Emergence of rare carbapenemases (FRI, GES-5, IMI, SFC, and SFH-1) in Enterobacterales isolated from surface waters in Japan

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ABSTRACT

Objectives: Carbapenemase-producing Enterobacterales (CPE) pose serious threats to public health. Compared with clinical CPE, the genetic characteristics of environmental CPE are not well understood. This study aimed to characterize the genetic determinants of carbapenem resistance in CPE isolated from environmental waters in Japan.

Methods: Eighty-five water samples were collected from rivers and a lake in Japan. CPE were identified using selective media, and genome sequencing was performed for the obtained isolates (n = 21).

Results: Various rare/novel carbapenemases were identified: GES-5 in *Raoultella planticola* (n = 1), FRI-8 and FRI-11 in *Enterobacter* spp. (n = 8), IMI-22 and IMI-23 in *Serratia ureilytica* (n = 3), and SFC-1, SFC-2 and SFH-1 in *Serratia fonticola* (n = 9). Genomes of 11 isolates could be closed, allowing the elucidation of the genetic contexts of the carbapenemase genes. The $bla_{\text{GES-5}}$ gene was located within a class 1 integron, In2071 (cassette array, $bla_{\text{GES-5}}$-*aacA3-aadA16*), on a 33 kb IncP6 plasmid. The $bla_{\text{FRI-8}}$ genes were carried on IncFII(Yp) plasmids ranging in size from 191 kb to 244 kb, and the $bla_{\text{FRI-11}}$ genes were carried on 70 kb and 74 kb IncFII(pECLA)/IncR plasmids. The $bla_{\text{IMI-22}}$ and $bla_{\text{IMI-23}}$ genes were colocated on a 107 kb plasmid. The bla_{SFC} and $bla_{\text{SFH-1}}$ genes were found on putative genomic islands inserted at tRNA-Phe genes in chromosomes.

Conclusions: This study revealed the presence of rare/novel carbapenemases among CPE in aquatic environments, suggesting that the environment may act as a potential reservoir of these minor carbapenemases.

INTRODUCTION

The worldwide spread of carbapenemase-producing Enterobacterales (CPE) is a major threat to public health. CPE can be resistant to carbapenems and many other classes of commonly used drugs, which significantly limits treatment options.[1, 2] The most clinically relevant carbapenemases detected in CPE are the 'big five' carbapenemases (Ambler class A KPC enzymes, class B enzymes of IMP, VIM, and NDM, and class D OXA-48-like enzymes), while Enterobacterales producing other 'minor' carbapenemases have also been sporadically reported.[3]

Historically, antibiotic resistance has been considered to be a clinical problem, but a growing number of studies have suggested that the environment represents an important reservoir for antibiotic resistant bacteria and antibiotic resistance genes.[4-6] Previous studies have reported the occurrence of CPE in environmental waters, with the aforementioned 'big five' carbapenemases being the predominant types.[7, 8] Minor carbapenemases, such as GES and IMI,

53 were also detected among environmental CPE,[9-11] raising the possibility that environmental
54 waters may act as reservoirs for these minor carbapenemases.

55 Previous studies have shown that Japanese clinical CPE usually carry bla_{IMP} and sporadically
56 carry other carbapenemase genes such as bla_{KPC}, bla_{NDM}, and bla_{OXA-48}.[12, 13] However, the genetic
57 characteristics of CPE in the natural environment, in particular in surface waters, are not well
58 understood in Japan.[14] Here, we isolated CPE from river and lake water in Japan and
59 characterized the obtained CPE isolates in terms of resistance determinants, genetic contexts
60 of the carbapenemase genes, and phylogeny.

61

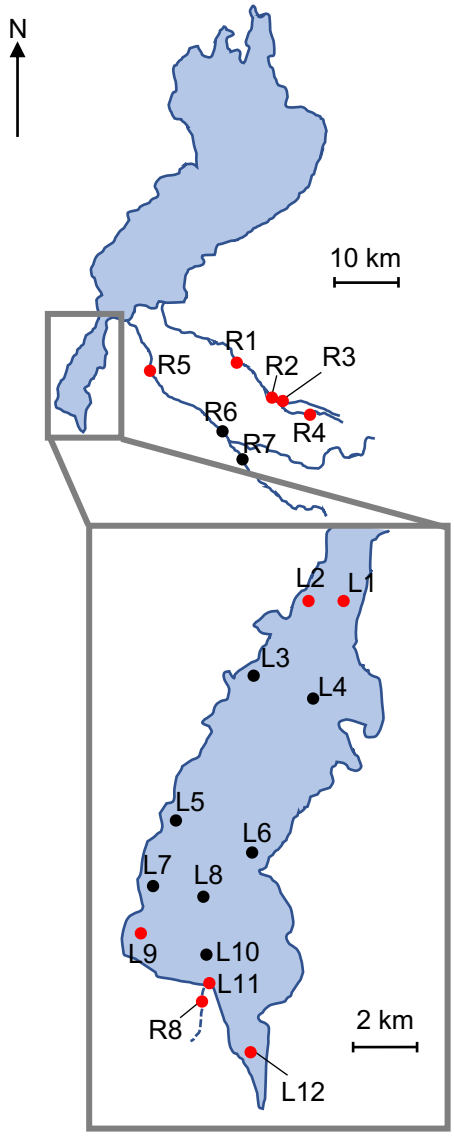
62 **MATERIALS AND METHODS**

63 ***Sample collection and bacterial isolation***

64 In total, 72 water samples were collected from 12 sites in Lake Biwa and 13 water samples
65 were collected from 8 sites in its surrounding rivers between February and November 2020
66 (**Figure 1**). Samples were processed using the membrane filter method with chromID CARBA
67 agar (bioMérieux, Marcy-l'Étoile, France). For each sample, up to three colonies showing
68 *Escherichia coli* profiles (pink to burgundy) or KESC (*Klebsiella*, *Enterobacter*, *Serratia*,
69 *Citrobacter*) profiles (bluish green to bluish grey) with different colony morphologies were
70 isolated. The isolates were restreaked on fresh chromID CARBA agar and incubated again.
71 Streaking on chromID CARBA agar and incubation were repeated until pure colonies were

72 obtained. The oxidase test was performed using a cytochrome oxidase test strip (Nissui, Tokyo,

73 Japan), and isolates with negative test results were stored at -85°C in 35% glycerol.



74

75 **Figure 1**. Map of sampling sites. Sampling sites in Lake Biwa are indicated with L1 to L12,

76 and sampling sites in rivers are indicated with R1 to R8. R8 is in a small river and located near

77 L11. Red filled circles indicate sampling points where CPE isolates were detected.

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80 ***Antibiotic susceptibility testing***

81 Antibiotic susceptibility was assessed by microdilution using a Dry Plate Eiken (Eiken,
82 Tokyo, Japan). The results were interpreted using CLSI criteria.[15] The Carba NP test was
83 performed to test the carbapenemase activity.[16] *Klebsiella pneumoniae* ATCC BAA-1705 was
84 used for the positive control, and *K. pneumoniae* ATCC BAA-1706 was used for the negative
85 control.

