

Emergence of *bla*_{OXA-1}- and *mph*(A)-Producing *Vibrio furnissii* Isolated from Hospital Sewage

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Abstract: Cephalosporins and azithromycin are clinical antibiotics used to treat infections. Co-resistance to cephalosporins and azithromycin has been observed in some *Enterobacterales*, but it has only rarely been reported in *Vibrio* species. In this study, we isolated a cephalosporin- and azithromycin-resistant *V. furnissii* strain, VFN3, from hospital sewage. Whole-genome sequencing results showed that the strain VFN3 possesses an IncA/C2 plasmid, pVFN3-*bla*_{OXA-193K}. This conjugative plasmid carries several clinically relevant drug resistance genes, including *mph*(A) and *bla*_{OXA-1}. We also found that in the strain VFN3, *mph*(A) and *bla*_{OXA-1} are surrounded by insertion sequences and class I integrons, respectively. These data suggest that mobile elements mediate the transfer of *mph*(A) and *bla*_{OXA-1}. This is the first reported *Vibrio* species that possesses an *mph*(A)- and *bla*_{OXA-1}-bearing conjugative plasmid. The emergence of this conjugative multi-drug-resistance plasmid is of great concern to public health.

Keywords: *mph*(A), *bla*_{OXA-1}, conjugative plasmid, *Vibrio furnissii*

Short Report

Cephalosporins and azithromycin are antimicrobial drugs commonly used to fight diseases caused by Gram-positive and Gram-negative pathogens, such as respiratory illness, skin infections, and diarrhea.^{1,2} However, a few bacteria have evolved to withstand both cephalosporin and azithromycin. For example, *Aeromonas* sp., *Escherichia coli*, and *Salmonella enterica* serotype Typhimurium strains encoding both *mph*(A) and *bla*_{OXA-1} have been reported.³⁻⁶ These two drug resistance genes have also been observed in clinical *Vibrio fluvialis* isolates. These isolates also possess other drug resistance genes, such as *bla*_{OXA-7}, *bla*_{CTX-M-3}, and *bla*_{NDM-1}, but the genetic backgrounds of these antibiotic resistance genes are unclear.⁷ *V. furnissii* is an emerging human pathogen. This marine bacterium can lead to acute gastroenteritis, bacteremia, cellulitis, and skin lesion.⁸⁻¹⁰ Hospital sewage is generally regarded as a reservoir of antibiotic-resistant bacteria, and the concentration of azithromycin in hospital sewage could be high.¹¹ However, little information about the prevalence of azithromycin-resistant *Vibrio* species from hospital sewage is available. In this study, we report one *V. furnissii* isolate recovered from hospital sewage in Zhuhai, a coastal city in China, carrying both *mph*(A) and *bla*_{OXA-1}, which are azithromycin and cephalosporin resistance genes, respectively.

Zhuhai People's hospital has one main hospital and three branch hospitals. In order to find out the distribution of azithromycin-resistant bacterial species in each hospital, 8 hospital sewage samples (2 samples/site) were collected from the main hospital area and its three branch hospitals within Zhuhai in April 2021. Then, 5 mL of each sample was added to 95 mL Luria-Bertani (LB) broth in a 250-mL flask with 100 mg/L azithromycin and incubated at 37°C overnight (X). Individual 100 µL aliquots of bacterial culture from each flask were spread on LB agar plates with 100 mg/L azithromycin. We used 100 mg/L azithromycin was due to the fact that a minimal inhibitory concentration (MIC) ≥32 mg/L has been proposed as the azithromycin resistance breakpoints, and a previous study showed that some diarrhoeagenic *E. coli* strains can withstand high concentration azithromycin (MIC >100 mg/L).¹² After incubation at

