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Identification of a novel chromosome-encoded fosfomycin resistance gene fosC3 in Aeromonas caviae

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Background: Owing to the rapid emerging of multidrug-, even pandrugresistant pathogens, and lack of new antibiotics, the older antibiotic, fosfomycin, has been reused in recent years in the clinical practice, especially for treatment of uropathogen infections. With the increased use of fosfomycin, bacterial resistance to it has also increased drastically. Elucidating the resistance mechanism to the antimicrobial has become an urgent task.

Methods: The putative fosfomycin resistance gene *fosC3* was cloned, and minimal inhibitory concentrations were determined by the agar dilution method. Enzyme kinetic parameters were measured by high-performance liquid chromatography. Bioinformatics analysis was applied to understand the evolutionary characteristics of FosC3.

Results: The *A. caviae* strain DW0021 exhibited high level resistance to several antimicrobials including kanamycin, streptomycin, chloramphenicol, florfenicol, tetracycline, and especially higher to fosfomycin (> 1,024 µg/mL), while genome annotation indicated that no function-characterized resistance gene was associated with fosfomycin resistance. A novel functional gene designated *fosC3* responsible for fosfomycin resistance was identified in the chromosome of *A. caviae* DW0021. Among the function-characterized proteins, FosC3 shared the highest amino acid similarity of 58.65% with FosC2. No mobile genetic element (MGE) was found surrounding the *fosC3* gene. The recombinant pMD19-*fosC3/* DH5 α displayed a MIC value of 32 µg/mL to fosfomycin, which revealed a 128-fold increase of MIC value to fosfomycin compared to the control pMD19/*E. coli* DH5 α (0.25 µg/mL). FosC3 was phylogenetically close to FosC2 and exhibited a k_{cat} and K_m of 82,442 ± 1,475 s⁻¹, 70.99 ± 4.31 µM, respectively, and a catalytic efficiency of (1.2 ± 0.3) × 10³ µM⁻¹·s⁻¹.

Conclusion: In this work, a novel functional fosfomycin thiol transferase, FosC3, which shared the highest protein sequence similarity with FosC2, was identified in *A. caviae*. The fosfomycin inactivation enzyme FosC3 could effectively inactivate fosfomycin by chemical modification. It is implied that such mechanism facilitates *A. caviae* to respond to fosfomycin exposure, thereby enhancing survival. However, *fosC3* was not related with any MGE, which differs from many other fosfomycin thiol transferase genes. As a result, *fosC3* is not expected to be transmitted to other species through horizontal gene transfer mechanism. Our findings will contribute to the resistance mechanism of the common pathogenic *A. caviae*.

KEYWORDS

fosfomycin, Aeromonas caviae, FosC3, enzyme kinetics, antibiotic resistance

Introduction

Classified under the Gammaproteobacteria, order Aeromonadales, and family Aeromonadaceae, *Aeromonas* spp. are Gram-negative facultative anaerobes and isolated from numerous sources such as animals, water, soil, and food. As a result, both immunocompromised and immunocompetent humans are more likely to be infected by *Aeromonas* (Pessoa et al., 2022). Common diseases are gastroenteritis, bacteremia, septicemia, etc. (Fernández-Bravo and Figueras, 2020).

The wastewater discharged from hospitals and aquaculture farms is a significant source of multidrug-resistant pathogens and *Aeromonas* spp. in these aquatic environments exhibit a broad spectrum of antibiotic resistance profile (Pessoa et al., 2022). In addition, antibiotic resistance genes could be transmitted between *Aeromonas* via mobile genetic elements such as integrons and plasmids (Bello-López et al., 2019).

The resistance to fosfomycin tends to rise in the settings with use of more fosfomycin and among multi-drug resistant pathogens (Falagas et al., 2019). Three major fosfomycin resistance mechanisms are as follows: (i) reduced permeability to fosfomycin in consequence of mutated fosfomycin intake genes (uhpT etc.), (ii) mutations in the fosfomycin target gene murA, which takes part in the biosynthesis of peptidoglycan, and (iii) fosfomycin-inactivating enzymes (FosA etc.) (Zurfluh et al., 2020). Fosfomycin-inactivating enzymes are generally associated with mobile genetic elements and therefore have a critical impact in the horizontal transfer of fosfomycin resistance (Güneri et al., 2022). Antibiotic modification by fosfomycin-modifying enzymes is one of the acquired resistance mechanisms (Falagas et al., 2019). Inactivation of fosfomycin could be achieved through (i) addition of the sulphydryl group to C1 of the epoxide ring in fosfomycin (FosA and FosC2); (ii) nucleophilic addition of l-Cys or bacillithiol to fosfomycin (FosB); (iii) addition of H₂O to the C1 position of epoxide ring in fosfomycin (FosX); (iv) phosphorylation of the phosphate group to monophosphate (FomA and FosC) and conversion of monophosphate to diphosphate (FomB) in fosfomycin (Falagas et al., 2019).