86 ***Cloning experiments***

87 Novel carbapenemase genes ($bla_{\text{FRI-11}}$ in JBIWA008, $bla_{\text{SFC-2}}$ in JSRIV001, $bla_{\text{IMI-22}}$ and $bla_{\text{IMI-}}$
88 $_{23}$ in JBIWA004) were amplified using the primers listed in **Table S1**, cloned into the pCR2.1-
89 TOPO vector using the TOPO TA cloning kit (Thermo Fisher Scientific, Waltham, MA, USA),
90 and transformed into *E. coli* DH5α (TaKaRa Bio, Inc., Shiga, Japan). Antibiotic susceptibilities
91 of the recipient and transformants were assessed by microdilution as described above.

92 ***Genome sequencing and assembly***

93 DNA was extracted from each isolate using a DNeasy Blood and Tissue kit (Qiagen, Hilden,
94 Germany) for DNBSEQ short-read sequencing. Libraries were prepared with the MGIEasy FS
95 PCR-free DNA library prep set (MGI Tech, Shenzhen, China), and paired-end sequencing
96 (2×150-bp) was performed on DNBSEQ-G400RS. Reads were subsampled using seqtk (v1.3,
97 https://github.com/lh3/seqtk), trimmed using fastp (v0.20.0),[17] and assembled using Unicycler
98 (v0.4.8) with the --no_correct option.[18]

99 Selected isolates (n=12) were also subjected to Oxford Nanopore Technologies (ONT) long-
100 read sequencing. DNA was isolated as described previously,[19] and libraries were prepared using
101 the SQK-LSK109 kit (Oxford Nanopore Technologies, Oxford, UK) and the Short Read
102 Eliminator XS kit (Circulomics, Inc., Baltimore, MD, USA). Sequencing was performed using
103 ONT GridION X5 with a FLO-MIN106 flow cell. ONT reads were filtered using Filtlong
104 (v0.2.0, https://github.com/rrwick/Filtlong) to remove reads with low quality or short length.
105 *De novo* hybrid assemblies using trimmed DNBSEQ reads and filtered ONT reads were
106 performed using Unicycler (v0.4.8)[18] with the --no_correct option. Genomes that could not be
107 completed by Unicycler were subjected to long-read assembly by Flye (v2.8)[20] with the --
108 plasmids option followed by long-read polishing using Medaka (v1.2.6,
109 https://github.com/nanoporetech/medaka) and short-read polishing using Pilon (v1.23).[21]

110 ***Genomic analysis***

111 Species identification was performed by comparing the obtained genome sequences to the
112 genome sequences of the type strains. The average nucleotide identity (ANI) analysis was
113 performed using OrthoANIu,[22] and digital DNA-DNA hybridization (dDDH) analysis was
114 performed using the GGDC website (formula 2).[23] A ≥96% ANI value or ≥ a 70% DDH value
115 was used for defining the species.[23, 24] Sequence types (STs) were assigned to *Enterobacter*
116 isolates using the multilocus sequence typing (MLST) scheme established previously.[25] Whole-
117 genome SNP-based trees were built using kSNP3.[26] The program Kchooser was used to select

118 the optimum k-mer length, and SNP loci occurring in at least 80% of the strains were used for
119 building trees.

120 Plasmid replicons, relaxase types, mate-pair formation types, and transferability of plasmids
121 were analysed using PlasmidFinder 2.1 and MOB-typer.[27, 28] Annotation was performed using
122 ISfinder,[29] the RAST server,[30] DFAST,[31] ResFinder 4.1,[32] Abricate (v1.0.1,
123 https://github.com/tseemann/abricate), INTEGRALL,[33] and the BLAST algorithm
124 (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Genomic islands (GIs) were detected using
125 IslandViewer 4.[34] New allele numbers for carbapenemase genes were assigned by NCBI
126 through submission of sequence data according to instructions on the following website:
127 https://www.ncbi.nlm.nih.gov/pathogens/submit-beta-lactamase/.

128 ***Accession numbers***

129 The complete genomes and sequence read files were deposited in GenBank and the NCBI
130 SRA under BioProject PRJNA725976. The BioSample accession number for each isolate is
131 also available in **Table 1**.

132

133 **Table 1**. Characteristics of 21 CPE isolates recovered from surface waters in the present study.

Isolate[a]	Sampling site	Collection date	Species[b]	Carbapenemase gene(s)	Location of carbapenemase gene(s)[c]	BioSample accession number
JBIWA001	L12	2/5/2020	*Raoultella planticola*	*bla*$_{GES-5}$	pJBIWA001_5 (IncP6, 32,930 bp)	SAMN18916998
JBIWA002	L12	2/5/2020	*Enterobacter asburiae* (ST1585)	*bla*$_{FRI-8}$	pJBIWA002_1 (IncFII(Yp), 243,962 bp)	SAMN18916999
JBIWA003	L12	3/4/2020	*Enterobacter quasiroggenkampii* (ST929)	*bla*$_{FRI-8}$	pJBIWA003_1 (IncFII(Yp), 221,552 bp)	SAMN18917000
JBIWA004	L12	3/4/2020	*Serratia ureilytica*	*bla*$_{IMI-22}$, *bla*$_{IMI-23}$	pJBIWA004_1 (IncFII(pRSB107), pSM22, 106,702 bp)	SAMN18917001
JBIWA005	L12	3/4/2020	*Enterobacter* genomospecies 8 (ST930)	*bla*$_{FRI-8}$	pJBIWA005_1 (IncFII(Yp), 191,037 bp)	SAMN18917002

