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Origin of the plasmid-mediated fosfomycin resistance gene *fosA3*

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Background: *fosA3* is the most commonly reported plasmid-mediated fosfomycin resistance gene among Enterobacteriaceae.

Objectives: To identify the origin of *fosA3*.

Methods: The chromosome of *Kluyvera georgiana* clinical strain YDC799 was fully sequenced with single-molecule real-time sequencing. Comparative genetic analysis was performed for *K. georgiana* YDC799, *K. georgiana* type strain ATCC 51603 and representative *fosA3*-carrying plasmids. *fosA* genes were cloned in *Escherichia coli* to confirm function.

Results: *K. georgiana* YDC799 harboured *fosA* (designated $fosA^{KG}$) and $bla_{CTX\text{-}M\text{-}8}$ on the chromosome. The genetic environments surrounding *fosA3* and bounded by IS*26* were nearly identical with the corresponding regions of *K. georgiana* YDC799 and ATCC 51603. The amino acid sequence of $FosA^{KG}$ from YDC799 and *K. georgiana* ATCC 51603 shared 99% and 94% identity with FosA3, respectively. Cloned $FosA^{KG}$ conferred fosfomycin resistance with an MIC of >1024 mg/L for *E. coli*.

Conclusions: The plasmid-mediated *fosA3* gene was likely mobilized from the chromosome of *K. georgiana* by an IS*26*-mediated event.

Introduction

Fosfomycin exhibits a broad-spectrum activity against Gram-positive and -negative bacteria by covalent modification of the *N*-acetylglucosamine enolpyruvyl transferase (MurA) active site.[1,2] FosA enzymes are Mn^{2+} and K^{+}-dependent glutathione S-transferases that inactivate fosfomycin in some Gram-negative bacteria rendering them resistant to this agent.[3] The *fosA* gene can be encoded on plasmids or chromosomes. The most commonly reported plasmid-mediated *fosA* is *fosA3*, which is widely distributed among ESBL-producing *Escherichia coli* in East Asia.[4,5] However, the origin of *fosA3* is unknown. Based on the sequence similarity of FosA3 with FosA encoded on the draft genome of *Kluyvera georgiana* ATCC 51603, we hypothesized that this species may serve as the reservoir of *fosA3*.

Methods

Bacterial strains

The following strains were used in this study: *E. coli* YD472, which carries *fosA3*;[6] *K. georgiana* YDC799, which was identified in an autopsy culture at the University of Pittsburgh Medical Center in 2017; and *K. georgiana* ATCC 51603, which was purchased from ATCC.

Susceptibility testing

Fosfomycin MICs were determined by the agar dilution method using Mueller–Hinton agar supplemented with and without 25 mg/L glucose-6-phosphate according to CLSI guidelines.[7] *E. coli* ATCC 25922 was used as the quality control strain. Inhibition of FosA activity by sodium phosphonoformate, an inhibitor of FosA,[8,9] was examined using the disc diffusion method.[10]

PCR and cloning of fosA

PCR was performed in *E. coli* YD472, *K. georgiana* YDC799 and *K. georgiana* ATCC 51603 using the following set of primers: FosA3_BamHI_F, GCGGATCCATGCTGCAGGGATTGAATC and FosA3_HindIII_R, GCAAGCTTAAG CTGAACTAACCCGTCA. To confirm the role of the promoter region in the expression of FosA, we used the following forward primer to generate PCR products of *fosA* with their promoter region: FosA3pr_BamHI_F, GCGGATCCATTTTATCGGGCGTATGAA. The PCR products were digested with restriction enzymes BamHI and HindIII, and ligated with cloning vector pBC-SK (Thermo Scientific, Waltham, MA, USA), which were then introduced into *E. coli* TOP10 (Thermo Scientific). Transformants were identified by growth on lysogeny broth agar plates containing 30 mg/L chloramphenicol and 20 mg/L fosfomycin, and the sequences were confirmed by Sanger sequencing.

