically recovered from hospital-acquired infections.

Among several fosfomycin-modifying enzymes described, the FosA enzymes (FosA, FosA2, FosA3, FosA4, FosA5, and FosA6) are the most prevalent enzymes among Gram-negative organisms. They are found mainly in plasmids from *Enterobacteriaceae* but are also observed in *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (1, 2). Recently, the origins of some plasmid-mediated *fosA* genes have been proposed; *fosA2* in Tn*2961* originated from the chromosome of *Enterobacter cloacae* (3), *fosA4* occurred in an *Escherichia coli* isolate (4), *fosA5* and *fosA6* originated from the chromosome of *Klebsiella pneumoniae* (5, 6), and, more recently, Ito et al. proposed a chromosome-encoded *fosA* gene from *Kluyvera georgiana* YDC799 as the origin of plasmid-encoded *fosA3*, with 99% amino acid identity (7).

Upon whole-genome sequencing (WGS) with the Illumina platform, we searched for the putative chromosome-encoded *fosA* gene(s) in *Kluyvera georgiana* 14751, which was isolated from a bloodstream infection (SENTRY Antimicrobial Surveillance Program) in Louisville, Kentucky, USA, in 2002. *De novo* assembly of reads was achieved using the Velvet package (velveth and velvetg programs) (https://www.ebi.ac.uk/~zerbino/velvet/), resulting in a contigs.fa file with 1,589 nodes, an N_{50} value of 20,178, a longest contig of 105,293 bp, and a total assembly of 4,993,089 bp, using 1,264,850/1,278,330 reads. The *fosA* genes were screened in the contigs.fa file using NCBI BLAST.

Node 845 (8,626 bp; coverage of 23.09×) contained a *fosA* gene (*fosA*^{K14751}) displaying 99% nucleotide identity (409/412 bp, including a single cytosine nucleotide insertion at position 339) with plasmid-encoded *fosA4* (GenBank accession numbers CP023167.1 and CP016184.1, among others) and ~93% nucleotide identity with *fosA3* from *K. georgiana* YDC799 (GenBank accession number CP022114.1), which was previ-

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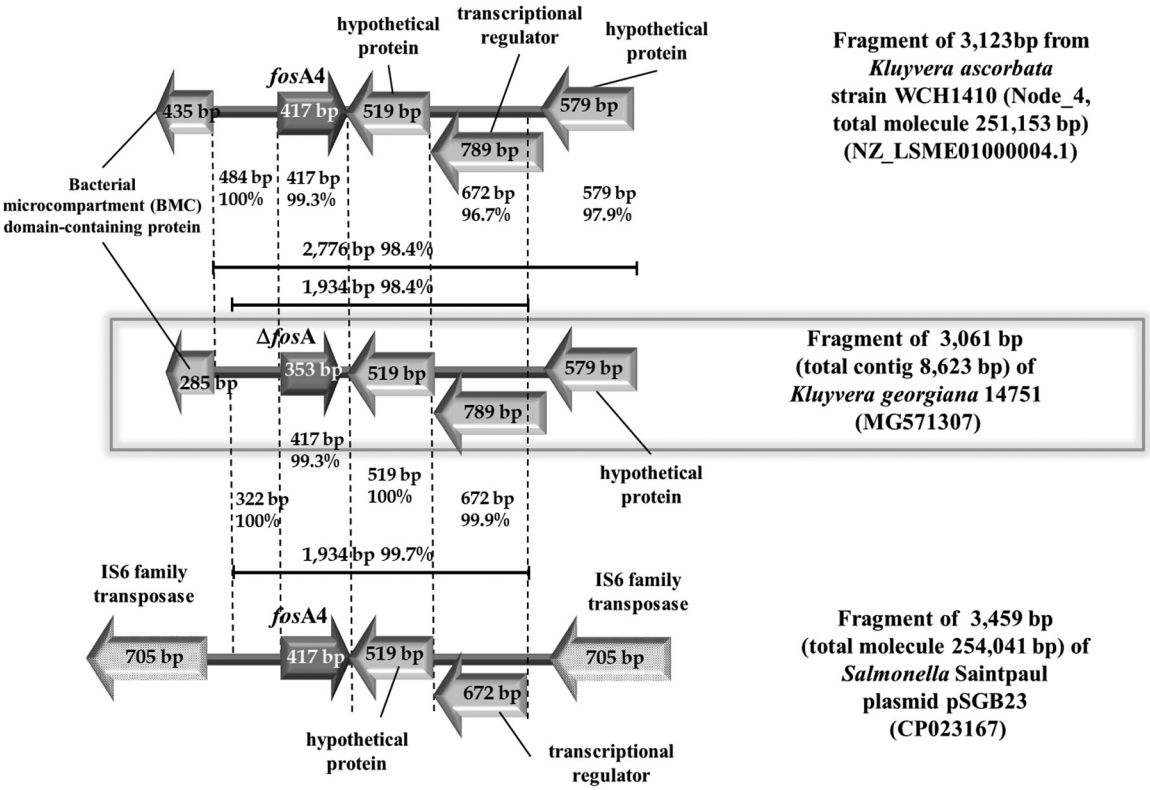