JBIWA006	L9	9/2/2020	*Enterobacter asburiae* (ST1586)	*bla*$_{FRI-11}$	UD	SAMN19031978
JBIWA007	L2	9/2/2020	*Enterobacter asburiae* (ST1586)	*bla*$_{FRI-11}$	pJBIWA007_1 (IncFII(pECLA), IncR, 69,715 bp)	SAMN18917003
JBIWA008	L1	9/2/2020	*Enterobacter* sp. (ST1587)	*bla*$_{FRI-11}$	pJBIWA008_3 (IncFII(pECLA), IncR, 73,531 bp)	SAMN18917004
JBIWA009	L1	9/2/2020	*Enterobacter asburiae* (ST1586)	*bla*$_{FRI-11}$	UD	SAMN19031979
JBIWA010	L11	9/2/2020	*Enterobacter* sp. (ST1587)	*bla*$_{FRI-11}$	UD	SAMN19031980
JBIWA011	L2	10/5/2020	*Serratia ureilytica*	*bla*$_{IMI-22}$, *bla*$_{IMI-23}$	UD	SAMN19031981
JBIWA012	L1	10/5/2020	*Serratia ureilytica*	*bla*$_{IMI-22}$, *bla*$_{IMI-23}$	UD	SAMN19031982

JSRIV001	R8	3/4/2020	*Serratia fonticola* (clade III)	*bla*$_{SFC-2}$, *bla*$_{SFH-1}$	chromosome	SAMN18917005
JSRIV002	R4	3/17/2020	*Serratia fonticola* (clade III)	*bla*$_{SFC-2}$, *bla*$_{SFH-1}$	chromosome	SAMN18917006
JSRIV003	R2	3/17/2020	*Serratia fonticola* (clade III)	*bla*$_{SFC-2}$, *bla*$_{SFH-1}$	UD	SAMN19031973
JSRIV004	R2	3/17/2020	*Serratia fonticola* (clade III)	*bla*$_{SFC-2}$, *bla*$_{SFH-1}$	chromosome	SAMN18917007
JSRIV005	R1	3/17/2020	*Serratia fonticola* (clade III)	*bla*$_{SFC-2}$, *bla*$_{SFH-1}$	UD	SAMN19031974
JSRIV006	R1	3/17/2020	*Serratia fonticola* (clade III)	*bla*$_{SFC-1}$, *bla*$_{SFH-1}$	chromosome	SAMN18917008
JSRIV007	R3	3/17/2020	*Serratia fonticola* (clade III)	*bla*$_{SFC-2}$, *bla*$_{SFH-1}$	UD	SAMN19031975
JSRIV008	R5	3/17/2020	*Serratia fonticola* (clade III)	*bla*$_{SFC-2}$, *bla*$_{SFH-1}$	UD	SAMN19031976
JSRIV009	R5	3/17/2020	*Serratia fonticola* (clade III)	*bla*$_{SFC-2}$, *bla*$_{SFH-1}$	UD	SAMN19031977

134 [a]Genomes of isolates in bold were sequenced by both DNBSEQ and ONT sequencers. Among
135 the 12 isolates in bold, all the genomes except JBIWA006 were completed. Also see **Table S2**
136 for the accession numbers of the closed replicons and **Table S3** for the SRA accession numbers
137 of the incomplete genomes.
138 [b]STs/clades are shown in parentheses.
139 [c]UD: undetermined.
140
141
142 **RESULTS AND DISCUSSION**
143 ***Detection of CPE in water samples***
144 Twelve isolates and nine isolates were obtained from lake water samples and river water
145 samples, respectively (**Table 1**). Lake isolates were identified as *Enterobacter* spp. (n = 8),
146 *Serratia ureilytica* (n = 3), and *Raoultella planticola* (n = 1), and all nine river isolates were
147 identified as *Serratia fonticola* clade III (discussed in detail below) by ANI and dDDH analyses
148 (**Table 1**).
149 Among the 21 isolates, 12 were subjected to both DNBSEQ short-read sequencing and ONT
150 long-read sequencing. Nine genomes were completed by Unicycler, and two of the remaining
151 three genomes were completed by Flye. The genome of JBIWA006 could not be completed by
152 either approach. The completed genomes contained one to 12 plasmids (see **Table S2** for

153 details). The draft genome assemblies of the remaining isolates had 28 to 217 contigs and
154 189,689 bp to 2,472,998 bp N50 values after discarding contigs shorter than 100 bp (see **Table**
155 **S3** for details).

156 All the carbapenemase genes detected in this study were found to encode minor
157 carbapenemases (**Table 1**). Briefly, $bla_{\text{GES-5}}$ was detected in a *R. planticola* isolate, $bla_{\text{FRI-8}}$ and
158 $bla_{\text{FRI-11}}$ were detected in *Enterobacter* isolates, $bla_{\text{IMI-22}}$ and $bla_{\text{IMI-23}}$ were detected in *S.*
159 *ureilytica* isolates, and $bla_{\text{SFC-1}}$, $bla_{\text{SFC-2}}$ and $bla_{\text{SFH-1}}$ were detected in *S. fonticola* clade III
160 isolates. $bla_{\text{FRI-11}}$, $bla_{\text{IMI-22}}$, $bla_{\text{IMI-23}}$, and $bla_{\text{SFC-2}}$ are the genes encoding novel carbapenemase
161 variants, and their protein accession numbers are as follows: FRI-11 (WP_223174987.1), IMI-
162 22 (WP_223174988.1), IMI-23 (WP_223525081.1), and SFC-2 (WP_223174986.1).

163 Phenotypic resistance was determined by microdilution. All the isolates were nonsusceptible
164 to doripenem and meropenem, and all but GES-5-producing *R. planticola* (JBIWA001) were
165 nonsusceptible to imipenem (**Table S4**). The Carba NP test confirmed carbapenemase activity
166 in all isolates carrying bla_{SFC} and $bla_{\text{SFH-1}}$, whereas isolates carrying $bla_{\text{GES-5}}$, $bla_{\text{FRI-8}}$, and bla_{IMI}
167 were negative by the test. Results of the Carba NP test for the $bla_{\text{FRI-11}}$-carrying isolates were
168 inconsistent (two were positive, one was negative, and two were indeterminate). Previous
169 studies reported that Enterobacterales harbouring carbapenemases such as $bla_{\text{GES-5}}$ and bla_{IMI}
170 can be falsely negative in the Carba NP test.[35-37] Novel carbapenemase genes were cloned into
171 the pCR2.1-TOPO vector and transformed into *E. coli* DH5α. An increase in MICs of

172 carbapenems was detected in *E. coli* DH5α carrying the recombinant plasmids pCR2.1-TOPO-
173 bla_{SFC-2} and pCR2.1-TOPO-bla_{IMI-23} (**Table S5**). However, no detectable increase in MICs of
174 carbapenems was observed for *E. coli* DH5α carrying the recombinant plasmid pCR2.1-TOPO-
175 bla_{FRI-11} or pCR2.1-TOPO-bla_{IMI-22} under the conditions in the present study, which indicates
176 that FRI-11 and IMI-22 are enzymes with low carbapenemase activity.