Table 1. Characteristics of the *E. coli* and *K. georgiana* strains used in this study

Strains	Characteristics	MIC with G6P (mg/L)	MIC without G6P (mg/L)	Zone diameter (mm)	Zone diameter with PPF (mm)
E. coli YD472	contains *fosA3* on plasmid	>1024	>1024	6	6
K. georgiana YDC799	contains $fosA^{KG}$ on the chromosome	32	>1024	18	24
K. georgiana ATCC 51603	contains $fosA^{KG}$ on the chromosome	0.5	128	32	34
E. coli TOP10 (pFosA3)	carries *fosA3* from YD472 on pBC-SK	>1024	>1024	6	18
E. coli TOP10 ($pFosA^{KG\text{-}YDC799}$)	carries $fosA^{KG}$ from YDC799 on pBC-SK	>1024	>1024	6	18
E. coli TOP10 ($pFosA^{KG\text{-}ATCC\ 51603}$)	carries $fosA^{KG}$ from ATCC 51603 on pBC-SK	>1024	>1024	6	22
E. coli TOP10 (pBC-SK)	vector control	1	128	40	40
E. coli TOP10		1	64	38	38

MICs were determined by the agar dilution method with and without 25 mg/L glucose-6-phosphate (G6P). For disc testing, 1 mg of sodium phosphonoformate (PPF) was added to fosfomycin discs.

WGS

K. georgiana YDC799 was subjected to WGS using single-molecule real-time (SMRT) sequencing performed on an RS II instrument (Pacific Biosciences, Menlo Park, CA, USA) at the Yale Center for Genome Analysis.[11] A total of 56948 sub-reads with an average read length of 10258 bp were obtained. *De novo* assembly of the reads using the hierarchical genome assembly process (HGAP 3.0) available in the SMRT Analysis v2.3 software generated one contig with 94× coverage. This contig was then circularized and polished using the resequencing protocol in SMRT Analysis with two passes to reach the final consensus accuracy of 100%. The sequence was annotated through the Rapid Annotations using Subsystem Technology (RAST) Server.[12] The annotated sequence of YDC799 was compared with that of *K. georgiana* ATCC 51603 (LXEU01000090.1) as well as those of representative *fosA3*-carrying plasmids harboured by *E. coli* YD472 (KR078259.1), *E. coli* 5CRE51 (CP021177.1) and *E. coli* 21TF (JQ343849.1) by using NCBI BLAST analysis. *E. coli* 5CRE51 was identified in Taiwan and harbours *fosA3* and $bla_{NDM\text{-}9}$ on a single plasmid. *E. coli* 21TF was identified in Korea and harbours an IncN-type plasmid carrying *fosA3* and $bla_{CTX\text{-}M\text{-}14}$.[13] Antimicrobial resistance genes in YDC799 were identified by ResFinder.[14]

Results

Antimicrobial susceptibility

E. coli YD472 carrying *fosA3* exhibited a high fosfomycin MIC of >1024 mg/L. *K. georgiana* YDC799 and ATCC 51603 had fosfomycin MICs of 32 and 0.5 mg/L in the presence of glucose-6-phosphate, respectively (Table 1).[7] However, *E. coli* transformants harbouring *fosA3* and $fosA^{KG}$ (*fosA* from both *K. georgiana* YDC799 and ATCC 51603), either with or without native promoters, were all highly resistant to fosfomycin with an MIC of >1024 mg/L. The expansion of the growth inhibition zone around the fosfomycin discs for *K. georgiana* YDC799 and *E. coli* transformants carrying $fosA^{KG}$ upon the addition of sodium phosphonoformate corroborated FosA activity (Table 1).[8,9]

Sequence of K. georgiana FosA ($FosA^{KG}$)