FIG 1 Schematic representation of the partial sequence of node 845 of the *Kluyvera georgiana* 14751 genome assembly (middle) and comparison with the partial sequence of node 4 of the genome assembly from *Kluyvera ascorbata* WCH1410 (top) and the partial sequence of plasmid pSGB23 from *Salmonella enterica* subsp. *enterica* serovar Saintpaul strain SGB23 (bottom).

ously proposed as the chromosomal origin of *fosA3* (7). Interestingly, we also detected a sequence containing a chromosome-encoded *fosA4* gene deposited in the nonredundant nucleotide databases as *Kluyvera ascorbata* WCH1410 (GenBank accession number NZ_LSME00000000.1), which we included in the analysis to compare both *fosA4* genes and the surrounding sequences.

A 1,933-bp sequence is almost identical (99.7% nucleotide identity) between *K. georgiana* 14751 and plasmid pSGB23 from *Salmonella enterica* subsp. *enterica* serovar Saintpaul, including a 322-bp region upstream of *fosA* (100% nucleotide identity), the *fosA* gene (99.3% nucleotide identity [409/412 bp]), and a 1,195-bp region downstream of *fosA* (99.9% identity). The last fragment includes a 519-bp open reading frame (ORF) (100% nucleotide identity) and a 789-bp ORF in which a 672-bp segment has 99.9% nucleotide identity with a transcriptional regulator in plasmid pSGB23. In *K. ascorbata* strain WCH1410, the corresponding 322-bp and 519-bp regions demonstrate 100% nucleotide identity, and *fosA* has 99.3% nucleotide identity with *K. georgiana* 14751 *fosA*; however, the gene is 100% identical to its plasmidic counterpart. There is also a 792-bp transcriptional regulator with ~96% nucleotide identity with that from *K. georgiana* 14751 (764/792 bp, including 3 gaps) (Fig. 1). Remarkably, chromosomal *fosA* from *K. georgiana* 14751 and the *fosA* genetic environment display greater identity with plasmid-borne *fosA4* and the neighboring sequences (99.7% nucleotide identity [1,928/1,933 bp]) than with the equivalent chromosomal segment from *K. ascorbata* WCH1410 (98.7% nucleotide identity [1,901/1,933 bp]).

