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Emergence of plasmid-mediated RND-type efflux pump gene cluster *tmexCD-toprJ* in *Shewanella xiamenensis* in a water environment

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Abstract

The emergence of the mobile resistance-nodulation-division (RND)-type efflux pump *tmexCD-toprJ* gene cluster that confers multidrug resistance (MDR), including tigecycline resistance, in gram-negative bacteria poses a global public health threat. However, the spread of such clinically important antimicrobial resistance genes (ARGs) in the natural environment has not yet been well investigated. In this study, we investigated MDR aquatic bacteria in Vietnam. A carbapenem- and tigecycline-resistant *Shewanella xiamenensis* isolate NUITM-VS2 was obtained from urban drainage in Hanoi, Vietnam, in October 2021. *S. xiamenensis* NUITM-VS2 showed resistance to most antimicrobials tested, including tigecycline, tetracyclines, carbapenems, cephalosporins, fluoroquinolone, and aminoglycosides. Whole-genome analysis was performed by long- and short-read sequencing, resulting in the complete genome sequence consisting of one chromosome and five plasmid sequences. ARGs and plasmid replicons in the genome were detected using ResFinder with the custom ARG database, including all known tigecycline resistance genes, and PlasmidFinder, respectively. A 152.2-kb IncC plasmid, pNUITM-VS2_2, co-carried two mobile tigecycline resistance genes, *tet*(X4) and *tmexC3.1D3.1-toprJ1*. In addition, a 24.8-kb untypeable plasmid, pNUITM-VS2_4, carried the carbapenemase gene $bla_{\text{NDM-1}}$. pNUITM-VS2_2 was transferred to *Escherichia coli* by conjugation, which simultaneously conferred high-level resistance against many antimicrobials, including tigecycline. To the best of our knowledge, this is the first report of the detection of the mobile RND-type efflux pump gene cluster *tmexCD-toprJ* in *Shewanella* species. Our results provide genetic evidence of the

47 complexity of the dynamics of clinically important ARGs among aquatic bacteria,

48 which could be important reservoirs for ARGs in the natural environment.

49

50 **Main text**

51 **Introduction**

52 Tigecycline is a semi-synthetic antimicrobial agent, which was the first drug of
53 the glycylcycline class developed for the last-line treatment of infections caused
54 by multidrug-resistant (MDR) bacterial pathogens, especially carbapenem-
55 resistant *Enterobacterales*. However, due to the widespread use of this
56 antimicrobial in clinical settings, novel antimicrobial resistance genes (ARGs),
57 including the mobile tigecycline-inactivation enzyme gene *tet*(X) and the
58 resistance-nodulation-division (RND)-type efflux pump gene cluster *tmexCD-*
59 *toprJ*, both conferring high-level tigecycline resistance, have rapidly been
60 disseminated between diverse gram-negative bacteria (1). This not only poses
61 an economic burden to the healthcare system but also increases mortality risks
62 in patients by disabling the availability of last-resort antimicrobial agents, such as
63 tigecycline.

64 *Shewanella* species are gram-negative anaerobes found in marine and
65 freshwater environments, which belong to the family Shewanellaceae within the
66 class Gammaproteobacteria. *Shewanella* are natural reservoirs of bla_{OXA-48}-like
67 carbapenemase genes, such as bla_{OXA-48} and $bla_{OXA-181}$, encoded on the
68 chromosomes (2, 3). In 2022, we reported a *Shewanella xiamenensis* isolate
69 obtained from a water environment in Vietnam, which harbored bla_{OXA-48} and
70 *tet*(X4) on the chromosome (4).

71 In 2021, we reported a *Klebsiella aerogenes* isolate harboring a self-
72 transferable incompatibility group C (IncC)–IncX3 hybrid plasmid co-carrying
73 bla_{NDM-4}, *tet*(X4), and *tmexCD3-toprJ1* [initially named *tmexCD3-toprJ3* (5) and

74 later renamed (6)] from a water environment in Vietnam (7). Although the
75 *Flavobacteriaceae* family is estimated as the natural reservoir of *tet*(X) genes (8),
76 limited information is available on the distribution of *tmexCD-toprJ* in the natural
77 environment.