Up to date, at least 10 types of *fos* genes have been discovered, e.g., *fosA*, *fosB*, *fosC*, and so on (Yang et al., 2019). *fosC2* is mainly responsible for fosfomycin resistance in Enterobacteriaceae (Yang et al., 2019). In this work, we identified and characterized a novel chromosome-encoded fosfomycin resistance gene designated *fosC3* from a *A. caviae* strain DW0021.

Materials and methods

Bacterial strains and plasmids

The bacteria and plasmids used in this work were listed in Table 1. DW0021 was isolated from a soil sample at an animal farm in Wenzhou, China. The taxonomic classification analysis was a combination of 16S rRNA gene homology and whole-genome average nucleotide identity (ANI). The recommended 95% threshold of ANI was used for species delimitation (Richter and Rosselló-Móra, 2009).

Minimum inhibitory concentration determination

The antimicrobial agents used in antimicrobial susceptibility testing were in Table 2, which included streptomycin, kanamycin, chloramphenicol, florfenicol, tetracycline, fosfomycin and so on. The antimicrobial susceptibility testing was performed following the antimicrobial susceptibility testing standard M100 (34th Edition, 2024) from the Clinical and Laboratory Standards Institute.

Molecular cloning of the fosC3 gene

Primers were designed using Primer Premier¹ (PREMIER Biosoft International, Palo Alto, CA) and SnapGene² (Table 3). The open reading frame (ORF) of *fosC3* with the promoter region was amplified and inserted into the T-Vector pMD19 with the T4 DNA ligase (Takara Bio, Inc., Dalian, China). The constructed recombinant plasmid was transformed into *E. coli* DH5 α using the calcium chloride method and cultured on Luria-Bertani agar plates containing 100 µg/mL ampicillin. The inserted sequences were verified by sequencing (Shanghai Sunny Biotechnology Co., Ltd., Shanghai, China).

Expression and purification of the FosC3 enzyme

The ORF of fosC3 was PCR-amplified and inserted into the pCold I vector with the cleavage sites of thrombin, restriction endonucleases EcoRI and XbaI (Table 3). The recombinant plasmid was transformed into E. coli BL21. FosC3 was overexpressed and purified as described previously (Qing et al., 2004). After the OD₆₀₀ reached 0.6 at 37°C, FosC3 enzyme induction occurred in the presence of 0.1 mM isopropyl- β -d-thiogalactoside, followed by 20 h cultivation at 16°C. Bacteria were centrifugated $(5,000 \times g, 10 \text{ min})$ at 4°C, resuspended in lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 3 mM β -mercaptoethanol, 0.5% Nonidet-P-40, pH 8.0), and disintegrated by sonication. The cell debris was eliminated through centrifugation $(12,000 \times g, 30 \text{ min})$ at 4°C. Subsequently, the lysates were incubated with pre-equilibrated nickel-nitrilotriacetic acid (Ni-NTA) agarose resin (Beyotime Biotechnology, Shanghai, China) for 8 h at 4°C under slow agitation. The recombinant protein purification was achieved using standard Ni-NTA affinity chromatography. The His6 tag was removed by incubation with thrombin for 4 h at 37°C. The concentration of the purified FosC3 protein was measured by SDS-PAGE and the BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, United States).

¹ https://www.premierbiosoft.com/primerdesign/index.html

² https://www.snapgene.com/

TABLE 1 Bacteria and plasmids used in this study.