Additionally, we performed a phylogenetic analysis by concatenation of several housekeeping genes (16S rRNA, *adk*, *gyrA*, *gyrB*, *recA*, *infB*, and *rpoB* genes) from *K. ascorbata* WCH1410, *K. ascorbata* ATCC 33433, *Kluyvera cryocrescens* NBRC102167, *K. georgiana* ATCC 51603, and *K. georgiana* 14751. The analysis was conducted by using ClustalX (http://clustalx.software.informer.com/2.1) to align all sequences, and the mo-

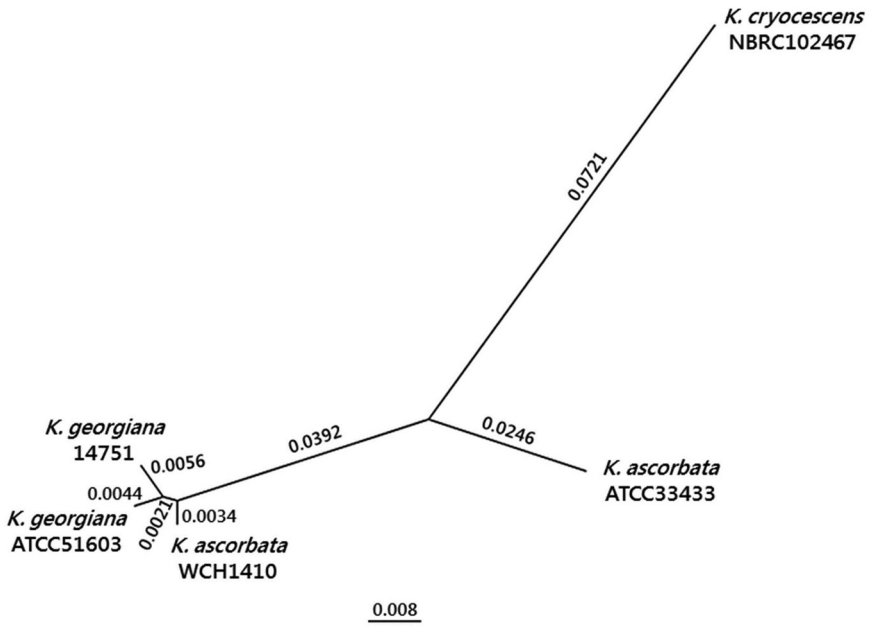

FIG 2 Phylogenetic tree (1,000 bootstraps) of housekeeping genes from *K. ascorbata* WCH1410, *K. ascorbata* ATCC 33433, *K. cryocrescens* NBRC102147, *K. georgiana* ATCC 51603, and *K. georgiana* 14751. Phylogenetic relationships are expressed in each branch as the substitutions per site (also expressed in the scale bar).

lecular evolution model was estimated with jModelTest2 (http://github.com//ddarriba/jmodeltest2/releases). The resulting phylogenetic tree was obtained with PhyML (http://www.atgc-montpellier.fr/phyml/versions.php), using the Bayesian information criterion (BIC) parameters suggested by the jModelTest software, with 1,000 bootstraps. The phylogenetic tree was visualized and edited using FigTree (http://tree.bio.ed.ac.uk/software/figtree).

Housekeeping genes from *K. ascorbata* WCH1410 showed greater identity with the homologous genes from *K. georgiana* ATCC 51603 (99.1% nucleotide identity) and *K. georgiana* 14751 (99.0% nucleotide identity) than with the corresponding genes from *K. ascorbata* ATCC 33433 (95.0% nucleotide identity), as shown in Fig. 2. We suggest that *K. ascorbata* WCH1410 might be, in fact, a *K. georgiana* isolate. Therefore, a taxonomic reevaluation of the entire genus is currently necessary (data not shown; M. M. Rodriguez, B. Ghiglione, M. Almuzara, P. Power, T. Naas, and G. Gutkind, unpublished data).

As a result of the previously mentioned single-nucleotide insertion generated at the 3′ end of the *fosA*^{K14751} gene, a shorter peptide seems to be translated due to the occurrence of a premature stop codon in the mRNA; this generates a deduced FosA^{K14751} enzyme with 95% amino acid identity with the main core of FosA4 (111/117 amino acids) from several species (GenBank accession numbers BAP18892.1, KXT28349.1, OJQ09299.1, OYF76970.1, OYI75904.1, ASZ39831.1, and PAY66171.1); the protein seems to conserve all proposed active site residues except for the last α-helix (Fig. 3).