78 In this study, we report an *S. xiamenensis* isolate obtained from the aquatic
79 environment in Vietnam harboring *tmexCD-toprJ*, representing the first such
80 report for *Shewanella* species. The isolate showed carbapenem and tigecycline
81 resistance, and harbored $bla_{\text{OXA-181}}$ on the chromosome, $bla_{\text{NDM-1}}$ on a 24.8-kb
82 untypeable plasmid, and *tmexC3.1D3.1-toprJ1* together with *tet*(X4) on a self-
83 transferable 152.2-kb IncC plasmid.

84

85 **Results and discussion**

86 An environmental carbapenem- and tigecycline-resistant bacterial isolate,
87 NUITM-VS2, was identified as *Shewallena* species using MALDI Biotyper. The
88 minimum inhibitory concentrations (MICs) of tigecycline, tetracyclines,
89 carbapenems, cephalosporins, and gentamycin were all above 128 mg/L (R); and
90 those of ciprofloxacin, tobramycin, and streptomycin were 4, 16, and 64 mg/L (R),
91 respectively; whereas that of amikacin was 0.5 mg/L (S) (Table 1).

92 Whole-genome analysis using the Illumina and Oxford Nanopore Technologies
93 (ONT) sequencing platforms resulted in the complete genome sequence
94 consisting of one chromosome and five plasmids, designated pNUITM-VS2_1,
95 pNUITM-VS2_2, pNUITM-VS2_3, pNUITM-VS2_4, and pNUITM-VS2_5
96 (accession nos. AP026732, AP026733, AP026734, AP026735, AP026736, and
97 AP026737), respectively. Genome annotation and average nucleotide identity
98 (ANI) analyses revealed that the 4.9-Mb chromosome with a 46.4% GC content
99 contains 4,100 coding sequences (CDSs), in which $bla_{OXA-181}$ was detected by
100 ResFinder, showed 97.3% identity to the genome sequence of *S. xiamenensis*
101 JCM 16212^T (accession no. BMOP01000000).

102 The 152.2-kb pNUITM-VS2_2 plasmid of *S. xiamenensis* NUITM-VS2
103 contained a replicon classified to IncC and co-carried two mobile tigecycline
104 resistance genes, *tet*(X4) and a variant of *tmexCD3-toprJ1*, whose sequence was
105 a perfect match to that reported in the *K. aerogenes* pNUITM-VK5_mdr plasmid
106 (accession no. LC633285) isolated from the same environmental location as
107 NUITM-VS2 in Hanoi, Vietnam, in February 2021 (7) (Fig. 1). The CDSs of
108 *tmexCD-toprJ* in pNUITM-VS2_2 and pNUITM-VK5_mdr showed high identity to

109 *tmexCD3-toprJ1* of *Proteus mirabilis* RGF134-1 (accession no. CP066833) (5)
110 isolated from a pig in China in 2019. The identity for *tmexC3* was 99.7%
111 (1,161/1,164 nt), with three amino acid substitutions (Q187H, T256M, and
112 A386T). For *tmexD3*, the identity was 99.9% (3,133/3,135 nt), including two
113 amino acid substitutions (V610L and L611F). For *toprJ3*, the identity was 100%
114 (1,434/1,434 nt). Therefore, we named the variant identified in this and previous
115 studies *tmexC3.1D3.1-toprJ1*, consisting of *tmexC3.1*, *tmexD3.1*, and *toprJ1*.
116 Moreover, the carbapenemase gene bla_{NDM-1}, fluoroquinolone resistance gene
117 *qnrVC6*, and aminoglycoside resistance genes *aac(3)-IId* and *aac(6')-IIa* were
118 encoded in another 24.8-kb untypeable plasmid, pNUITM-VS2_4, that is highly
119 identical to the *S. xiamenensis* 26.8-kb untypeable plasmid pSx1 (100% identity
120 with 96% of the region of accession no. CP013115) isolated from the environment
121 in Algeria in 2012 (9). pSx1 was previously classified as an IncP-2 plasmid (10),
122 which was subsequently found to be a misidentification (11). The replicon type of
123 pNUITM-VS2_4 and pSx1 could not be identified by PlasmidFinder; thus, this
124 plasmid type is presumed to be unique to *Shewanella* species.
125 On pNUITM-VS2_2 in *S. xiamenensis* NUITM-VS2, *tet*(X4) was flanked by two
126 mobile genetic elements, IS*Vsa3* and IS*91* family transposase, while
127 *tmexC3.1D3.1-toprJ1* was flanked by Δ*tnpA* and IS*L3*. Interestingly, pNUITM-
128 VS2_2 was almost identical to the IncC backbone of the *K. aerogenes* IncC–
129 IncX3 hybrid plasmid pNUITM-VK5_mdr (100% identity with 38% of the region of
130 accession no. LC633285) (7) (Fig. 1). The rest of the IncX3 backbone of pNUITM-
131 VK5_mdr carried additional ARGs, such as bla_{NDM-4} and *qnrS1*, conferring
132 resistance to carbapenem and fluoroquinolone, respectively, suggesting that the

