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Proposing *Kluyvera georgiana* as the origin of the plasmid-mediated resistance gene *fosA4*

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Running title: *Kluyvera georgiana* as the origin of *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. Nonetheless, analysis of another genome deposit (*K. ascorbata* WCH1410) that could be recategorized as *K. georgiana* after phylogenetic analysis, revealed a *fosA* 100% identical to the plasmid-borne *fosA4*. Collectively, we suggest that *Kluyvera georgiana* represents the most probable origin of *fosA4*.

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Text

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 last few years due to its activity against multidrug and extremely drug-resistant microorganisms, typically recovered from hospital-acquired infections.

Among several fosfomycin-modifying enzymes described, the FosA (FosA, FosA2, FosA3, FosA4, FosA5 and FosA6) are the most prevalent enzymes among gram-negatives, mainly found in plasmids from *Enterobacteriaceae*, but also described in *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (1, 2).

Recently, the origin of some plasmid-mediated *fosA* genes has been proposed: *fosA2* in Tn*2961* originated from *Enterobacter cloacae*'s chromosome (3), *fosA4* in an *Escherichia coli* isolate (4), *fosA5* and *fosA6* from the chromosome of *Klebsiella pneumoniae* (5, 6), and more recently, Ito *et al*. proposed a chromosome-encoded *fosA* from *Kluyvera georgiana* YDC799 as the origin of plasmid-encoded *fosA3*, with 99% amino acid identity (7).

Upon WGS by Illumina platform, we searched for the putative chromosome-encoded *fosA* gene(s) in *Kluyvera georgiana* 14751, isolated from a bloodstream infection (SENTRY Antimicrobial Surveillance Program) in Louisville, 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, n_{50} = 20,178, longest contig of 105,293 bp, and a total assemble 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.

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The node 845 (8,626 bp; 23,090,183 coverage; GenBank: MG571307) contained the *fosA* gene (*fosA*^{K14751}) displaying 99% nucleotide (nt) identity (409/412 bp, including a single cytosine nucleotide insertion at position 339) with plasmid-encoded *fosA4* (GenBank: CP023167.1, CP016184.1, etc), and *ca.* 93% nucleotide identity with *fosA3* from *K. georgiana* YDC799 (GenBank: CP022114.1), previously proposed as the chromosomal origin of *fosA3* (7).

Interestingly, we also detected a sequence deposited as *Kluyvera ascorbata* WCH1410 in the non-redundant nucleotide databases containing a chromosome-encoded *fosA4* gene (GenBank: NZ_LSME00000000.1), which we included in the analysis to compare both *fosA4* genes and surrounding sequences.

A 1,933-bp sequence is almost identical between *K. georgiana* 14751 and plasmid pSGB23 from *Salmonella* Saintpaul (99.7% nt identity), including a 322-bp region upstream *fosA* (100% nt identity), the *fosA* gene (99.3% nt identity, 409/412 bp), and a 1,195-bp region downstream *fosA* with 99.9% identity. This last fragment includes a 519-bp orf (100% nt identity) and a 789-bp orf from which 672 bp have 99.9% nt identity with a transcriptional regulator in plasmid pSGB23. In *K. ascorbata* strain WCH1410, the corresponding 322-bp and 519-bp regions share 100% nt identity, and *fosA* has 99.3% nt identity with *K. georgiana* 14751 *fosA*; however, it is 100% identical to the plasmidic counterpart. There is also a 792-bp transcriptional regulator with *ca.* 96% nt identity with *K. georgiana* 14751 (764/792, including 3 gaps; Figure 1).

Remarkably, chromosomal *fosA* from *K. georgiana* 14751, and the *fosA* genetic environment, display higher identity with plasmid-borne *fosA4* and neighboring sequences (99.7%, 1,928/1,933 bp) compared to the equivalent chromosome segment from *K. ascorbata* WCH1410 (98.7%, 1,901/1,933 bp).