Strain and plasmid	Description	References
		References
DW0021	The wild-type strain of A. caviae DW0021	This study
DH5a	<i>E. coli</i> DH5 α as a host for cloning of the <i>fosC3</i> gene	Our laboratory collection
BL21	E. coli BL21 as a host for expression of the fosC3 gene	Our laboratory collection
ATCC 25922	E. coli ATCC 25922 as the quality control for antimicrobial susceptibility testing	Our laboratory collection
pMD19- <i>fosC</i> 3/DH5α	DH5α carrying the recombinant plasmid pMD19-fosC3	This study
pCold I-fosC3/BL21	BL21 carrying the recombinant plasmid pCold I-fosC3	This study
pMD19	Cloning vector for the <i>fosC3</i> gene with its upstream promoter region, AMP ^r	Our laboratory collection
pMD19-fosC3	A recombinant plasmid of pMD19 carrying the <i>fosC3</i> gene with its upstream promoter region	This study
pCold I	Expression vector for the ORF of the <i>fosC3</i> gene, AMP'	Our laboratory collection
pCold I-fosC3	A recombinant plasmid of pCold I carrying the ORF of the fosC3 gene	This study

AMP^r, Ampicillin resistance.

TABLE 2 MICs (μ g/mL) of A. caviae DW0021, the cloned fosC3 gene and the controls.

Antibiotics	DH5α	pMD19/DH5α	<i>E. coli</i> ATCC 25922	pMD19- <i>fosC3/</i> DH5α	<i>A. caviae</i> DW0021*	Genes
Tobramycin	2	2	2	4	128 ^R	aph(3")-Ib, aph(6)-
Streptomycin	8	8	8	16	128 ^{NA}	Id, aac(6')-Ib9 × 2
Kanamycin	4	4	4	4	512 ^R	
Chloramphenicol	8	8	8	16	128 ^R	
Florfenicol	8	8	8	16	64 ^{NA}	cmlA5, floR
Tetracycline	4	4	4	4	128 ^R	tet(E), tet(A)
Fosfomycin	0.25	0.25	0.25	32	>1,024 ^R	fosC3
Ceftazidime	2	4	2	4	64 ^R	
Cefotaxime	2	2	2	2	2 ^s	bla _{OXA-10} , bla _{OXA-504} ,
Aztreonam	1	1	1	1	1 ^s	bla _{MOX-3}
Meropenem	1	1	1	1	1 ^s	-
Levofloxacin	1	1	0.5	1	32 ^R	qnrVC4
Sulfonamides	2	2	4	4	512 ^R	sul1

*Interpretive criteria were adapted from CLSI document M100 for Enterobacteriaceae. NA, Interpretive criteria not available; S, Sensitive; R, Resistant.

Enzyme kinetic parameter determination of the FosC3 enzyme

The kinetic assay was based on high-performance liquid chromatography (HPLC) and the methods described previously (Rigsby et al., 2005). The components of reaction mixture were listed in Table 4. After 5 min of incubation at 37°C, the reaction was terminated by 900 μ L solution contain 90 and 10% volume of mobile phase A (100 mM KH₂PO₃-H₃PO₃) and B (methanol), respectively. Then the mixture was centrifugated at 12,000 rpm for 10 min. 700 μ L supernatant was injected into a 250 mm × 4.6 mm Elite C-18 column (GL Sciences, Shanghai, China) with a flow rate of 800 μ L/min. Analysis was done by the Accela UHPLC system (Thermo Fisher Scientific, Rockford, IL, United States) under the 200 nm wavelength.

Whole genome sequencing, genome assembly, and annotation

The whole-genome sequencing was processed on the Illumina NovaSeq (paired-end, 2×150 bp) and PacBio RS II (20 kbp library) platforms (Shanghai Personal Biotechnology Co., Ltd., Shanghai, China), respectively. The genome sequence was obtained through Unicycler assembly pipeline (Wick et al., 2017). The long reads were assembled by miniasm (Li, 2016) and the genome was polished with Illumina short reads by Racon (Vaser et al., 2017). Protein-, tRNA-and rRNA-coding sequences (CDSs) were found by Prodigal (Hyatt et al., 2010), ARAGORN (Laslett and Canback, 2004) and Barrnap³, respectively. The promoter region was predicated by BPROM (Solovyev, 2011). The annotation of protein sequences was based on alignment of the predicted CDSs to the NCBI nr database (Sayers et al., 2021), the Swiss-Prot database (Bateman et al., 2023), and the Comprehensive Antibiotic Resistance Database (CARD) (Alcock et al., 2022) using DIAMOND blastp (Buchfink et al., 2021). The genome of DW0021 was served as the reference genome and the other 9 A. caviae genomes sharing the highest nucleotide identity with DW0021 were used for comparison (Table 5). Comparison of different A. caviae genomes was visualized by CGView Comparison Tool (Grant et al., 2012). The molecular weight and isoelectric point (pI) of protein sequences were calculated by EMBOSS pepstats (Rice et al., 2000).