177 **Genomic analysis of *Raoultella planticola* isolate**

178 River isolate JBIWA001 showed 99.15% ANI and 93.90% dDDH values with the type strain
179 of *R. planticola*. The assembly resulted in a chromosome (5,626,881 bp) and 12 plasmids
180 ranging from 1,443 bp to 136,413 bp (see **Table S2** for details). The bla_{GES-5} gene was on a
181 32,930 bp plasmid, pJBIWA001_5, with an IncP6 replicon and a MOBP-type relaxase. bla_{GES-}
182 $_{5}$ was found to be part of a novel class 1 integron, In2071. In2071 contains a bla_{GES-5}-*aacA3-*
183 *aadA16* cassette array, followed by the 3′-conserved segment (3′-CS) (*qacEΔ1*, *sul1*, orf5, orf6),
184 IS*1326*, *tniBΔ2*, *tniA*, and IRt (inverted repeat at *tni* end). A BLASTN search against the
185 GenBank database using pJBIWA001_5 as a query sequence identified some closely related
186 plasmids. pN260-3, which was carried by an *E. roggenkampii* strain detected in a patient with
187 cholecystitis in Japan in 2019,[38] showed the highest query coverage (97%) and also showed the
188 high percent identity (>96%) (see **Figure S1A** for the comparison of these two plasmids).
189 pN260-3 also carried bla_{GES-5} but within a different class 1 integron structure (*intI1*-bla_{GES-5}-
190 *aac(6′)-31-intI1Δ-qacG2-aadA5-qacEΔ1-sul1*) (**Figure S1B**).

191 **Genomic analysis of *Enterobacter* isolates**

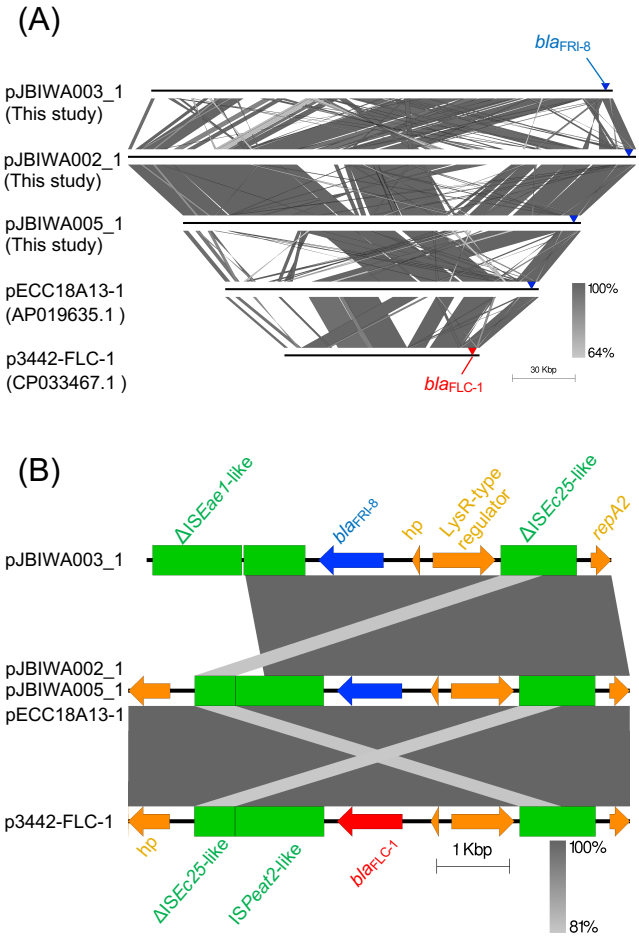
192 The *Enterobacter* genus comprises 22 species and 22 additional genomospecies.[39] The lake
193 *Enterobacter* isolates in the present study were identified as *E. asburiae* (n = 4), *E.*
194 *quasiroggenkampii* (n = 1), and *Enterobacter* genomospecies 8 (n = 1) based on the ANI and
195 dDDH analyses (also see **Figure S2** for the *Enterobacter* phylogenetic tree). The remaining
196 two isolates, JBIWA008 and JBIWA010, showed < 96% ANI values and < 70% dDDH values
197 against all type strains and reference strains (closest to *E. asburiae*, 95.2% ANI and 63.0%
198 dDDH). It should be noted that although we adopted a 96% ANI cutoff value, which is the
199 cutoff value used in a recent study defining *Enterobacter* species,[40] a 95% ANI cutoff value is
200 also used for defining the species and 95-96% ANI values can be an inconclusive zone.[40, 41]
201 However, dDDH values were lower than the 70% cutoff and thus these two isolates were
202 considered to belong to a novel genomospecies. MLST assigned two novel STs (ST1585 and
203 ST1586) to *E. asbriae* isolates, ST929 to an *E. quasiroggenkampii* isolate, ST930 to an
204 *Enterobacter* genomospecies 8 isolate, and a novel ST (ST1587) to two isolates belonging to
205 the novel genomospecies. Interestingly, *Enterobacter* ST929 and ST930 were first reported in
206 our previous study analysing wastewater CPE isolates in the same region.[42] The location where
207 ST929 and ST930 isolates were recovered in the present study is near the outlets of wastewater
208 treatment plants (WWTPs), indicating the origin of these isolates could be those WWTPs.

209 We could determine the complete sequences of three *bla*$_{FRI-8}$-carrying plasmids, namely
210 pJBIWA002_1 (243,962 bp) from *E. asbriae* isolate JBIWA002, pJBIWA003_1 (221,552 bp)
211 from *E. quasiroggenkampii* isolate JBIWA003, and pJBIWA005_1 (191,037 bp) from
212 *Enterobacter* genomospecies 8 isolate JBIWA005. A BLASTN search against the GenBank
213 database using *bla*$_{FRI-8}$ as a query sequence identified one *bla*$_{FRI-8}$-carrying plasmid,
214 *Enterobacter* sp. 18A13 plasmid pECC18A13-1 (150,509 bp), in which *bla*$_{FRI-8}$ was first
215 detected.[43] The BLASTN search also identified the *bla*$_{FLC-1}$-carrying plasmid p3442-FLC-1
216 (93,658 bp) from the *E. cloacae* complex strain E3442.[44] FLC-1 (FRI-like carbapenemase-1)
217 shares 99.66% amino acid identity with FRI-8 and was suggested that it be reclassified as an
218 FRI variant.[3] All five plasmids (pJBIWA002_1, pJBIWA003_1, pJBIWA005_1, pECC18A13-
219 1, p3442-FLC-1) carried an IncFII(Yp)-like replicon and were typed as Y10:A-:B- by plasmid
220 MLST. All five plasmids also carried a MOBP-type relaxase, though pECC18A13-1 also
221 carried a MOBF-type relaxase. Although the five plasmids carried some sequences in common,
222 insertions/deletions contributed to variable region diversification (**Figure 2A**). Analysis of
223 genetic contexts of *bla*$_{FRI-8}$ and *bla*$_{FLC-1}$ revealed the presence of an IS*Peat2*-like insertion
224 sequence (IS) downstream of *bla*$_{FRI-8}$/*bla*$_{FLC-1}$ and a LysR-type transcriptional regulator
225 upstream of *bla*$_{FRI-8}$/*bla*$_{FLC-1}$ (**Figure 2B**). A truncated IS*Eae1*-like element disrupted the
226 IS*Peat2*-like IS in pJBIWA003_1, changing the downstream structure in this plasmid. RAST
227 identified a coding sequence encoding a hypothetical protein between *bla*$_{FRI-8}$/*bla*$_{FLC-1}$ and a