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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, *K. cryocrescens* NBRC102167, *K. georgiana* ATCC 51603, and *K. georgiana* 14751. The analysis was conducted using ClustalX (http://clustalx.software.informer.com/2.1/) to align all sequences and the molecular evolution model was estimated with JModelTest2 (http://gihub.com//ddarriba/jmodeltest2/releases). The resulting phylogenetic tree was obtained with PhyML program (http://www.atgc-montpellier.fr/phyml/versions.php), using the BIC criterion parameters suggested by the JModelTest software, with 1,000 bootstraps. The phylogenetic tree was visualized and edited using the FigTree program (http://tree.bio.ed.ac.uk/software/figtree/).

Housekeeping genes from *K. ascorbata* WCH1410 showed higher identity with the homologous genes from *K. georgiana* ATCC 51603 (99.1% nt identity) and *K. georgiana* 14751 (99.0% nt identity) than with the corresponding genes from *K. ascorbata* ATCC 33433 (95.0% nt identity), as shown in Figure 2. We could assume that *K. ascorbata* WCH1410 might be, in fact, a *K. georgiana* isolate. Therefore, a taxonomic reevaluation of the entire genus is today necessary (data not shown, Rodríguez *et.al*. in press).

As the result of the previously mentioned single nucleotide insertion generated at the 3'-end of the *fosA*^{K14751}, 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) from several species (Sequences ID: BAP18892.1; KXT28349.1; OJQ09299.1; OYF76970.1; OYI75904.1, ASZ39831.1; PAY66171.1); the protein seems to conserve all proposed active site residues except for the last α-helix (Figure 3).

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To test if the expressed FosA protein has activity towards 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´-AAGCTTCATGCTGCAGGGATTGAA3´) 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 service. Fosfomycin susceptibility tests were performed using fosfomycin disk (200 µg) with glucose-6-phosphate (50 µg) according to CLSI guidelines (8). *Escherichia coli* clones producing FosA were susceptible to fosfomycin showing the same inhibition zone than the control strains, suggesting that the C-terminus deletion in the FosA protein (including the conserved Arg122 residue; Figure 3) has indeed a deleterious impact on fosfomycin resistance.

While our own sequence does not provide resistance due to the single nucleotide insertion frameshift resulting in the premature termination of the protein, based on the 100% identity of *fosA* of WCH1410 with *fosA* and the analysis of genetic contexts described above, we still consider that the origin of plasmid-borne *fosA4* gene can be traced back to *K. georgiana*, along with other resistance genes (9-11). The role of *Kluyvera* members as donors of chromosomal genes to be recruited by plasmid platforms is noteworthy. A 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 therefore occur either (most probably) before or after recruitment.

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177 **Figure legends**

178

179 **Figure 1.** Schematic representation of partial sequences of node 845 from *Kluyvera*
180 *georgiana* 14751 genome assembly (middle) and comparison with partial sequence of
181 node_4 of genome assembly from *Kluyvera ascorbata* WCH1410 (up), and partial
182 sequence of plasmid pSGB23 from *Salmonella* Saintpaul strain SGB23 (bottom).

183

184 **Figure 2.** Phylogenetic tree (1,000 bootstraps) of housekeeping genes from *K.*
185 *ascorbata* WCH1410, *K. ascorbata* ATCC33433, *K. cryocrescens* NBRC102147, *K.*
186 *georgiana* ATCC51603, and *K. georgiana* 14751.

187

188 **Figure 3.** Amino acid sequence alignment of FosA proteins from *Kluyvera georgiana*
189 14751, *K. georgiana* ATCC 51603, FosA4 from *Salmonella* Saintpaul plasmid pSGB23
190 (ID: ASZ39831.1), 100% amino acid identity with FosA4 from *Kluyvera ascorbata*
191 WCH1410, and FosA3 (PDB 5VB0) and from *K. georgiana* YDC799
192 (ID: ASG63672.1). Putative secondary domains are shown in the upper frame.