We ran a BLASTn search of *fosA3* against the assembled genome sequences of *K. georgiana* YDC799 (this study; CP022114.1) and *K. georgiana* ATCC 51603 (NZ_LXEU00000000.1). As a result, we identified sequences with high similarity to *fosA3* in *K. georgiana* YDC799 and *K. georgiana* ATCC 51603, which were named $fosA^{KG\text{-}YDC799}$ and $fosA^{KG\text{-}ATCC\ 51603}$, respectively. The deduced amino acid sequences of FosA from *K. georgiana* YDC799 ($FosA^{KG\text{-}YDC799}$) and *K. georgiana* ATCC 51603 ($FosA^{KG\text{-}ATCC\ 51603}$) shared 99% (136/138 amino acids) and 94% (130/138 amino acids) identity with FosA3, respectively (Figure 1). The next closest FosA sequence available in GenBank was that of *Kluyvera ascorbata* WCH1410 with an identity of 93% (129/138 amino acids). Therefore, FosA3 is most closely related to $FosA^{KG}$ from *K. georgiana* among known chromosomally encoded FosA. All active site residues were conserved (H7, T9, W46, C48, S50, H67, K93, N95, S97, E98, G99, S101, Y103, E113 and R122) across the FosA3 and $FosA^{KG}$ proteins (Figure 1).

Location and genetic environments of $fosA^{KG}$ in K. georgiana YDC799 and ATCC 51603

Since the only publicly available *K. georgiana* genome sequence (strain ATCC 51603) consists of 163 contigs, we performed SMRT sequencing of *K. georgiana* YDC799 to determine the location and genetic environment of $fosA^{KG}$. SMRT sequencing with a single cell resulted in a closed chromosome after *de novo* assembly. There was no evidence of extra-chromosomal genetic elements, including plasmids. The YDC799 chromosome was ∼5.0 Mbp in size with a GC content of 55.0% and encoded 7557 genes, including hypothetical ones.

The 3.8 kb region containing $fosA^{KG}$ in *K. georgiana* YDC799 and ATCC 51603 shared 95% nucleotide identity and consisted of a truncated transcriptional regulator gene, *eutM*, *etuM*, $fosA^{KG}$, two additional ORFs and a second transcription regulator gene (Figure 2). Although the upstream transcriptional regulator gene in *K. georgiana* YDC799 was truncated, it shared 95% identity with that of *K. georgiana* ATCC 51603. The chromosomal sequences of the two *K. georgiana* strains diverged beyond this 3.8 kb region containing $fosA^{KG}$. In particular, an IS of the IS*110* family, members of which share a highly conserved tetrad motif with reverse transcriptase,[15] was located downstream of $fosA^{KG}$ in YDC799. In addition, the regions further upstream and downstream of $fosA^{KG}$ in YDC799 shared 96% (5641 bp) and 95% (12 955 bp) identity with those of *K. georgiana* ATCC 51603 at the nucleotide level, respectively.

The region downstream of $fosA^{KG}$ of the *K. georgiana* YDC799 chromosome shared 98% (1275 bp), 98% (2497 bp) and 98% (1897 bp) nucleotide identity with the corresponding regions downstream of plasmid-mediated *fosA3* in *E. coli* YD472, *E. coli* 5CRE51 and *E. coli* 21TF, respectively (Figure 2). These high levels of identity were only interrupted by ISs (two tandem copies of IS*26*