37°C overnight, colonies grown on the plates were obtained. To identify the azithromycin-resistant bacterial species, 16S rRNA genes were amplified and sequenced. Most of the azithromycin-resistant isolates were identified as *Enterobacterales*. However, one strain, VFN3, was identified as *Vibrio* species. It was recovered from the southern branch of Zhuhai People's Hospital sewage, which is less than 1.0 km from the coast. Given that azithromycin-resistant *Enterobacterales* species have been reported^{3,4,6,12} and azithromycin-resistant *Vibrio* species are rare, we focused on this strain in the following analysis. First, the broth microdilution method was used for antimicrobial susceptibility testing, and the results were interpreted based on the CLSI guidelines (for other antibiotics)¹³ and a published paper (for azithromycin).¹² The MIC data showed the strain VFN3 to be resistant to ampicillin, azithromycin, ceftiofur, ceftazidime, ciprofloxacin, chloramphenicol, ceftriaxone, gentamicin, kanamycin, streptomycin, and tetracycline but susceptible to tigecycline, meropenem, aztreonam and colistin (Table 1). Next, to investigate the mechanism by which strain VFN3 is resistant to azithromycin, its genome was sequenced. The short-read and long-read sequencing data were obtained using Illumina HiSeq 2500 and an Oxford Nanopore MinION sequencer. To obtain the complete genome sequences of strain VFN3, both short-read and long-read data were combined to perform hybrid *de novo* assembly with Unicycler v0.4.4.¹⁴ RAST server (<https://rast.nmpdr.org>) was used for genome annotation. ResFinder (<https://cge.cbs.dtu.dk/services/ResFinder/>) was used to identify potential antibiotic resistance genes. PlasmidFinder (<https://cge.cbs.dtu.dk/services/PlasmidFinder/>) was utilized to screen the plasmid replicon type. BLAST Ring Image Generator (BRIG) and Easyfig were used for sequence comparisons and visualizations.^{15,16}

Strain VFN3 harbors two chromosomes and one plasmid. Based on Ribosomal MLST (<https://pubmlst.org/species-id>) with whole-genome sequences, strain VFN3 was identified as *V. furnissii*. In strain VFN3, chromosome I is 3,404,211 bp

Table 1 Minimal Inhibitory Concentrations (MICs) of Strains Against Different Antimicrobials Used in This Study

Antibiotic	MIC (μg/mL)		
	<i>V. furnissii</i> VFN3	<i>E. coli</i> C600 Transconjugant	<i>E. coli</i> C600
Tigecycline	≤0.25	≤0.25	≤0.25
Tetracycline	32	32	4
Meropenem	≤0.25	≤0.25	≤0.25
Ceftiofur	8	8	0.5
Ceftriaxone	8	8	≤0.25
Ceftazidime	16	16	≤0.25
Azithromycin	150	150	0.5
Chloramphenicol	128	128	8
Ciprofloxacin	32	16	≤0.25
Kanamycin	16	16	4
Gentamicin	16	16	0.5
Streptomycin	64	64	4
Aztreonam	2	2	1
Ampicillin	>128	>128	8
Colistin	≤0.25	≤0.25	≤0.25
Trimethoprim-sulfamethox	120	120	1
Amoxicillin-clavulanic acid	64	32	4

with 50.50% GC content and chromosome II is 1,588,410 bp with 50.90% GC content. No drug resistance gene was detected in the chromosomes. However, we found an IncA/C2 replicon-type plasmid, pVFN3-blaOXA-193K (193,896 bp and 52.79%GC content), carrying several antibiotic resistance genes, including *tet(D)*, *floR*, *aac(3)*, *sul2*, *sul1*, *bla_{OXA-1}*, *mph(A)*, *aac(6')lb-cr*, and *catB3* (Figure 1). To the best of our knowledge, pVFN3-blaOXA-193K is the first reported plasmid that carries both *mph(A)* and *bla_{OXA-1}* in *Vibrio* species. BLAST analysis showed that pVFN3-blaOXA-193K shares 99% identity with pEC-13-49-NDM-1 at 80% coverage, pKP-16-57-NDM-1 at 80% coverage, pPV835TEM24 at 77% coverage, and pVC1447 at 77% coverage, hosted by *E. coli*, *Klebsiella pneumoniae*, *Proteus vulgaris* and *V. cholerae*, respectively (Figure 1). Unlike those of pVFN3-blaOXA-193K, pEC-13-49-NDM-1 and pKP-16-57-NDM-1, neither pPV835TEM24 nor pVC1447 encodes *mph(A)*. Further analysis showed that *mph(A)* in pVFN3-blaOXA-193K is surrounded by insertion sequences, IS6100 and IS5075. Similar genetic structures were observed in pEC732_IMP14, pSA70-2, pKP27_NDM1 and the chromosome of *Morganella morganii* strain GDMM86 (Figure 2A). These data indicated that the insertion sequences could mediate *mph(A)* transfer. We also found that, in pVFN3-blaOXA-193K, *bla_{OXA-1}* is located in a class I integron with the structure *tnpA-intI1-aac(6')lb-cr-bla_{OXA-1}-catB3-arr3-qacEΔ1-sul1-ISCRI1*. Similar genetic structures were observed in pRIVM_C014947_1, p50935_IncHI2, and pSL131_IncHI2, hosted by *K. pneumoniae*, *Citrobacter freundii*, and *S. enterica*, respectively. In the chromosome of *M. morganii* strain ZJG944, *bla_{OXA-1}* was also detected, and a similar genetic structure with that of pVFN3-blaOXA-193K was observed (Figure 2B). These results suggest that class I integron facilitates the spread of *bla_{OXA-1}*.