228 LysR-type transcriptional regulator. BLASTN analysis showed this coding sequence to be
229 absent near the other *bla*$_{FRI}$ genes.

230 We were able to determine the complete sequences of two *bla*$_{FRI-11}$-carrying plasmids, namely
231 pJBIWA007_1 (69,715 bp) from *E. asburiae* isolate JBIWA007 and pJBIWA008_3 (73,531
232 bp) from *Enterobacter* sp. isolate JBIWA008. Online BLASTN analysis identified a 108,672
233 bp plasmid, pJF-587, from *E. asburiae*,[45] which showed >90% coverage to pJBIWA007_1 and
234 pJBIWA008_3. pJF-587 carried *bla*$_{FRI-2}$, which shares 98.5% nucleotide identity with *bla*$_{FRI-11}$.
235 All three plasmids carried IncFII(pECLA) and IncR replicons. Although pJF-587 contained a
236 MOBF-type relaxase and was predicted to be conjugative by MOB-typer, pJBIWA007_1 and
237 pJBIWA008_3 did not contain a relaxase and were predicted to be non-mobilizable. Alignment
238 of the three plasmids revealed conjugation genes present on pJF-587 were deleted in
239 pJBIWA007_1 and pJBIWA008_3 (**Figure 3A**). The genetic contexts of *bla*$_{FRI-11}$/*bla*$_{FRI-2}$ were
240 slightly different among the three plasmids. A DNA segment containing *ltrA* was present in
241 pJBIWA008_3, while this region was absent in pBIWA007_1 (**Figure 3B**). In pJF-587, this
242 segment was inverted compared to pJBIWA008_3, and an IS*Sm3*-like element was absent.
243 Although complete plasmid sequences were not available for JBIWA006, JBIWA009, and
244 JBIWA010, we could determine the genetic contexts of *bla*$_{FRI-11}$ in these isolates using short-
245 read assemblies: a pBIWA007_1-type genetic context was detected in JBIWA006 and

246 JBIWA009, while a pJBIWA008_3-type genetic context was detected in JBIWA010.



247

248 **Figure 2.** (A) Alignment of plasmids pJBIWA003_1, pJBIWA002_1, pJBIWA005_1,

249 pECC18A13-1, and p3442-FLC-1. Positions of *bla*$_{FRI-8}$ and *bla*$_{FLC-1}$ are indicated by blue and

250 red triangles, respectively. (B) Genetic contexts of *bla*$_{FRI-8}$ and *bla*$_{FLC-1}$. Blue arrows indicate

251 *bla*$_{FRI-8}$, a red arrow indicates *bla*$_{FLC-1}$, green boxes indicate insertion sequences, and orange

252 arrows indicate other coding sequences. hp: hypothetical protein. The alignments were

253 generated with Easyfig.[46]

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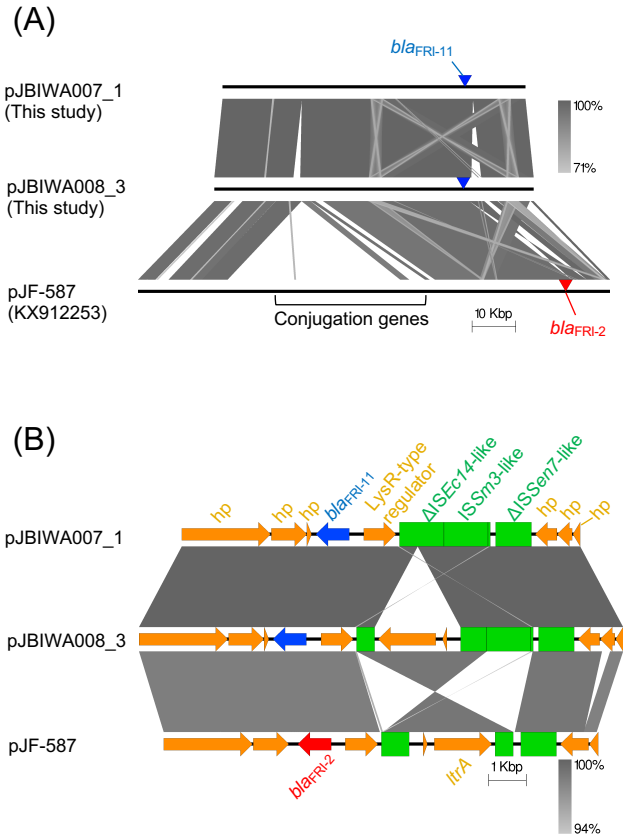

256

257 **Figure 3.** (A) Alignment of pJBIWA007_1, pJBIWA008_3, and pJF-587. Positions of *bla*$_{\text{FRI-}}$

258 $_{11}$ and *bla*$_{\text{FRI-2}}$ are indicated by blue and red triangles, respectively. (B) Genetic contexts of

259 *bla*$_{\text{FRI-11}}$ and *bla*$_{\text{FRI-2}}$. Blue arrows indicate *bla*$_{\text{FRI-11}}$, a red arrow indicates *bla*$_{\text{FRI-2}}$, green boxes

260 indicate insertion sequences, and orange arrows indicate other coding sequences.

261

262

263 **Genomic analysis of *Serratia* isolates**

264 Although three *Serratia* isolates recovered from the lake could be classified as *S. ureilytica*,

265 nine *Serratia* isolates from rivers could not be classified based on the ANI and dDDH analyses.