133 MDR IncC–IncX3 hybrid plasmid pNUITM-VK5_mdr resulted from fusion of a
134 tigecycline-resistance IncC plasmid such as pNUITM-VS2_2 with a carbapenem-
135 resistance IncX3 plasmid.

136 A bacterial conjugation assay using *Escherichia coli* J53 as the recipient strain
137 showed that *S. xiamenensis* NUITM-VS2 transferred pNUITM-VS2_2 to J53 at a
138 frequency of 6.4 x 10^{-5} after overnight co-culture at 37°C. PCR confirmed that the
139 transconjugant strain (J53/pNUITM-VS2_2) co-harbored *tet*(X4) and
140 *tmexC3.1D3.1-toprJ1*, and was resistant to tigecycline and tetracyclines (Table
141 1).

142

143 **Conclusions**

144 In conclusion, the complete genome sequence of the carbapenem- and
145 tigecycline-resistant isolate *S. xiamenensis* NUITM-VS2 obtained from a water
146 environment in Vietnam was characterized. This isolate co-carried two mobile
147 tigecycline resistance genes, *tet*(X4) and *tmexC3.1D3.1-toprJ1*, in the self-
148 transferable 152.2-kbp IncC plasmid. This plasmid corresponds to the IncC
149 backbone of the IncC–IncX3 hybrid plasmid pNUITM-VK5_mdr identified in *K.*
150 *aerogenes* in our previous report that was isolated from the same area (7),
151 suggesting that they might share a common ancestor. Our results provide genetic
152 evidence that clinically important ARGs have been disseminated though plasmids,
153 such as broad-host-range IncC plasmids, and their fusion plasmids among
154 environmental bacteria, such as *Shewanella* species, in aquatic environments.

155

156 **Materials and methods**

157 **Bacterial isolation and antimicrobial susceptibility testing**

158 Carbapenem- and tigecycline-resistant *S. xiamenensis* NUITM-VS2 was
159 isolated from the Kim-Nguu River in Hanoi, Vietnam, in October 2021.
160 Environmental water sample was collected and cultured using Luria-Bertani (LB)
161 broth containing 4 mg/L of meropenem at 37°C overnight, and then further
162 selected and isolated using CHROMagar COL-APSE (CHROMagar
163 Microbiology) containing 4 mg/L of tigecycline. Bacterial species identification
164 was performed using MALDI Biotyper (Bruker). Antimicrobial susceptibility testing
165 using *Escherichia coli* ATCC 25922 as quality control was performed with agar
166 dilution according to the CLSI 2020 guidelines. MIC breakpoints of antimicrobials
167 (S: susceptible; I: intermediate; R: resistant) for other non-*Enterobacterales* were
168 also determined according to the CLSI 2020 guidelines.

169

170 **Whole-genome sequencing and subsequent bioinformatics analysis**

171 Whole-genome sequencing of *S. xiamenensis* NUITM-VS2 was performed
172 using MiSeq (Illumina) with MiSeq Reagent Kit v2 (300-cycle) and MinION
173 (Oxford Nanopore Technologies; ONT) with the R9.4.1 flow cell. The library for
174 Illumina sequencing (paired-end, insert size of 300–800 bp) was prepared using
175 the Nextera XT DNA Library Prep Kit, and the library for MinION sequencing was
176 prepared using the Rapid Barcoding Kit (SQK-RBK004). ONT reads were base-
177 called using Guppy v5.0.11, in the super-accuracy mode. Hybrid de novo
178 assembly with both Illumina and ONT reads was performed using Unicycler
179 v0.4.8.0 (https://github.com/rrwick/Unicycler) (12) with default parameters.