³ https://github.com/tseemann/barrnap

Primer ¹	Sequence $(5' \rightarrow 3')^2$	Restriction endonuclease	Vector	Annealing temperature (°C)	Amplicon size (bp)
pro-fosC3-F	ATCGATGGGGTTGACCCCCGCAAAG		pMD19	Ľ	841
pro-fosC3-R	TGGGGCAGGCAGTCGTCACTAC		pMD19	/0	841
orf-fosC3-F	CCGGAATTCCTGGTGCCGCGGCGGCGGCGGCGGCTGCAGCCTGAACCATCTCAC	EcoRI+ Thrombin	pCold I	ç	438
orf-fosC3-R	TGC <u>TCTAGA</u> TCAGTCGAACCAGATAAGACCCTCATAGGG	XbaI	pCold I	C0	438
Primers started with "pro" The underlined sequences	were used to clone the <i>fosC3</i> gene with its promoter region; primers started with "orf" were used to clone the indicated the restriction endonuclease sites.	ORF of the $fosC3$ gene.			

TABLE 4 Components of reaction mixture for enzyme kinetic parameter determination.

Component	Concentration	Volume (µL)
H ₂ O	/	48
MnCl ₂	2.5 mM	2
Tris-HCl	1 M	10
KCl	1 M	10
GSH	10 mM	10
Enzyme	0.015 mg/mL	10
Fosfomycin	concentration gradients ^a	10

 $^{*}250,\,500,\,1,000,\,1,500,\,2,000,\,3,000,\,4,000,\,6,000\,\mu M.$

Phylogenetic tree reconstruction and model building

Entrez Direct⁴ and GNU Parallel (Tange, 2021) were used to retrieve sequences from the NCBI databases. Samtools (Danecek et al., 2021) and SeqKit (Shen et al., 2016) were used to manipulate fasta sequences. FosC3 and the other sequences were aligned with L-INS-i strategy by MAFFT (Katoh and Standley, 2013) and printed by r-msa (Bodenhofer et al., 2015). The maximum-likelihood tree of FosC3 and its homologous sequences was reconstructed with LG + G4 substitution model, tested by 1,000 bootstrap replicates and visualized by IQ-TREE 2 (Minh et al., 2020), UFBoot2 (Hoang et al., 2018) and ggtree (Yu, 2020), respectively. The structure of FosC3 and FosC3 bound fosfomycin was modeled by AlphaFold 3 and SWISS-MODEL using the fosfomycin resistance protein with bound fosfomycin (SMTL ID: 5v3d.1) as template (Waterhouse et al., 2018; Abramson et al., 2024).

Gene synteny analysis

The *fosC3* gene, along with its flanking regions, were used as queries and searched against the NCBI non-redundant nucleotide database to infer synteny. Comparison of gene clusters was generated by clinker (Gilchrist and Chooi, 2021).

Nucleotide sequence accession numbers

The GenBank accession numbers of the sequences of the *A. caviae* DW0021 chromosome, plasmid pAECA21-3829 and *fosC3* gene were CP128475, CP128476, and OR187734, respectively.

Results and discussion

Antibiotic resistance pattern and genomic analysis of DW0021

Among 13 antimicrobials tested, A. caviae DW0021 showed high MIC values ($\geq 32 \,\mu g/mL$) to 10 of them, including kanamycin

⁴ https://ftp.ncbi.nlm.nih.gov/entrez/entrezdirect/

Bacterium	Accession	Size (bp)	CDSs	tRNA	rRNA (5S, 16S, 23S)	Resistance genes	Virulence factors	Plasmids
DW0021	CP128475.1	4,589,869	4,086	126	11, 10, 10	11	56	1
FAHZZU2447	CP100392.1	4,540,521	4,254	124	11, 10, 10	11	59	2
NCTC12244	LS483441.1	4,586,140	4,048	123	11, 10, 10	2	76	0
FDAARGOS_72	CP062787.1	4,527,600	3,741	122	11, 10, 10	2	73	0
FDAARGOS_75	CP062801.1	4,551,146	3,871	122	11, 10, 10	2	73	0
211,703	CP092181.1	4,783,384	4,270	124	11, 10, 10	14	76	0
KAM376	AP024402.1	4,664,715	4,441	120	11, 10, 10	20	58	7
71,442	CP084350.1	4,444,683	3,922	122	11, 10, 10	7	41	0
R25-6	CP025705.1	4,702,913	4,146	123	11, 10, 10	11	48	1
NUITM-VA2	AP025280.1	5,035,951	4,473	123	11, 10, 10	31	41	0

TABLE 5 Comparison of the 9 genomes of A. caviae.