266 The pairwise ANI values and dDDH values among the nine river isolates were all above 96%

267 and 70%, respectively, indicating the river isolates belong to a single species. The river isolates
268 seem to be related to *S. fonticola* (**Figure S3**), but the ANI and dDDH values between the river
269 isolates and the type strain of *S. fonticola* ranged from 95.5% to 95.7% and 65.1% to 65.4%,
270 respectively, which are lower than the 96% ANI and 70% dDDH cutoff values. These results
271 suggest the 9 river isolates could belong to a novel species whose closest relative is *S. fonticola*.
272 It should be noted that genome sequences for type strains were not available for some *Serratia*
273 species (*Serratia aquatilis*, *Serratia bozhouensis*, *Serratia entomophila*, *Serratia myotis*, and
274 *Serratia profundus*), and ANI and dDDH analyses could not be performed for these species.
275 However, 16S rRNA gene sequences were available for them and all showed < 98.7% identity
276 with the 16S rRNA gene sequences of the river isolates, which implies they belong to different
277 species.[41]

278 In order to gain further insight into the phylogenetic characteristics of river *Serratia* isolates,
279 we downloaded RefSeq assemblies labelled *S. fonticola* and built a phylogenetic tree (**Figure**
280 **4**, also see **Figure S4** for phylogenetic characteristics of these strains in relation to other
281 *Serratia* species). Interestingly, three clades were identified in the phylogenetic tree, namely
282 clade I (containing the genomes of type strains of *S. fonticola*), clade II, and clade III
283 (containing the genomes of river isolates). Mean within-clade ANI values were > 98% for all
284 clades, and within-clade pairwise ANI values always exceeded 96%. On the other hand, mean
285 between-clade ANI values were < 96%, and between-clade pairwise ANI values were always

286 < 96% (**Table S6**). As there are limitations in the use of the website-based GGDC dDDH
287 calculation tool to compare large numbers of genomes, we performed dDDH analysis only for
288 comparisons including the type strain from clade I or the reference strains from clade II and
289 clade III (**Table S7**). The results show that within-clade pairwise dDDH values were always
290 above 70% while between-clade pairwise dDDH values were always below 70%. These results
291 suggest clades I-III could represent different species, although further studies are needed to
292 confirm this.

293 River *Serratia* isolates carried two types of carbapenemase genes, bla_{SFH} and bla_{SFC}. bla_{SFH-1}
294 (a subclass B2 carbapenemase gene) and bla_{SFC-1} (a class A carbapenemase gene) have been
295 described only once in *S. fonticola* clade II strain UTAD54 (GCF_000476315.1) from
296 Portugal.[3, 47-49] Although no variants were reported previously for either of these two
297 carbapenemases, online BLASTN analysis revealed that *S. fonticola* clade III strain AU-AP2C
298 (GCF_000477615.1)[50] carried a variant of bla_{SFC} (the product shows 99.4% amino acid identity
299 to SFC-1). All river isolates except JSRIV006 carried bla_{SFH-1} and a variant of bla_{SFC} that is
300 different from bla_{SFC-1} and bla_{SFC} of AU-AP2C (the product shows 99.4% amino acid identities
301 to SFC-1 and SFC of AU-AP2C). Novel alleles SFC-2 and SFC-3 were assigned to SFC
302 enzymes in river isolates and AU-AP2C, respectively. JSRIV006, in contrast, carried bla_{SFH-1}
303 and bla_{SFC-1}, though a synonymous mutation was detected in bla_{SFH-1} when compared with
304 bla_{SFH-1} in UTAD54. Although detailed genetic contexts of the bla_{SFH} and bla_{SFC} genes are not

305 known, these genes were previously shown to be located on the chromosome of UTAD54 by
306 probe hybridization.[47, 48] The *bla*$_{SFH-1}$ and *bla*$_{SFC}$ genes in the river isolates sequenced to closure
307 in this study (JSRIV001, JSRIV002, JSRIV004, JSRIV006) were also located on chromosomes
308 and situated close to each other (separated by 76 kbp to 131 kbp). Given the sporadic
309 phylogenetic distribution (**Figure 4**), the two genes were suspected to be located on GIs.[51]
310 Comparative analysis of the chromosomes of four river isolates and the chromosome of *S.*
311 *fonticola* clade III strain GS2 (GCF_001514455.1) revealed that the two genes were located on
312 putative GIs inserted at tRNA-Phe genes. (**Figure 5A**). Importantly, IslandViewer 4 predicted
313 almost the same regions as potential GIs (**Figure S5**). Perfect (5′-
314 TCGATTCCGAGTCCGGGCACCA-3′, detected in JSRIV004) or imperfect (5′-
315 TCGATTCCGAGTCC-GGCACCA-3′, detected in JSRIV001, JSRIV002, and JSRIV006)
316 repeat sequences corresponding to the 3′-end of the tRNA-Phe gene were detected at the right
317 ends of the GIs. Some previous studies also reported imperfect repeats at the right ends of GIs.[52,
318 53] An integrase gene was detected ~200 bp downstream of the tRNA-Phe gene in each GI
319 (**Figure 5B**), which indicates that the GIs were integrated at tRNA-Phe genes by site-specific
320 recombination mediated by these integrases. Genetic contexts of *bla*$_{SFH-1}$ were highly conserved
321 and the *bla*$_{SFH-1}$ gene was surrounded by a ΔIS*Ec18*-like element and an IS*1N*-like element in
322 all four completed chromosomes (**Figure 5B**). Although the complete genome was not
323 available for strain UTAD54, analysis of a contig carrying *bla*$_{SFH-1}$ revealed that the gene was

324 embedded within the same genetic context and also carried by a putative GI which was inserted
325 at the tRNA-Phe gene (**Figure 5B**). In draft assemblies of JSRIV003, JSRIV005, JSRIV007,
326 JSRIV008 and JSRIV009, contigs were broken near bla_{SFH-1} and genetic contexts of bla_{SFH-1}
327 could not be determined. Genetic surroundings of bla_{SFC} were also highly conserved, though
328 variations were observed in regions >3,000 bp away from bla_{SFC} (**Figure 5C**). bla_{SFC} was
329 surrounded by genes such as *smvA* (encoding a methyl viologen resistance protein), *acrR*
330 (encoding a transcriptional regulator), and genes encoding hypothetical proteins. The genetic
331 surroundings of bla_{SFC} (<3,000 bp from bla_{SFC}) were also conserved in draft assemblies of
332 JSRIV003, JSRIV005, JSRIV007, JSRIV008 and JSRIV009. Although bla_{SFH} and bla_{SFC} seem
333 to be mobilized together within the same GIs, strain AU-AP2C carried only bla_{SFC-3} and lacked
334 bla_{SFH}. A contig carrying bla_{SFC-3} in the draft genome assembly of AU-AP2C was long enough
335 for the comparative analysis. In AU-AP2C, a putative GI was inserted at the tRNA-Phe gene,
336 and bla_{SFC-3} was also carried on this putative GI (**Figure S6A**). However, the region containing
337 the integrase gene and bla_{SFH-1} was replaced by another segment as shown in **Figure S6B**.
338 Three *S. ureilytica* isolates from the lake, JBIWA004, JBIWA011 and JBIWA012, carried
339 bla_{IMI-22} and bla_{IMI-23}. Among the three isolates, the genome of JBIWA004 was sequenced to
340 closure. The two bla_{IMI} genes in JBIWA004 were located on a 106,702 bp plasmid, named
341 pJBIWA004_1, with IncFII(pRSB107) and pSM22 replicons. Online BLASTN analysis
342 revealed the plasmid p2-125 from *Serratia liquefaciens* showed the highest coverage (55%)