To test whether the expressed FosA protein has activity toward fosfomycin, we cloned the *fosA* gene from *K. georgiana* 14751 in a pK19 vector in frame with the vector's promoter, using the primers fosA4_HindIII_F (5′-AAGCTTCATGCTGCAGGGATTGAA-3′) and fosA4_EcoRI_R (5′-CGGCAGTAAGCTGAACGAATTCGTCA-3′), and transformed the recombinant plasmid in *E. coli* TOP10 cells. The sequence was confirmed by DNA sequencing at Macrogen (Korea). Fosfomycin susceptibility tests were performed using fosfomycin disks (200 μg) with glucose-6-phosphate (50 μg), according to CLSI guidelines (8). *E. coli* clones producing FosA were susceptible to fosfomycin and showed the same inhibition zones as the control strains, suggesting that the C-terminal

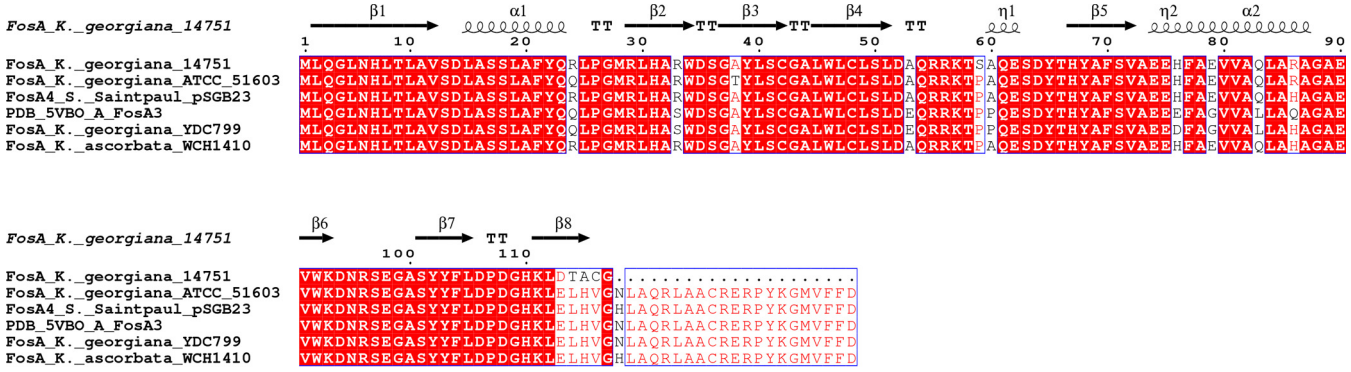

FIG 3 Amino acid sequence alignment of FosA proteins from *Kluyvera georgiana* 14751 and *K. georgiana* ATCC 51603, FosA4 from *Salmonella enterica* subsp. *enterica* serovar Saintpaul plasmid pSGB23 (GenBank accession number ASZ39831.1), FosA4 from *Kluyvera ascorbata* WCH1410 (100% amino acid identity), FosA3 from *Escherichia coli* (PDB accession number 5VB0), and FosA3 from *K. georgiana* YDC799 (GenBank accession number ASG63672.1). Putative secondary domains are shown above the sequences.

deletion in the FosA protein (including the conserved Arg122 residue) (Fig. 3) indeed has a deleterious impact on fosfomycin resistance.

While our own sequence does not provide resistance (due to the single-nucleotide insertion and frameshift, resulting in premature termination of the protein), we still consider, based on the 100% identity of *K. ascorbata* WCH1410 *fosA* with *fosA* and the analysis of genetic contexts described above, that the origin of the plasmid-borne *fosA4* gene, as well as other resistance genes, can be traced back to *K. georgiana* (9–11). The role of *Kluyvera* members as donors of chromosomal genes to be recruited by plasmid platforms is noteworthy. Therefore, compartmentalized evolution (as expected for microorganisms in soil, water, or sewage environments, with no epidemiological link) through which microevolution within different, originally chromosomal genes may occur, either (most probably) before or after recruitment, is proposed.