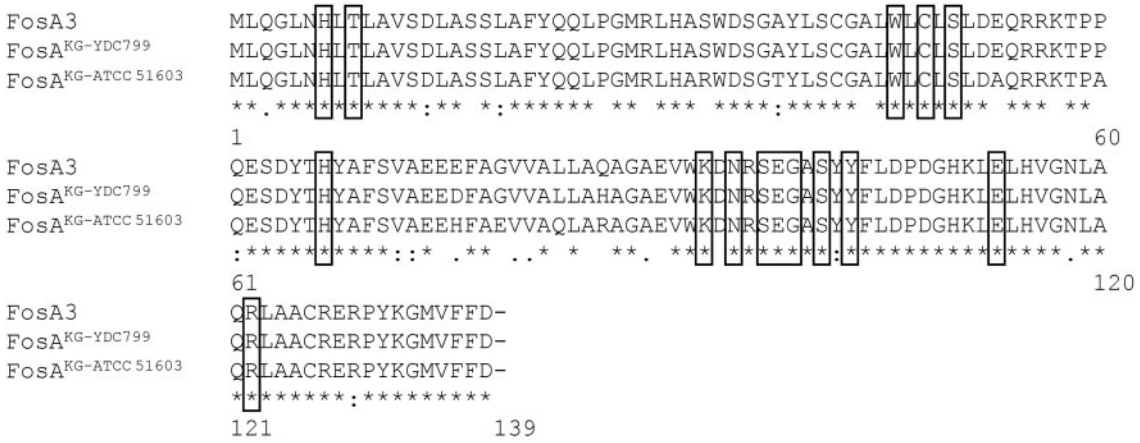

Figure 1. Amino acid alignment of FosA proteins in *E. coli* YD472, *K. georgiana* YDC799 and *K. georgiana* ATCC 51603. FosA$^{KG\text{-}ATCC\ 51603}$, FosA from *K. georgiana* ATCC 51603; FosA$^{KG\text{-}YDC799}$, FosA from *K. georgiana* YDC799.

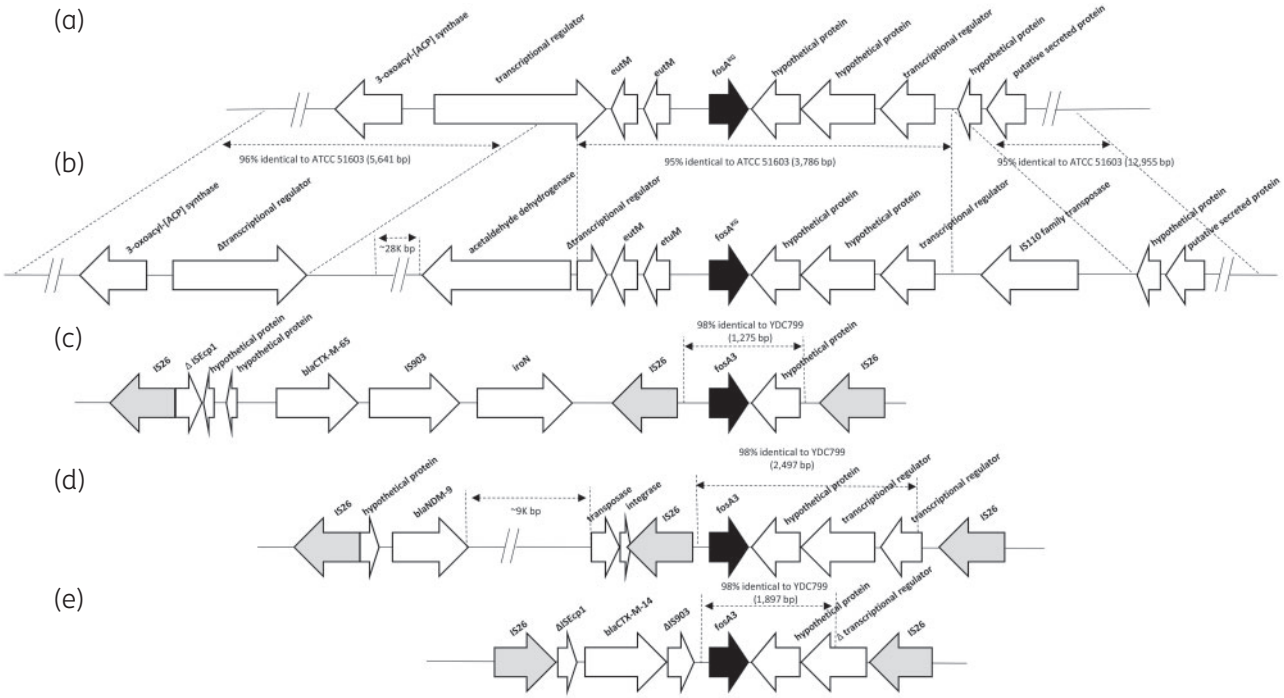

Figure 2. Genetic environment of FosA and the neighbouring regions in *K. georgiana* ATCC 51603 (a), *K. georgiana* YDC799 (b), *E. coli* YD472 (c), *E. coli* 5CRE51 (d) and *E. coli* 21TF (e).