A conjugation assay was performed to test the transferability of pVFN3-blaOXA-193K. Briefly, strain VFN3 and *E. coli* C600 (rifampin resistance) were incubated in LB broth at 37°C overnight. Next, 100 μL of strain VFN3 and 500 μL of *E. coli* C600 were mixed. After centrifugation for 2 min at 12,000 rpm, cells were resuspended in 100 μL LB broth, and co-cultures were incubated at 37°C for 18 h. Transconjugants were selected using Eosin-Methylene Blue agar plates with 100 mg/L

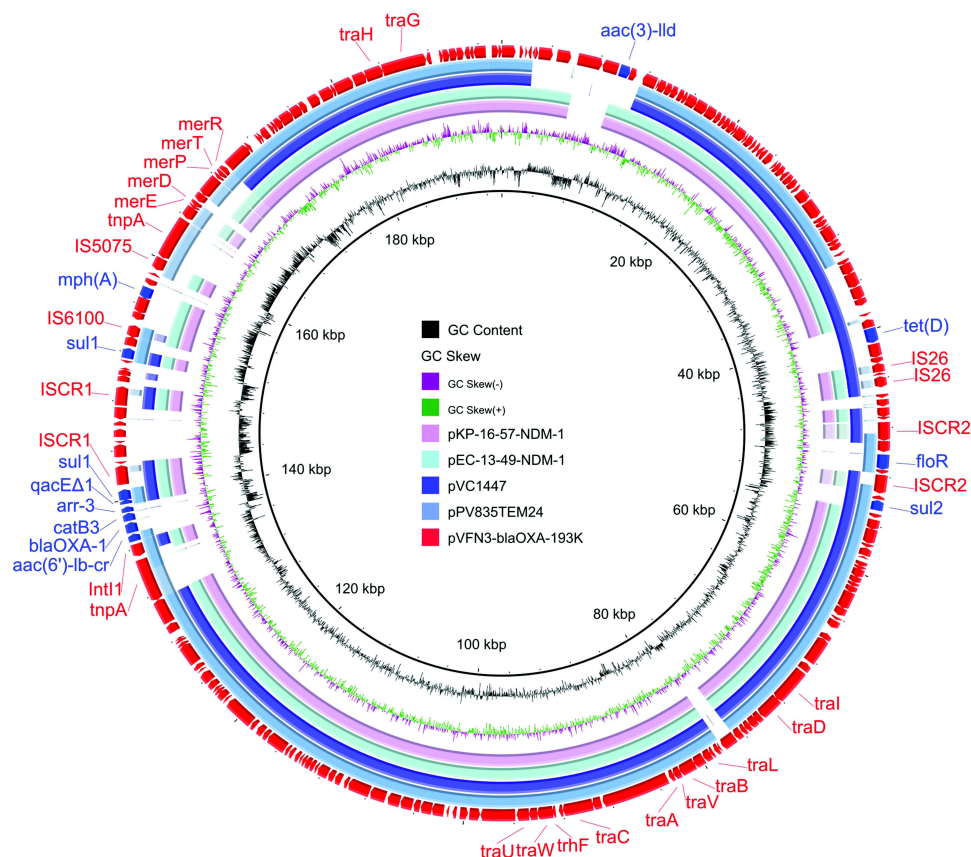


Figure 1 Circular comparisons of pVFN3-blaOXA-193K and related plasmids, pEC-13-49-NDM-1, pKP-16-57-NDM-1, pPV835TEM24, and pVC1447, available in the NCBI database (<https://www.ncbi.nlm.nih.gov/>). The outermost circle denotes pVFN3-blaOXA-193K, with arrows indicating coding genes.

