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

Ryota GOMI[1,*], Yasufumi MATSUMURA[2], Michio TANAKA[2], Masaru IHARA[3], Yoshinori SUGIE[3], Tomonari MATSUDA[3], and Masaki YAMAMOTO[2]

[1]Department of Environmental Engineering, Graduate School of Engineering, Kyoto University, Katsura, Nishikyo-ku, 615-8540, Kyoto, Japan

[2]Department of Clinical Laboratory Medicine, Kyoto University Graduate School of Medicine, 54 Shogoin-kawahara-cho, Sakyo-ku, 606-8507, Kyoto, Japan

[3]Research Center for Environmental Quality Management, Kyoto University, 1-2 Yumihama, Otsu, 520-0811, Shiga, Japan

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[*]Corresponding author

E-mail address: gomi.ryota.34v@kyoto-u.jp; Tel: +81-75-383-3354; Fax: +81-75-383-3358

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

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

61 **MATERIALS AND METHODS**

62 **Sample collection and bacterial isolation**

63 In total, 72 water samples were collected from 12 sites in Lake Biwa and 13 water samples
64 were collected from 8 sites in its surrounding rivers between February and November 2020
65 (**Figure 1**). All samples were collected in sterile 1-L sampling bottles. Samples (100 mL to 600
66 mL) were processed using the membrane filter method with chromID CARBA agar
67 (bioMérieux, Marcy-l'Étoile, France). After 20 h of incubation at 37 °C, for each sample, up
68 to three colonies showing *Escherichia coli* profiles (pink to burgundy) or KESC (*Klebsiella*,
69 *Enterobacter*, *Serratia*, *Citrobacter*) profiles (bluish green to bluish grey) with different colony
70 morphologies were isolated. The oxidase test was performed using a cytochrome oxidase test
71 strip (Nissui, Tokyo, Japan), and isolates with negative test results were stored at -85°C in 35%
72 glycerol. Colonies suspected to be non-Enterobacterales species were not saved because some

73 non-Enterobacterales species prevalent in surface waters are intrinsically resistant to
74 carbapenems.[15, 16]

75 **Antibiotic susceptibility testing**

76 Antibiotic susceptibility was assessed by microdilution using a Dry Plate Eiken (Eiken,
77 Tokyo, Japan) using 29 antibiotics as described in the Supplementary Materials and methods.
78 The results were interpreted using CLSI criteria.[17] The Carba NP test was performed to test the
79 carbapenemase activity.[18] *Klebsiella pneumoniae* ATCC BAA-1705 was used for the positive
80 control, and *K. pneumoniae* ATCC BAA-1706 was used for the negative control.

81 **Genome sequencing and assembly**

82 Genome sequencing and assembly were performed as described in the Supplementary
83 Materials and methods.

84 **Genomic analysis**

85 Species identification was performed based on the genome sequence data, i.e., by comparing
86 the obtained genome sequences to the genome sequences of the type strains. The average
87 nucleotide identity (ANI) analysis was performed using OrthoANIu,[19] and digital DNA-DNA
88 hybridization (dDDH) analysis was performed using the GGDC website (formula 2).[20] A ≥96%
89 ANI value or ≥ a 70% DDH value was used for defining the species.[20, 21] STs were assigned to
90 *Enterobacter* isolates using the MLST scheme established previously.[22] Whole-genome SNP-
91 based trees were built using kSNP3.[23]

92 Plasmid replicons, relaxase types, mate-pair formation types, and transferability of plasmids
93 were analysed using PlasmidFinder 2.1 and MOB-typer.[24, 25] Annotation was performed using
94 ISfinder,[26] the RAST server,[27] DFAST,[28] ResFinder 4.1,[29] Abricate (v1.0.1,
95 https://github.com/tseemann/abricate), INTEGRALL,[30] and the BLAST algorithm
96 (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Genomic islands (GIs) were detected using
97 IslandViewer 4.[31] New allele numbers for carbapenemase genes were assigned by NCBI
98 through submission of sequence data according to instructions on the following website:
99 https://www.ncbi.nlm.nih.gov/pathogens/submit-beta-lactamase/.