for *E. coli* YDC472 and 5CRE51, and IS*26* and a truncated IS*903* in the case of *E. coli* 21TF). These data strongly suggest that *K. georgiana* served as the origin of *fosA3* and that IS*26* played a pivotal role in the acquisition of the plasmid-mediated *fosA3* gene.[13] Furthermore, truncation of the *fosA3*-containing regions by IS*26* and other ISs at various locations suggests that mobilization of *fosA3* may represent multiple independent events rather than a single event in the past.

Discussion

There is an increasing interest in using fosfomycin to treat infections due to MDR Gram-negative pathogens, in particular ESBL-producing *E. coli*. In this context, the emergence and dissemination of plasmid-mediated *fosA* in *E. coli* and other Enterobacteriaceae represents a major threat against its clinical utility. The origin of some plasmid-mediated *fosA* genes has been identified. For instance, *fosA*^{Tn2921} originated from the chromosome of *Enterobacter cloacae*, and *fosA5* and *fosA6* originated from the chromosome of *Klebsiella pneumoniae*.[10,16] However, the origin of *fosA3* has not been identified to date. Here, we provide evidence that *fosA3* was likely mobilized from the chromosome of *K. georgiana*. It has previously been postulated that *fosA3* may have originated from *K. pneumoniae* due to moderate sequence identity (~78%) of the *fosA* genes themselves as well as downstream of *fosA3* and chromosomal *fosA* of *K. pneumoniae* 342.[4,13]

However, the higher identity of $fosA^{KG}$ with *fosA3* including their genetic environments (~98%) suggests that the chromosome of *K. georgiana* is the more likely origin of *fosA3*.

While both *K. georgiana* strains used in this study were susceptible to fosfomycin, there was a significant difference in their MICs (32 mg/L for YDC799 versus 0.5 mg/L for ATCC 51603). The fosfomycin MICs for the transformants harbouring $fosA^{KG\text{-}YDC799}$ and $fosA^{KG\text{-}ATCC\ 51603}$ with the native promoter regions were over 1024 mg/L. While the reason for the discordance is unclear, we assume that $fosA^{KG\text{-}ATCC\ 51603}$ may be poorly expressed in ATCC 51603, a hypothesis that is also supported by the lack of inhibition of FosA activity by phosphonoformate.

Kluyvera is a genus of the Enterobacteriaceae family and members of this genus are generally considered to be non-pathogenic although there are rare reports of them as human pathogens.[17] Of interest, the β-lactamase gene $bla_{KLUG\text{-}1}$ located on the chromosome of *K. georgiana*, including YDC799, has been identified as the origin of $bla_{CTX\text{-}M\text{-}8}$, an ESBL gene frequently identified in *E. coli* in South America.[18] Our findings therefore underscore the importance of *Kluyvera* spp. as a reservoir of resistance genes that can be transferred to Gram-negative pathogens and render them resistant to clinically important antimicrobial agents.

In summary, our study identified the origin of plasmid-mediated *fosA3* as the chromosomal $fosA^{KG}$ gene in *K. georgiana.* While *fosA3* is currently prevalent in East Asia, this finding suggests that its mobilization may occur elsewhere independently as fosfomycin use increases globally.

Nucleotide sequence accession number

The finished chromosome sequence of *K. georgiana* YDC799 was submitted to GenBank under accession number CP022114.1.

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Transparency declarations

Y. D. has served on advisory boards for Meiji, Allergan, Curetis, The Medicines Company and Roche. All other authors: none to declare.

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