TABLE 6 Genome features of A. caviae DW0021.

Property	Chromosome	Plasmid (pAECA21-3829)
Accession number	CP128475	CP128476
Size (bp)	4,589,869	3,829
GC content (%)	61.43	55.68
CDS	4,086	6
Known protein	2,868	0
Hypothetical protein	1,218	6
Average protein length	322	113
tRNA	126	0
rRNA	31	0

(512 µg/mL), tobramycin (128 µg/mL), streptomycin (128 µg/mL), chloramphenicol (128 µg/mL), florfenicol (64 µg/mL), tetracycline (128 µg/mL), and especially higher to fosfomycin (>1,024 µg/mL) (Table 2).

To analyze the molecular resistance mechanism, the complete genome of A. caviae DW0021 was sequenced, and it consisted of a chromosome and a plasmid designated pAECA21-3829. The chromosome was 4,589,869 bp in length with 61.43% GC content, encoding 4,086 proteins, 126 tRNAs and 31 rRNAs. pAECA21-3829 was 3,829 bp in length, harboring 6 CDSs (Table 6). The 16S rRNA gene sequence analysis indicated that DW0021 was phylogenetically closer to Aeromonas spp., and genome wide sequence comparison of DW0021 with those genomes available in the NCBI genome database revealed that it shared the highest whole-genome ANI (97.99%) with the A. caviae type strain NCTC12244 (genome assembly accession number: GCA_900476005.1). Therefore, the strain DW0021 was classified into A. caviae and named A. caviae DW0021. The annotation result of antimicrobial resistance genes was listed in Table 7. Thirteen antimicrobial resistance genes ($\geq 80\%$ similarity with the function-characterized resistance genes in the CARD database) that related to six classes of antimicrobial agents were annotated, including aminoglycoside [aph(3")-Ib, aph(6)-Id, and two copies of aac(6')-Ib9], β -lactam (bla_{OXA-10} , $bla_{OXA-504}$, and *bla*_{MOX-3}), fluoroquinolone (*qnrVC4*), phenicol (*cmlA5* and *floR*), sulfonamide (sul1), and tetracycline [tet(E) and tet(A)]. All the antimicrobial resistance genes were encoded on the chromosome, while the plasmid pAECA21-3829 was free of an antimicrobial resistance gene.

Comparative genomic analysis of DW0021 and the other *A. caviae* strains was shown in Figure 1. It revealed that the genome of *A. caviae* DW0021 was similar to the other 9 *A. caviae* genomes in large part.

fosC3 showing resistance to fosfomycin

Although *A. caviae* DW0021 exhibited high level MIC to fosfomycin, the 13 predicted resistance genes from the whole genome were not associated with the resistance to the antimicrobial. A novel fosfomycin resistance mechanism would be present in *A. caviae* DW0021, which may be affiliated with an unidentified antimicrobial resistance gene. To confirm the speculation, the annotation result of the genome sequence was examined and the deduced protein sequence encoded by one *fosC2*-like gene sharing 99.25% coverage and 59.09% identity with FosC2 (BAJ10053.1) was found. The *fosC2*-like gene (designated *fosC3* in this work) was cloned and confirmed to be functional. The recombinant pMD19-*fosC3*/DH5 α displayed a 128-fold increase of MIC value to fosfomycin (32 µg/mL) compared to pMD19/DH5 α (0.25 µg/mL), however, no significantly reduced susceptibility to the other antimicrobial agents identified (Table 2).