100 **Cloning experiments**

101 Novel carbapenemase genes ($bla_{\text{FRI-11}}$ in JBIWA008, $bla_{\text{SFC-2}}$ in JSRIV001, $bla_{\text{IMI-22}}$ and $bla_{\text{IMI-}}$
102 $_{23}$ in JBIWA004) were amplified using the primers listed in **Table S1**, cloned into the pCR2.1-
103 TOPO vector using the TOPO TA cloning kit (Thermo Fisher Scientific, Waltham, MA, USA),
104 and transformed into *E. coli* DH5α (TaKaRa Bio, Inc., Shiga, Japan). Antibiotic susceptibilities
105 of the recipient and transformants were assessed by microdilution as described above.

106 **Accession numbers**

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

110

111 **RESULTS AND DISCUSSION**

112 **Detection of CPE in water samples**

113 Twelve isolates and nine isolates were obtained from lake water samples and river water
114 samples, respectively (**Table 1**). Lake isolates were identified as *Enterobacter* spp. (n = 8),
115 *Serratia ureilytica* (n = 3), and *Raoultella planticola* (n = 1), and all nine river isolates were
116 identified as *Serratia fonticola* clade III (discussed in detail below) by ANI and dDDH analyses
117 (**Table 1**).

118 All 21 isolates were subjected to DNBSEQ short-read sequencing, and 12 isolates were also
119 subjected to Oxford Nanopore Technologies (ONT) long-read sequencing. Among the 12
120 isolates with both short-read and long-read data, all genomes except for the genome of
121 JBIWA006 were completed by either Unicycler[32] or Flye.[33] The completed genomes contained
122 one to 12 plasmids (see **Table S2** for details). The draft genome assemblies of the remaining
123 isolates had 28 to 217 contigs and 189,689 bp to 2,472,998 bp N50 values after discarding
124 contigs shorter than 100 bp (see **Table S3** for details).

125 All the carbapenemase genes detected in this study were found to encode minor
126 carbapenemases, namely, $bla_{\text{GES-5}}$, bla_{FRI}, bla_{IMI}, bla_{SFC}, which all encode Ambler class A and
127 functional group 2f enzymes, and $bla_{\text{SFH-1}}$, which encodes an Ambler subclass B2 and functional
128 group 3b enzyme (GES: Guiana Extended-Spectrum β-lactamase, FRI: French imipenemase,
129 IMI: Imipenem-hydrolysing β-lactamase, SFC: *Serratia fonticola* carbapenemase, SFH:

130 *Serratia fonticola* carbapenem hydrolase).[34, 35] Briefly, bla_{GES-5} was detected in a *R. planticola*
131 isolate, bla_{FRI-8} and bla_{FRI-11} were detected in *Enterobacter* isolates, bla_{IMI-22} and bla_{IMI-23} were
132 detected in *S. ureilytica* isolates, and bla_{SFC-1}, bla_{SFC-2} and bla_{SFH-1} were detected in *S. fonticola*
133 clade III isolates (**Table 1**). bla_{FRI-11}, bla_{IMI-22}, bla_{IMI-23}, and bla_{SFC-2} are the genes encoding novel
134 carbapenemase variants, and their protein accession numbers are as follows: FRI-11
135 (WP_223174987.1), IMI-22 (WP_223174988.1), IMI-23 (WP_223525081.1), and SFC-2
136 (WP_223174986.1).