343 and also showed the high percent identity (96%) to pJBIWA004_1, while other plasmids
344 showed <30% coverage. Alignment of pJBIWA004_1 and p2-125 revealed that the two
345 plasmids shared conjugation regions, but the region containing the *bla*$_{IMI}$ genes was only
346 present in pJBIWA004_1 (**Figure 6A**). Comparison of the genetic contexts of the two *bla*$_{IMI}$
347 genes and that of *bla*$_{IMI-1}$ in *Enterobacter cloacae* complex strain N11-1168 showed that the
348 regulator *imiR* gene and a gene encoding a hypothetical protein, which are usually present
349 upstream and downstream of *bla*$_{IMI}$ genes, were truncated or deleted in pJBIWA004_1 (**Figure**
350 **6B**). *bla*$_{IMI-22}$ and *bla*$_{IMI-23}$ were surrounded by remnants of ISs, which might have contributed
351 to their mobilization. Although only draft genome assemblies were available for JBIWA011
352 and JBIWA012, the *bla*$_{IMI-22}$ and *bla*$_{IMI-23}$ genes were located on a single contig in each assembly.
353 Moreover, those contigs showed >94% coverage and >99.9% identity to pJBIWA004_1,
354 indicating that JBIWA011 and JBIWA012 also carried *bla*$_{IMI-22}$ and *bla*$_{IMI-23}$ on pJBIWA004_1-
355 like plasmids.
356

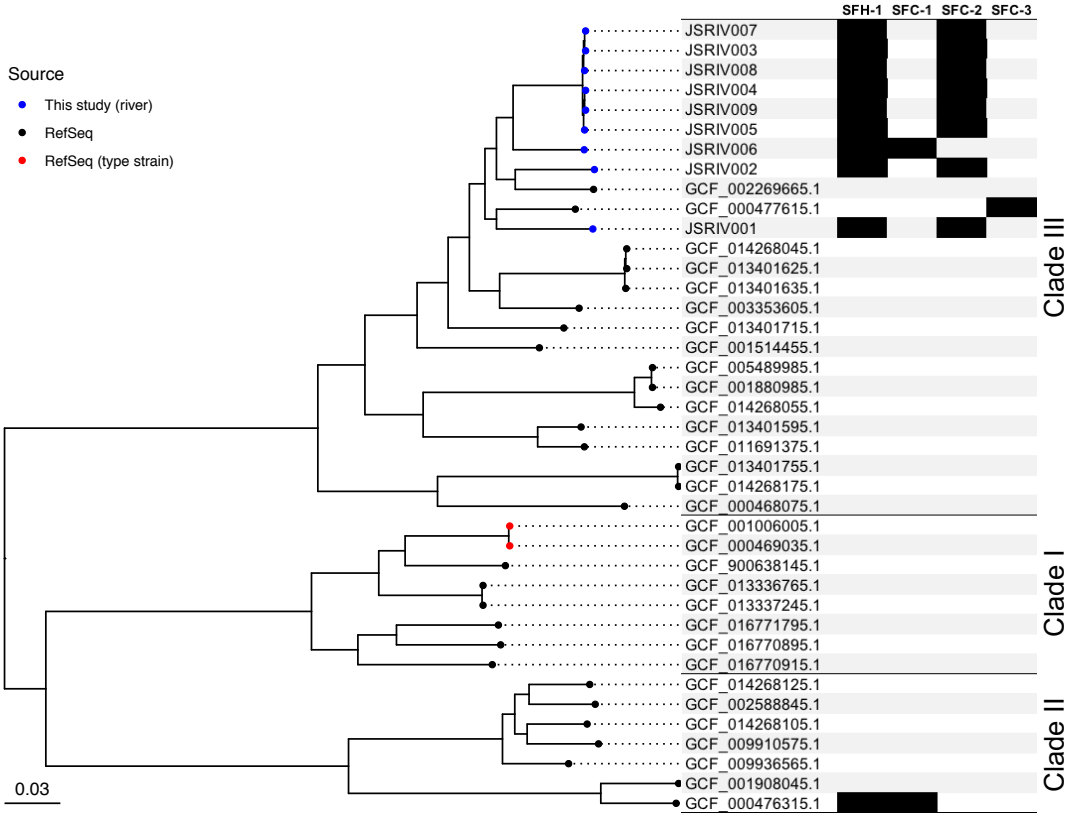

Figure 4. Phylogenetic tree constructed using RefSeq assemblies labelled *S. fonticola* and assemblies of nine river *Serratia* isolates. The RefSeq assemblies were downloaded in March 2021. The tree was constructed based on 177,349 SNPs occurring in at least 80% of the genomes. Four RefSeq assemblies (GCF_006714955.1, GCF_006715025.1, GCF_007827525.1, GCF_014205655.1) were removed because these assemblies showed < 85% ANI values with the type strain of *S. fonticola*. The mid-point rooted tree was visualized with ggtree.[54] The black blocks represent the presence of a carbapenemase gene.