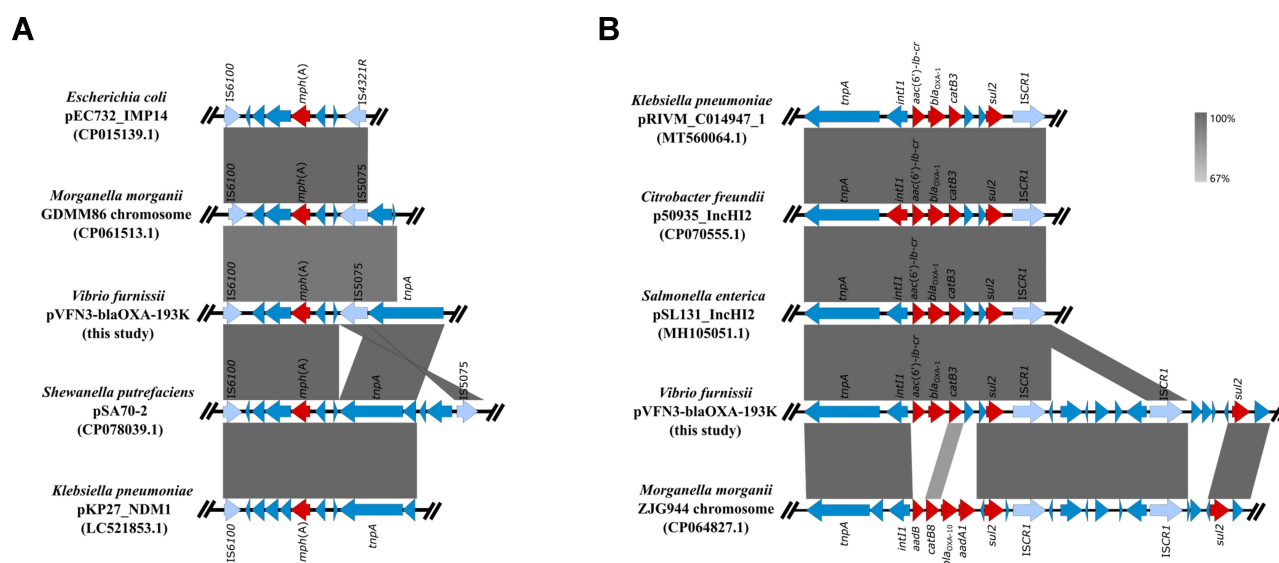


Figure 2 Sequence comparisons of *mph(A)* and *bla_{OXA-1}* genetic environments. **(A)** Linear comparisons of the *mph(A)* genetic environment in pVFN3-blaOXA-193K with other related *mph(A)* encoding bacteria. **(B)** Linear comparisons of the *bla_{OXA-1}* genetic environment in pVFN3-blaOXA-193K with other related *bla_{OXA-1}* encoding bacteria. Red arrows indicate the resistance genes.

azithromycin plus 200 mg/L rifampicin. The transconjugant was confirmed by PCR. Conjugation assay revealed that pVFN3-blaOXA-193K can transfer to *E. coli* C600, rendering the transconjugant resistant to many drugs (Table 1).

To determine whether other *V. furnissii* strains possess azithromycin resistance gene, we downloaded all genome sequences of *V. furnissii* strains deposited in NCBI in April 2022. Using ResFinder, we found that no *V. furnissii* strain encodes azithromycin resistance gene except strain VFN3.

In conclusion, this study isolated the azithromycin- and cephalosporin-resistant *V. furnissii* strain VFN3 from hospital sewage. This strain harbors an ≈193kb conjugative plasmid, pVFN3-blaOXA-193K. In this IncA/C2 plasmid, *mph(A)*, *bla_{OXA-1}*, and other antibiotic resistance genes were identified. This multi-drug resistant pathogen may pose a threat to public health. Further research is needed to investigate the prevalence of pathogens carrying multi-drug-resistance plasmids in hospital sewage.

Data Sharing Statement

The complete sequences of strain VFN3 were submitted to the NCBI database with accession numbers CP089603-CP08960.

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Disclosure

The authors declare no competing interests in this work.

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