137 The carbapenemases and bacterial species detected in this study are different from those
138 usually observed in clinical settings. One possible reason for this is that these CPE isolates are
139 better adapted to the environment than those usually observed in clinical settings. There are
140 three municipal wastewater treatment plants (WWTPs) near the study area, and the effluents
141 (although biologically treated) from those WWTPs could be the potential sources of CPE
142 detected in the present study. Moreover, insufficiently treated wastewater could be released
143 from those WWTPs on rainy days, which could also have contributed to the dissemination of
144 CPE. The actual human health risks associated with the presence of these CPE isolates in
145 surface waters remain unclear. However, there seem to be some overlaps between the CPE
146 detected in this study and CPE isolated in clinical settings in terms of species and
147 carbapenemase types. For example, FRI-producing *Enterobacter* spp. have been previously
148 reported in patients at hospitals.[36, 37] On the other hand, bla_{SFC} and bla_{SFH}, which were prevalent

149 among the river *Serratia* isolates, have been described only once in environmental *S. fonticola*

150 and not in clinical settings.[3, 38-40] Further studies are needed to elucidate the health risks posed

151 by these CPE in surface waters.

152 **Susceptibility testing and cloning experiments**

153 Phenotypic resistance was determined by microdilution. All the isolates were nonsusceptible

154 to doripenem and meropenem, and all but GES-5-producing *R. planticola* (JBIWA001) were

155 nonsusceptible to imipenem (**Table S4**). The Carba NP test confirmed carbapenemase activity

156 in all isolates carrying bla_{SFC} and bla_{SFH-1}, whereas isolates carrying bla_{GES-5}, bla_{FRI-8}, and bla_{IMI}

157 were negative by the test. Results of the Carba NP test for the bla_{FRI-11}-carrying isolates were

158 inconsistent (two were positive, one was negative, and two were indeterminate). Previous

159 studies reported that Enterobacterales harbouring carbapenemases such as bla_{GES-5} and bla_{IMI}

160 can be falsely negative in the Carba NP test.[41-43] Novel carbapenemase genes were cloned into

161 the pCR2.1-TOPO vector and transformed into *E. coli* DH5α. An increase in MICs of

162 carbapenems was detected in *E. coli* DH5α carrying the recombinant plasmids pCR2.1-TOPO-

163 bla_{SFC-2} and pCR2.1-TOPO-bla_{IMI-23} (**Table S5**). However, no detectable increase in MICs of

164 carbapenems was observed for *E. coli* DH5α carrying the recombinant plasmid pCR2.1-TOPO-

165 bla_{FRI-11} or pCR2.1-TOPO-bla_{IMI-22} under the conditions in the present study, which indicates

166 that FRI-11 and IMI-22 are enzymes with low carbapenemase activity.

167 **Genomic analysis of a *Raoultella planticola* isolate**

168 River isolate JBIWA001 showed 99.15% ANI and 93.90% dDDH values with the type strain
169 of *R. planticola*. The assembly resulted in a chromosome (5,626,881 bp) and 12 plasmids
170 ranging from 1,443 bp to 136,413 bp (see **Table S2** for details). The bla_{GES-5} gene was on a
171 32,930 bp plasmid, pJBIWA001_5, with an IncP6 replicon and a MOBP-type relaxase. bla_{GES-}
172 $_{5}$ was found to be part of a novel class 1 integron, In2071. In2071 contains a bla_{GES-5}-*aacA3-*
173 *aadA16* cassette array, followed by the 3′-conserved segment (3′-CS) (*qacEΔ1*, *sul1*, orf5, orf6),
174 IS*1326*, *tniBΔ2*, *tniA*, and IRt (inverted repeat at *tni* end) (see Supplementary Results and
175 **Figure S1** for comparative analysis of pJBIWA001_5 and a closely related plasmid).

176 **Genomic analysis of *Enterobacter* isolates**

177 The *Enterobacter* genus comprises 22 species and 22 additional genomospecies.[44] The lake
178 *Enterobacter* isolates in the present study were identified as *E. asburiae* (n = 4), *E.*
179 *quasiroggenkampii* (n = 1), and *Enterobacter* genomospecies 8 (n = 1) based on the ANI and
180 dDDH analyses (also see **Figure S2** for the *Enterobacter* phylogenetic tree). The remaining
181 two isolates, JBIWA008 and JBIWA010, showed < 96% ANI values and < 70% dDDH values
182 against all type strains and reference strains (closest to *E. asburiae*, 95.2% ANI and 63.0%
183 dDDH). It should be noted that although we adopted a 96% ANI cutoff value, which is the
184 cutoff value used in a recent study defining *Enterobacter* species,[45] a 95% ANI cutoff value is
185 also used for defining the species and 95-96% ANI values can be an inconclusive zone.[45, 46]
186 However, dDDH values were lower than the 70% cutoff and thus these two isolates were