Accession number(s). The genomic sequence of *Kluyvera georgiana* 14751 was deposited in GenBank under accession number MG571307.

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REFERENCES

1. Thompson MK, Keithly ME, Sulikowski GA, Armstrong RN. 2015. Diversity in fosfomycin resistance proteins. Perspect Sci 4:17–23. https://doi.org/10.1016/j.pisc.2014.12.004.
2. Silver LL. 2017. Fosfomycin: mechanism and resistance. Cold Spring Harb Perspect Med 7:a025262. https://doi.org/10.1101/cshperspect.a025262.
3. Xu H, Miao V, Kwong W, Xia R, Davies J. 2011. Identification of a novel fosfomycin resistance gene (*fosA2*) in *Enterobacter cloacae* from the Salmon River, Canada. Lett Appl Microbiol 52:427–429. https://doi.org/10.1111/j.1472-765X.2011.03016.x.
4. Nakamura G, Wachino J, Sato N, Kimura K, Yamada K, Jin W, Shibayama K, Yagi T, Kawamura K, Arakawa Y. 2014. Practical agar-based disk potentiation test for detection of fosfomycin-nonsusceptible *Escherichia coli* clinical isolates producing glutathione *S*-transferases. J Clin Microbiol 52:3175–3179. https://doi.org/10.1128/JCM.01094-14.
5. Ma Y, Xu X, Guo Q, Wang P, Wang W, Wang M. 2015. Characterization of *fosA5*, a new plasmid-mediated fosfomycin resistance gene in *Escherichia coli*. Lett Appl Microbiol 60:259–264. https://doi.org/10.1111/lam.12366.

6. Guo Q, Tomich AD, McElheny CL, Cooper VS, Stoesser N, Wang M, Sluis-Cremer N, Doi Y. 2016. Glutathione-*S*-transferase FosA6 of *Klebsiella pneumoniae* origin conferring fosfomycin resistance in ESBL-producing *Escherichia coli*. J Antimicrob Chemother 71:2460–2465. https://doi.org/10.1093/jac/dkw177.
7. Ito R, Pacey MP, Mettus RT, Sluis-Cremer N, Doi Y. 2018. Origin of the plasmid-mediated fosfomycin resistance gene *fosA3*. J Antimicrob Chemother 73:373–376. https://doi.org/10.1093/jac/dkx389.
8. Clinical and Laboratory Standards Institute. 2017. Performance standards for antimicrobial susceptibility testing; 27th informational supplement. M100-S27. Clinical and Laboratory Standards Institute, Wayne, PA.
9. Olson AB, Silverman M, Boyd DA, McGeer A, Willey BM, Pong-Porter V, Daneman N, Mulvey MR. 2005. Identification of a progenitor of the CTX-M-9 group of extended-spectrum β-lactamases from *Kluyvera georgiana* isolated in Guyana. Antimicrob Agents Chemother 49:2112–2115. https://doi.org/10.1128/AAC.49.5.2112-2115.2005.
10. Poirel L, Kampfer P, Nordmann P. 2002. Chromosome-encoded Ambler class A β-lactamase of *Kluyvera georgiana*, a probable progenitor of a subgroup of CTX-M extended-spectrum β-lactamases. Antimicrob Agents Chemother 46:4038–4040. https://doi.org/10.1128/AAC.46.12.4038-4040.2002.
11. Rodriguez MM, Power P, Sader H, Galleni M, Gutkind G. 2010. Novel chromosome-encoded CTX-M-78 β-lactamase from a *Kluyvera georgiana* clinical isolate as a putative origin of CTX-M-25 subgroup. Antimicrob Agents Chemother 54:3070–3071. https://doi.org/10.1128/AAC.01615-09.