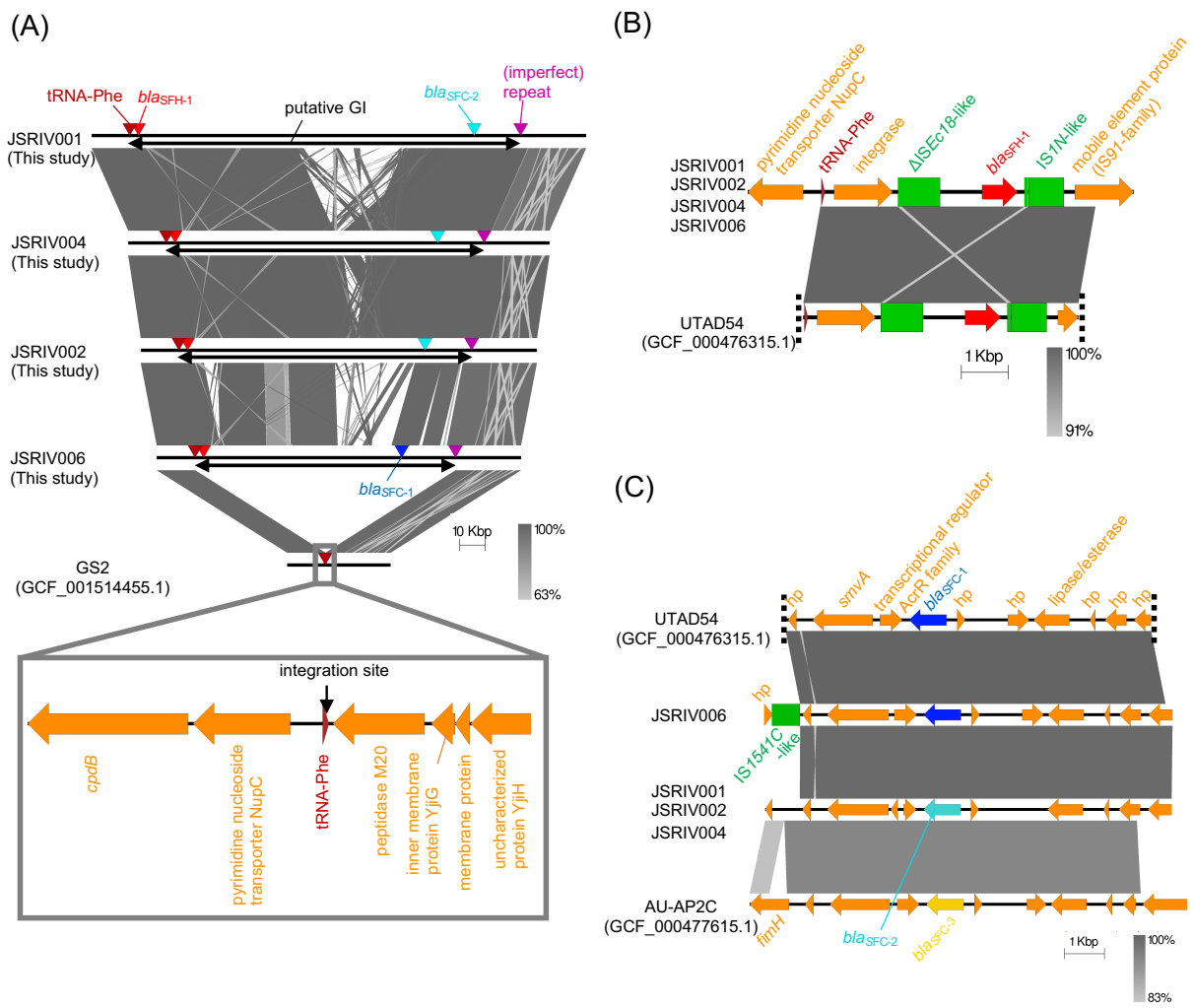

Figure 5. (A) Alignment of the putative GIs and the surrounding regions of river *Serratia* isolates sequenced to closure and the corresponding chromosomal region of *S. fonticola* clade III strain GS2. Positions of the bla_{SFC-1}, bla_{SFC-2}, bla_{SFH-1}, and tRNA-Phe genes, and (imperfect) repeats are indicated by blue, light blue, red, brown, and purple triangles, respectively. Double-headed arrows indicate genomic regions corresponding to GIs. Genetic contexts of (B) bla_{SFH} and (C) bla_{SFC}. Red arrows indicate bla_{SFH-1}, blue arrows indicate bla_{SFC-1}, a light blue arrow indicates bla_{SFC-2}, a yellow arrow indicates bla_{SFC-3}, green boxes indicate insertion sequences, and orange arrows indicate other coding sequences. Dashed lines indicate contig breaks.

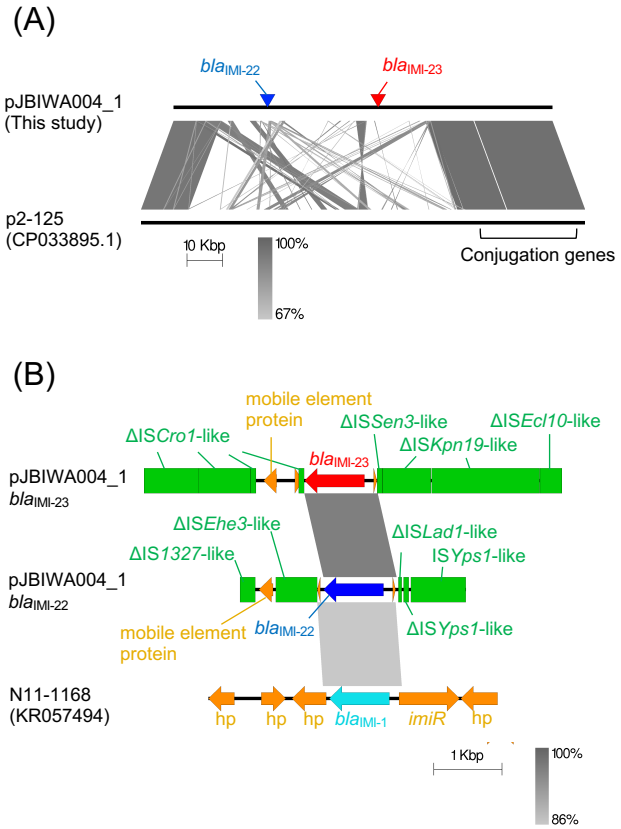

Figure 6. (A) Alignment of pJBIWA004_1 and p2-125. Positions of *bla*$_{\text{IMI-22}}$ and *bla*$_{\text{IMI-23}}$ are indicated by blue and red triangles, respectively. (B) Genetic contexts of *bla*$_{\text{IMI}}$ genes. A blue arrow indicates *bla*$_{\text{IMI-22}}$, a red arrow indicates *bla*$_{\text{IMI-23}}$, a light blue arrow indicates *bla*$_{\text{IMI-1}}$, green boxes indicate insertion sequences, and orange arrows indicate other coding sequences.

Conclusions

Here, we identified the prevalence of minor carbapenemases among environmental CPE isolates in Japan. Of note, *bla*$_{\text{SFH}}$ and *bla*$_{\text{SFC}}$, which have been described only in one *S. fonticola* isolate in Portugal, were prevalent among river *S. fonticola* isolates obtained from different

sampling sites. The results of this study indicate aquatic environments as potential reservoirs of minor carbapenemases and highlight the need to continuously monitor antibiotic resistance in the environment.

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Transparency declarations

None to declare.

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