187 considered to belong to a novel genomospecies. MLST assigned two novel STs (ST1585 and
188 ST1586) to *E. asburiae* isolates, ST929 to an *E. quasiroggenkampii* isolate, ST930 to an
189 *Enterobacter* genomospecies 8 isolate, and a novel ST (ST1587) to two isolates belonging to
190 the novel genomospecies. Interestingly, *Enterobacter* ST929 and ST930 were first reported in
191 our previous study analysing wastewater CPE isolates in the same region.[47] As discussed above,
192 the potential sources of these *Enterobacter* isolates could be the effluents from WWTPs.

193 We could determine the complete sequences of three $bla_{\text{FRI-8}}$-carrying plasmids, namely
194 pJBIWA002_1 (243,962 bp) from *E. asburiae* isolate JBIWA002, pJBIWA003_1 (221,552 bp)
195 from *E. quasiroggenkampii* isolate JBIWA003, and pJBIWA005_1 (191,037 bp) from
196 *Enterobacter* genomospecies 8 isolate JBIWA005. A BLASTN search against the GenBank
197 database using $bla_{\text{FRI-8}}$ as a query sequence identified one $bla_{\text{FRI-8}}$-carrying plasmid,
198 *Enterobacter* sp. 18A13 plasmid pECC18A13-1 (150,509 bp), in which $bla_{\text{FRI-8}}$ was first
199 detected.[37] The BLASTN search also identified the $bla_{\text{FLC-1}}$-carrying plasmid p3442-FLC-1
200 (93,658 bp) from the *Enterobacter cloacae* complex strain E3442.[48] FLC-1 (FRI-like
201 carbapenemase-1) shares 99.66% amino acid identity with FRI-8 and was suggested that it be
202 reclassified as an FRI variant.[3] All five plasmids (pJBIWA002_1, pJBIWA003_1,
203 pJBIWA005_1, pECC18A13-1, p3442-FLC-1) carried an IncFII(Yp)-like replicon and were
204 typed as Y10:A-:B- by plasmid MLST. All five plasmids also carried a MOBP-type relaxase,
205 though pECC18A13-1 also carried a MOBF-type relaxase. Although the five plasmids carried

206 some sequences in common, insertions/deletions contributed to variable region diversification

207 (**Figure 2A**). Analysis of genetic contexts of bla_{FRI-8} and bla_{FLC-1} revealed the presence of an

208 IS*Peat2*-like IS downstream of bla_{FRI-8}/bla_{FLC-1} and a LysR-type transcriptional regulator

209 upstream of bla_{FRI-8}/bla_{FLC-1} (**Figure 2B**). A truncated IS*Eae1*-like element disrupted the

210 IS*Peat2*-like IS in pJBIWA003_1, changing the downstream structure in this plasmid. RAST

211 identified a coding sequence encoding a hypothetical protein between bla_{FRI-8}/bla_{FLC-1} and a

212 LysR-type transcriptional regulator. BLASTN analysis showed this coding sequence to be

213 absent near the other bla_{FRI} genes.

214 We were able to determine the complete sequences of two bla_{FRI-11}-carrying plasmids, namely

215 pJBIWA007_1 (69,715 bp) from *E. asburiae* isolate JBIWA007 and pJBIWA008_3 (73,531

216 bp) from *Enterobacter* sp. isolate JBIWA008. Online BLASTN analysis identified a 108,672

217 bp plasmid, pJF-587, from *E. asburiae*,[49] which showed >90% coverage to pJBIWA007_1 and

218 pJBIWA008_3. pJF-587 carried bla_{FRI-2}, which shares 98.5% nucleotide identity with bla_{FRI-11}.