Generally, the bacteria with the fosfomycin-modifying genes (*fos* and *fom* genes) showed high MIC levels to fosfomycin. For example, *P. syringae* PB-5123 carrying a *fosC* gene demonstrated

Locus	Coverage (%)	Identity (%)	E-value	Gene	Related antimicrobial class
DW0021-chr_00343	100.0	99.6	4.72E-202	aph(3″)-Ib	Aminoglycoside
DW0021-chr_00344	100.0	99.0	1.81E-150	aph(6)-Id	Aminoglycoside
DW0021-chr_00347	95.5	99.5	5.08E-142	aac(6')-Ib9	Aminoglycoside
DW0021-chr_00350	99.0	100.0	2.23E-144	aac(6')-Ib9	Aminoglycoside
DW0021-chr_00349	100.0	100.0	1.76E-192	bla _{OXA-10}	β-lactam
DW0021-chr_04216	100.0	97.3	3.71E-193	bla _{OXA-504}	β-lactam
DW0021-chr_02969	100.0	95.3	2.25E-267	bla _{MOX-3}	β-lactam
DW0021-chr_00346	100.0	100.0	7.46E-80	qnrVC4	Fluoroquinolone
DW0021-chr_00348	100.0	100.0	8.39E-300	cmlA5	Phenicol
DW0021-chr_00357	100.0	99.3	1.04E-274	floR	Phenicol
DW0021-chr_00366	100.0	100.0	2.32E-199	sul1	Sulfonamide
DW0021-chr_00333	100.0	99.8	1.03E-286	tet(E)	Tetracycline
DW0021-chr_00354	92.8	98.7	1.24E-160	tet(A)	Tetracycline

TABLE 7 Annotation of antimicrobial resistance genes in A. caviae DW0021.



FIGURE 1

Genome map of Aeromonas caviae DW0021 and other A. caviae strains. Circles (from outside to inside) 1–2, indicated forward and reverse strand of the DW0021 chromosome (CP128475.1); 3–11 were the chromosomes of A. caviae KAM376 (AP024402.1), A. caviae FAHZZU2447 (CP100392.1), A. caviae FDAARGOS_75 (CP062801.1), A. caviae FDAARGOS_72 (CP062787.1), A. caviae NUITM-VA2 (AP025280.1), A. caviae 211703 (CP092181.1), A. caviae NCTC12244 (LS483441.1), A. caviae 71442 (CP084350.1), and A. caviae R25-6 (CP025705.1), respectively; 12–13 were GC content and GC skew of the DW0021 chromosome, respectively.

TABLE 8	Enzyme kinetics	of FosC3 and ot	her fosfomycin-	inactivating enzymes.
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Enzyme	κ_{cat} (s ⁻¹)	$k_{\rm cat}/K_{\rm m}~({\sf M}^{-1}~{ m s}^{-1})$	References
FosC3	82,442 ± 1,475	$(1.2 \pm 0.3) \times 10^3$	This study
FosA3	112.4 ± 5.3	$(9.0 \pm 1.0) \times 10^3$	
FosA11	56.1 ± 3.2	$(2.9 \pm 0.5) \times 10^3$	Lu et al. (2024)
FosX	34 ± 2	$(9.0 \pm 2.0) imes 10^4$	Fillgrove et al. (2007)
FosX	0.15 ± 0.02	$(5.0 \pm 0.6) \times 10^2$	Fillgrove et al. (2003)
FosB	NAª	$(1.7 \pm 0.3) \times 10^5$	Cao et al. (2001)
FosA	180 ± 6	$(4.1 \pm 0.8) imes 10^4$	Rigsby et al. (2007)
FosA	80 ± 2	$(2.1 \pm 0.1) \times 10^5$	Rife et al. (2002)
FosA	180 ± 6	$(9.0 \pm 1.4) \times 10^{5}$	Brown et al. (2009)
FosA	42.1 ± 4.5	$(3.7 \pm 1.0) \times 10^3$	
FosA3	99.4 ± 3.3	$(8.0 \pm 1.9) \times 10^3$	Klontz et al. (2017)
FosA	140 ± 15	$(1.0\pm1.3) imes10^4$	

^aNot available.