180 Genome annotation and average nucleotide identity (ANI) analyses were
181 performed using the DFAST server (https://dfast.nig.ac.jp) (13). ARGs and
182 plasmid replicons were detected using ResFinder v4.1
183 (https://bitbucket.org/genomicepidemiology/resfinder/) (14) with the custom ARG
184 database, including all known tigecycline resistance genes, and PlasmidFinder
185 v2.1 (https://bitbucket.org/genomicepidemiology/plasmidfinder/) (15) with the
186 default parameters, respectively. Type IV secretion system (T4SS)-associated
187 genes involved in conjugation were detected by CONJscan v1.0.5
188 (https://github.com/macsy-models/TXSS) with default parameters (16). Linear
189 comparison of sequence alignment was performed using BLAST and visualized
190 by Easyfig v.2.2.2 (http://mjsull.github.io/Easyfig/) (17).
191
192 **Bacterial conjugation assay**
193 A bacterial conjugation assay was performed as follows. LB broth cultures of
194 the donor *S. xiamenensis* NUITM-VS2 and the recipient azide-resistant *E. coli*
195 J53 (ATCC BAA-2731, F^- *met pro* Azi^r) were mixed in a 1:10 ratio, spotted onto
196 MacConkey agar, and then incubated at 37°C overnight. Subsequently, the
197 mixed cells, including transconjugants, were suspended in LB broth and then
198 plated onto MacConkey agar containing 1 mg/L of tigecycline and 100 mg/L of
199 sodium azide after 10-fold serial dilution and incubated at 37°C overnight.
200 *tet*(X) and *tmexCD-toprJ* in transconjugants were detected by colony PCR
201 using the following primer sets: tetX_F, CCCGAAAATCGWTTTGACAATCCTG;
202 tetX_R, GTTTCTTCAACTTSCGTGTCGGTAAC; tmexC_F,
203 TGGCGGGGATCGTGCTCAAGCGCAC; tmexC_R,

204 CAGCGTGCCCTTGCKCTCGATATCG. The PCR amplification consisted of the
205 initial denaturation for 3 min at 95°C, followed by 35 cycles of denaturation for
206 30 s at 95°C, annealing for 30 s at 54°C, and extension for 30 s at 72°C, and then
207 the final extension for 5 min at 72°C. Genomic DNAs of *K. aerogenes* strain
208 NUITM-VK5 co-harboring *tet*(X4) and *tmexC3.1D3.1-toprJ1* (7) was used as
209 positive controls. Purified water was used as a negative control.
210

211 **Nucleotide sequence accession numbers**

212 The complete genome sequence of *S. xiamenensis* NUITM-VS2 has been
213 deposited at GenBank/EMBL/DDBJ under accession nos. AP026732
214 (chromosome), AP026733 (pNUITM-VS2_1), AP026734 [pNUITM-VS2_2 co-
215 carrying *tet*(X4) and *tmexC3.1D3.1-toprJ1*], AP026735 (pNUITM-VS2_3),
216 AP026736 (pNUITM-VS2_4 carrying bla_{NDM-1}), and AP026737 (pNUITM-VS2_5).

217

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230

231 **Competing interests**

232 None declared.

233

234 **Ethical approval**

Not required.

237 **Table 1**

Strain	MIC (mg/L)												
	TIG	MIN	DOX	TET	IPM	MEM	CTX	CAZ	CIP	AMK	GEN	TOB	STR
S. xiamenensis NUITM-VS2	>128	>128	>128	>128	>128	>128	>128	>128	4	0.5	>128	16	64
E. coli J53/pNUITM-VS2_2	128	128	128	128	0.25	0.016	0.032	0.25	0.016	0.5	0.5	0.25	0.5
E. coli J53	0.25	2	4	1	0.25	0.016	0.032	0.25	0.016	0.5	0.5	0.25	0.5
E. coli ATCC 25922	0.25	1	2	1	0.25	0.016	0.064	0.5	0.008	1	0.5	0.5	0.5

238

239 **Legends**

240 **Table 1.** MICs of antimicrobials against *S. xiamenensis* NUITM-VS2 and the
241 transconjugant of *E. coli* J53. Abbreviations: TIG, tigecycline; MIN, minocycline;
242 DOX, doxycycline; TET, tetracycline; IPM, imipenem; MEM, meropenem; CTX,
243 cefotaxime; CAZ, ceftazidime; CIP, ciprofloxacin; AMK, amikacin; GEN,
244 gentamicin; TOB, tobramycin; STR, streptomycin.