219 All three plasmids carried IncFII(pECLA) and IncR replicons. Although pJF-587 contained a

220 MOBF-type relaxase and was predicted to be conjugative by MOB-typer, pJBIWA007_1 and

221 pJBIWA008_3 did not contain a relaxase and were predicted to be non-mobilizable. Alignment

222 of the three plasmids revealed conjugation genes present on pJF-587 were deleted in

223 pJBIWA007_1 and pJBIWA008_3 (**Figure 3A**). The genetic contexts of bla_{FRI-11}/bla_{FRI-2} were

224 slightly different among the three plasmids. A DNA segment containing *ltrA* was present in

225 pJBIWA008_3, while this region was absent in pBIWA007_1 (**Figure 3B**). In pJF-587, this
226 segment was inverted compared to pJBIWA008_3, and an IS*Sm3*-like element was absent.
227 Although complete plasmid sequences were not available for JBIWA006, JBIWA009, and
228 JBIWA010, we could determine the genetic contexts of $bla_{\text{FRI-11}}$ in these isolates using short-
229 read assemblies: a pBIWA007_1-type genetic context was detected in JBIWA006 and
230 JBIWA009, while a pJBIWA008_3-type genetic context was detected in JBIWA010.

231 **Genomic analysis of *Serratia* isolates**

232 Although three *Serratia* isolates recovered from the lake could be classified as *S. ureilytica*,
233 nine *Serratia* isolates from rivers could not be classified based on the ANI and dDDH analyses.
234 The pairwise ANI values and dDDH values among the nine river isolates were all above 96%
235 and 70%, respectively, indicating the river isolates belong to a single species. The river isolates
236 seem to be related to *S. fonticola* (**Figure S3**), but the ANI and dDDH values between the river
237 isolates and the type strain of *S. fonticola* ranged from 95.5% to 95.7% and 65.1% to 65.4%,
238 respectively, which are lower than the 96% ANI and 70% dDDH cutoff values. These results
239 suggest the 9 river isolates could belong to a novel species whose closest relative is *S. fonticola*.
240 It should be noted that genome sequences for type strains were not available for some *Serratia*
241 species (*Serratia aquatilis*, *Serratia bozhouensis*, *Serratia entomophila*, *Serratia myotis*, and
242 *Serratia profundus*), and ANI and dDDH analyses could not be performed for these species.
243 However, 16S rRNA gene sequences were available for them and all showed < 98.7% identity

244 with the 16S rRNA gene sequences of the river isolates, which implies they belong to different
245 species.[46]

246 In order to gain further insight into the phylogenetic characteristics of river *Serratia* isolates,
247 we downloaded RefSeq assemblies labelled *S. fonticola* and built a phylogenetic tree (**Figure**
248 **4**, also see **Figure S4** for phylogenetic characteristics of these strains in relation to other
249 *Serratia* species). Interestingly, three clades were identified in the phylogenetic tree, namely
250 clade I (containing the genomes of type strains of *S. fonticola*), clade II, and clade III
251 (containing the genomes of river isolates). Mean within-clade ANI values were > 98% for all
252 clades, and within-clade pairwise ANI values always exceeded 96%. On the other hand, mean
253 between-clade ANI values were < 96%, and between-clade pairwise ANI values were always
254 < 96% (**Table S6**). As there are limitations in the use of the website-based GGDC dDDH
255 calculation tool to compare large numbers of genomes, we performed dDDH analysis only for
256 comparisons including the type strain from clade I or the reference strains from clade II and
257 clade III (**Table S7**). The results show that within-clade pairwise dDDH values were always
258 above 70% while between-clade pairwise dDDH values were always below 70%. These results
259 suggest clades I-III could represent different species, although further studies are needed to
260 confirm this.