a MIC of 1,024 mg/mL to fosfomycin (García et al., 1995). Compared to the recipients, the transformant DH10B (pS-fosC2) with fosC2 showed reduced susceptibility (MIC > 256 µg/mL, increased > 512-fold) (Wachino et al., 2010), and the FosC2^{AS}producing E. coli recombinant also showed high level of resistance to fosfomycin (MIC > 256 µg/mL, increased > 64-fold) (Ortiz de la Rosa et al., 2022). Other reports also revealed high or increased MIC levels when a strain harbored a fosfomycin resistance gene or a mutated one. E. coli KAM32/pSP72/Vf-murA had an MIC of 3,000 µg/mL to fosfomycin (Kumar et al., 2009). Amino acid substituted MurA found in resistant isolates further raised the MIC for fosfomycin by more than 8-fold (\geq 1,024 mg/L) compared with the strains expressing wild-type MurA (Takahata et al., 2010). The fosfomycin-resistant subpopulations overexpressed murA also resulted in a 10-fold increase (from 0.064 to 0.64 mg/L) (Campos da Campos et al., 2020). Another gene called abrp conferred a 4-fold decreased susceptibility to fosfomycin (from 64 mg/L to 256 mg/L) in A. baumannii (Li et al., 2016). Mutations in the structure of glpT and uhpT showed increased MICs to fosfomycin (≥ 256 mg/L) (Takahata et al., 2010).

The 402 bp ORF of fosC3 gene encoded a 133 amino acid enzyme with a molecular weight of 14.87 kDa and pI of 5.96. To further study the properties of the novel enzyme, FosC3 was overexpressed (Supplementary Figure S1A) and purified (Supplementary Figure S1B). Enzyme kinetic assays of FosC3 based on HPLC (Supplementary Figure S2) manifested a k_{cat} and $K_{\rm m}$ of 82,442 ± 1,475 s⁻¹, 70.99 ± 4.31 µM, respectively, which indicated that FosC3 could inactivate fosfomycin with a catalytic efficiency (k_{cat}/K_m) of $(1.2 \pm 0.3) \times 10^3 \,\mu M^{-1} \cdot s^{-1}$. Most kinetic analyses of fosfomycin thiol transferases were conducted on FosAs (Table 8). FosA typically demonstrated a high catalytic efficiency $(\geq 10^3)$, which may be a result of selection pressures exerted by the clinical use of fosfomycin. Although the k_{cat} of FosC3 $(82,442 \pm 1,475 \text{ s}^{-1})$ was significantly more than that of other fosfomycin thiol transferases, no notable difference of catalytic efficiency was observed. The results indicate that the FosC3 was probably less or equal active than the FosA enzyme variants.

Comparative analysis of *fosC3* with other *fos* genes

fosC3 was a novel fosfomycin-modifying gene. The phylogenetic tree of FosC3 and the other function-characterized fosfomycin-modifying enzymes was depicted in Figure 2. In the phylogenetic tree, FosC3 was on a branch that was close to FosC2 and FosG. Among the function-characterized Fos proteins, FosC3 shared the highest amino acid similarity of 58.65% (99.25% coverage and 59.09% identity) with FosC2. FosC3 also shared > 50% similarities with FosG, FosK, FosA5, FosA6, FosA1, FosA1, FosA8, FosA2, FosA3, FosA7.5, FosA4, and FosA7, but < 40% with the rest fosfomycin thiol transferases.

FosC3 possessed the similar functional residues that were similar to FosA. Although FosA and FosC2 were two distinct enzymes, both could inactivate fosfomycin through glutathione S-transferase activity (Wachino et al., 2010). FosA proteins possessed residues that were responsible for the dimer interface loop (Figure 3, amino acid residues 53 to 58 in red frames), Mn²⁺ (residues 7H, 62H, 108E, blue frames) and K⁺ binding (93E, green frames), and fosfomycin binding (9 T, 60Y, 88 K, 92S, 96S, 98Y, purple frames) (Klontz et al., 2017). Multiple sequence alignment of FosC3 and its homologous fosfomycin-modifying enzymes revealed that FosC3 contained the similar residues (Figure 3) and may be able to inactivate fosfomycin through glutathione S-transferase activity (Wachino et al., 2010). The notable divergence was related to the dimer interface loop among fosfomycin thiol transferases, while those residues involved in Mn²⁺ coordination, K⁺ binding, and fosfomycin binding were identical. FosC3, FosC2, FosG and FosK were five residues shorter, and that of FosA and FosL1 were three and two, which implied that the loop could cross the dimer interface relatively directly (Klontz et al., 2017). The enzyme was predicted to a homo-dimer (Supplementary Figure S3) with residues 9T, 46W, 60Y, 88K, 92S, 98Y, 117R to bind with fosfomycin. FosC3 was also predicted to form metal complexes with K⁺ (90N, 92S, 94G, 96S) and Mn²⁺ (7H, 62H, 108E).