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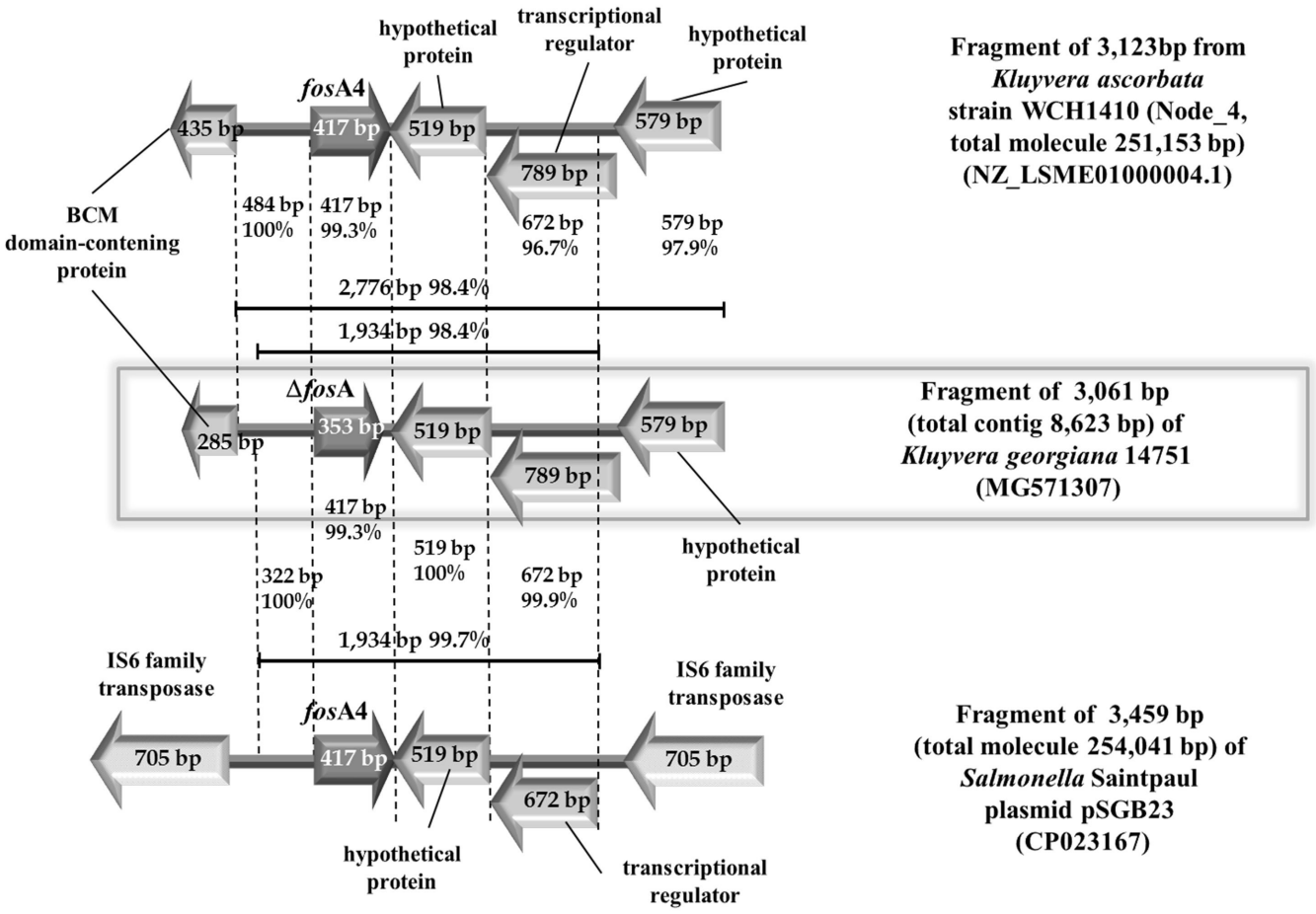