245

246 **Fig. 1.** Linear comparison of *tet*(X4) and *tmexC3.1D3.1-toprJ1*–carrying plasmids
247 *S. xiamenensis* pNUITM-VS2_2 and *K. aerogenes* pNUITM-VK5_mdr isolated in
248 Vietnam in 2021. Red, yellow, blue, green, and gray indicate carbapenem and
249 tetracycline resistance genes (CRG/TRG), other AMR genes (ARG), mobile gene
250 elements (MGE), type IV secretion system (T4SS)-associated genes involved in
251 conjugation, and other coding sequences (Other), respectively. The blue color in
252 comparison of sequences indicates % of identity.

253

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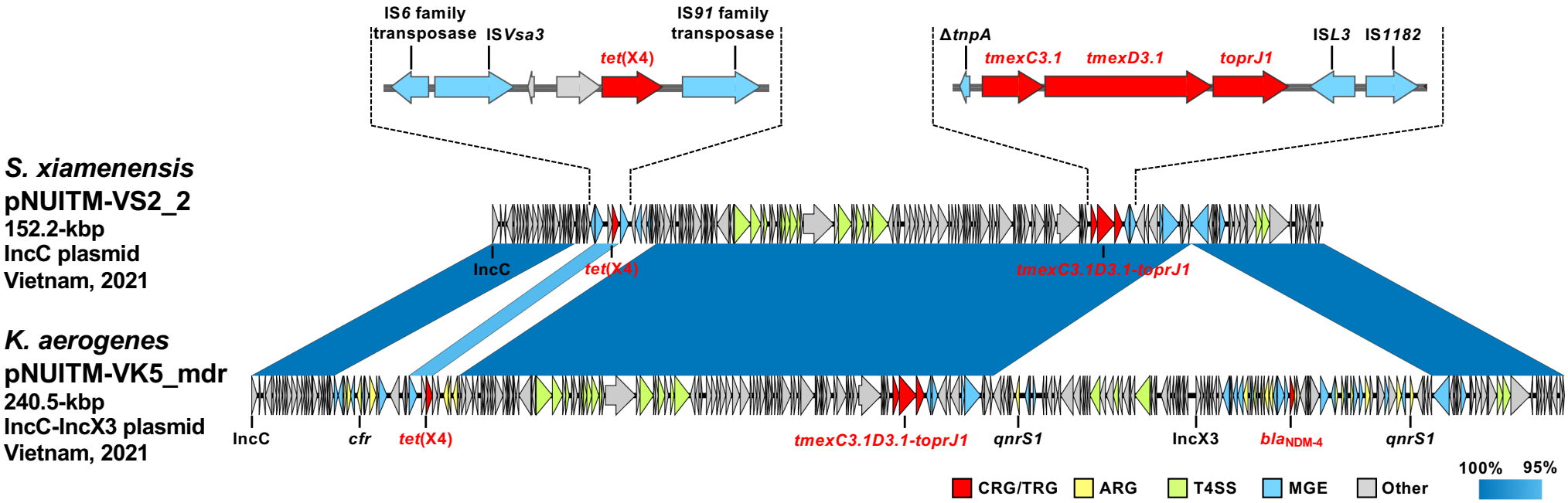
IS6 family transposase
ISVsa3
tet(X4)
IS91 family transposase
ΔtnpA
tmexC3.1
tmexD3.1
toprJ1
ISL3
IS1182
S. xiamenensis
pNUITM-VS2_2
152.2-kbp
IncC plasmid
Vietnam, 2021
IncC
tet(X4)
tmexC3.1D3.1-toprJ1
K. aerogenes
pNUITM-VK5_mdr
240.5-kbp
IncC-IncX3 plasmid
Vietnam, 2021
IncC
cfr
tet(X4)
tmexC3.1D3.1-toprJ1
qnrS1
IncX3
blaNDM-4
qnrS1
CRG/TRG
ARG
T4SS
MGE
Other
100%
95%

Fig. 1