Aeromonas spp. could be a reservoir of *fosC3* genes. It was found that 54 FosC3-like sequences (annotated as putative fosfomycin-modifying enzymes) sharing > 90.0% coverage and > 80.0% identity with FosC3 in the NCBI non-redundant database were all from



Aeromonas spp. Most of them were found in *A. caviae* (31/54, 57.4%), and the others were from *Aeromonas* spp. (16/54, 29.6%), *A. dhakensis* (4/54, 7.4%), *A. bivalvium* (2/54, 3.7%) and *A. enteropelogenes* (1/54, 1.9%), respectively (Supplementary Table S1).

The *fosC3* and *fosC3*-like genes were located within a conserved genomic region (Figure 4). No mobile genetic element was identified around the flanking regions of *fosC3* and *fosC3*-like genes (> 80% identity). *fosC3* was surrounded by upstream genes encoding FxsA family protein, aspartate ammonia-lyase, and anaerobic C4-dicarboxylate transporter, and downstream genes of *fosC3* were ribosomal protein and so on. The structure of the *fosC3*-related fragment was similar to several other chromosomal fragments of *A. caviae* strains. The result indicated that *fosC3* may be a gene in a conserved genomic region of *A. caviae* strains. *fosC2*^{AS} was also encoded in a region without MGE (Ortiz de la Rosa et al., 2022). However, other *fos* genes such as *fosC2* and *fosA* genes were found related with the MGEs. The *fosC2* gene

was found in a class 1 integron accompanied by *dfrA17* and *aadA5* encoded in a plasmid (Wachino et al., 2010). *fosA* was first discovered on Tn*2921* in a plasmid (Navas et al., 1990). Other plasmid-borne *fosA* genes such as *fosA3* (Wachino et al., 2010), *fosA5* (Ma et al., 2015), *fosA6* (Guo et al., 2016) and *fosA8* (Poirel et al., 2019) were also identified.

Conclusion

A novel gene *fosC3* that encoding fosfomycin thiol transferases was identified on the chromosome of *A. caviae* DW0021, and the kinetic and functional properties of the FosC3 enzyme were measured. FosC3 shared the highest amino acid similarity of 58.65% (99.25% coverage and 59.09% identity) with FosC2 and phylogenetically related to FosC2 and FosG. FosC3 was able to inactive fosfomycin with a catalytic efficiency (k_{cat}/K_m) of (1.2 ± 0.3) × 10³ µM⁻¹·s⁻¹. This work

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contributed to the study on mechanism of fosfomycin resistance in pathogenic *Aeromonas* species such as *A. caviae*.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Author contributions

JL: Methodology, Writing – original draft, Writing – review & editing, Data curation, Formal analysis. RZ: Data curation, Methodology, Writing – original draft, Formal analysis. YY: Data curation, Formal analysis, Methodology, Writing – original draft. HL: Conceptualization, Writing – review & editing. DL: Conceptualization, Methodology, Writing – review & editing. QB: Conceptualization, Methodology, Writing – review & editing. CF: Conceptualization, Formal analysis, Methodology, Visualization, Writing – original draft, Writing – review & editing.

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Alignments of the fosC3 and fosC3-like genes clusters. A grey linked region indicated a \geq 80% identity. fosC3 and fosC3-like genes were highlighted in red. Genes annotated with ORF were those without official gene symbols. hp, hypothetical genes.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2025.1577167/ full#supplementary-material

SUPPLEMENTARY FIGURE S1

(A) Over-expression of enzyme. lane 1, expression without IPTG induction; 2, sediment; 3, supernatant; 4, supernatant (pCold I-fosC3/BL21); 5, sediment (pCold I-fosC3/BL21); M, 180 kDa marker. (B) Purification of enzyme. Lane M, 10-180 kDa marker; lane 1-4, EK digestion for 12 h, 8 h, 4 h, and 0 h under 4°C, respectively; lane 5, FosC3.

SUPPLEMENTARY FIGURE S2

HPLC chromatogram. Retention time (0–6 min): 3.32 min: unknown peak, 3.97 min: product peak, 4.68 min: GSH peak, 5.05 min: unknown peak.

SUPPLEMENTARY FIGURE S3

(A) Cartoon representation of the structure of FosC3 and (B) FosC3 bound with fosfomycin (residues 9T, 46W, 60Y, 88K, 92S, 98Y, 117R). (C) Metal coordination with FosC3. K^+ : 90N, 92S, 94G, 96S and Mn²⁺: 7H, 62H, 108E.

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