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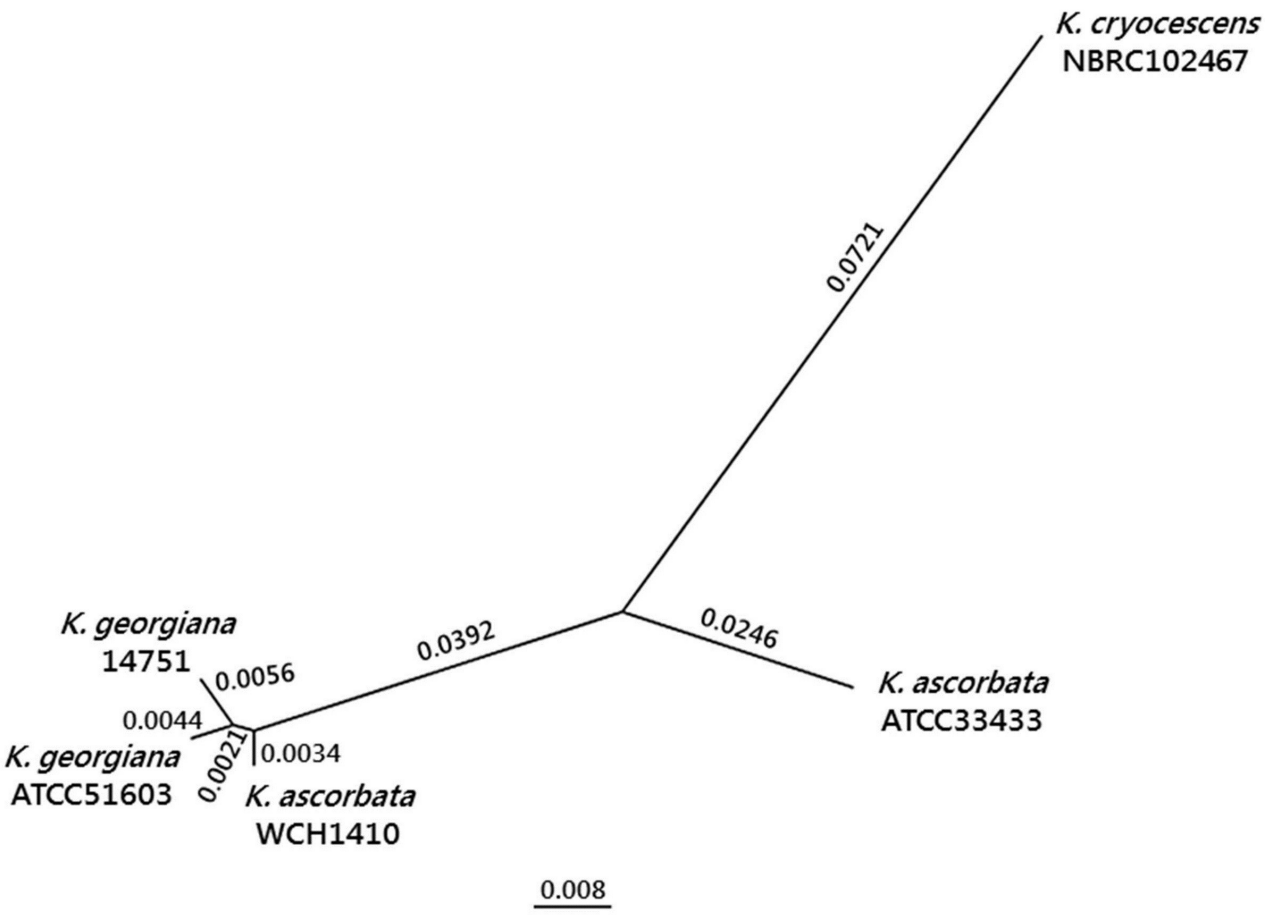

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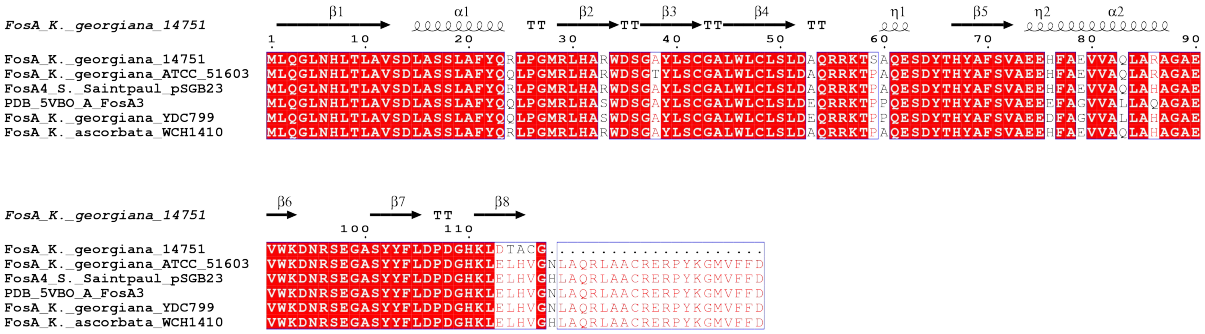

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