261 River *Serratia* isolates carried two types of carbapenemase genes, bla_{SFH} and bla_{SFC}. bla_{SFH-1}
262 and bla_{SFC-1} have been described only once in *S. fonticola* clade II strain UTAD54

263 (GCF_000476315.1) from Portugal.[3, 38-40] Although no variants were reported previously for
264 either of these two carbapenemases, online BLASTN analysis revealed that *S. fonticola* clade
265 III strain AU-AP2C (GCF_000477615.1)[50] carried a variant of bla_{SFC} (the product shows 99.4%
266 amino acid identity to SFC-1). All river isolates except JSRIV006 carried bla_{SFH-1} and a variant
267 of bla_{SFC} that is different from bla_{SFC-1} and bla_{SFC} of AU-AP2C (the product shows 99.4% amino
268 acid identities to SFC-1 and SFC of AU-AP2C). Novel alleles SFC-2 and SFC-3 were assigned
269 to SFC enzymes in river isolates and AU-AP2C, respectively. JSRIV006, in contrast, carried
270 bla_{SFH-1} and bla_{SFC-1}, though a synonymous mutation was detected in bla_{SFH-1} when compared
271 with bla_{SFH-1} in UTAD54. Although detailed genetic contexts of the bla_{SFH} and bla_{SFC} genes are
272 not known, these genes were previously shown to be located on the chromosome of UTAD54
273 by probe hybridization.[38, 39] The bla_{SFH-1} and bla_{SFC} genes in the river isolates sequenced to
274 closure in this study (JSRIV001, JSRIV002, JSRIV004, JSRIV006) were also located on
275 chromosomes and situated close to each other (separated by 76 kbp to 131 kbp). Given the
276 sporadic phylogenetic distribution (**Figure 4**), the two genes were suspected to be located on
277 GIs.[51] Comparative analysis of the chromosomes of four river isolates and the chromosome of
278 *S. fonticola* clade III strain GS2 (GCF_001514455.1) revealed that the two genes were located
279 on putative GIs inserted at tRNA-Phe genes. (**Figure 5A**). Importantly, IslandViewer 4
280 predicted almost the same regions as potential GIs (**Figure S5**). Perfect (5′-
281 TCGATTCCGAGTCCGGGCACCA-3′, detected in JSRIV004) or imperfect (5′-

282 TCGATTCCGAGTCC-GGCACCA-3′, detected in JSRIV001, JSRIV002, and JSRIV006)
283 repeat sequences corresponding to the 3′-end of the tRNA-Phe gene were detected at the right
284 ends of the GIs. Some previous studies also reported imperfect repeats at the right ends of GIs.[52,
285 53] An integrase gene was detected ~200 bp downstream of the tRNA-Phe gene in each GI
286 (**Figure 5B**), which indicates that the GIs were integrated at tRNA-Phe genes by site-specific
287 recombination mediated by these integrases. Genetic contexts of bla_{SFH-1} were highly conserved
288 and the bla_{SFH-1} gene was surrounded by a ΔIS*Ec18*-like element and an IS*1N*-like element in
289 all four completed chromosomes (**Figure 5B**). Although the complete genome was not
290 available for strain UTAD54, analysis of a contig carrying bla_{SFH-1} revealed that the gene was
291 embedded within the same genetic context and also carried by a putative GI which was inserted
292 at the tRNA-Phe gene (**Figure 5B**). In draft assemblies of JSRIV003, JSRIV005, JSRIV007,
293 JSRIV008 and JSRIV009, contigs were broken near bla_{SFH-1} and genetic contexts of bla_{SFH-1}
294 could not be determined. Genetic surroundings of bla_{SFC} were also highly conserved, though
295 variations were observed in regions >3,000 bp away from bla_{SFC} (**Figure 5C**). bla_{SFC} was
296 surrounded by genes such as *smvA* (encoding a methyl viologen resistance protein), *acrR*
297 (encoding a transcriptional regulator), and genes encoding hypothetical proteins. The genetic
298 surroundings of bla_{SFC} (<3,000 bp from bla_{SFC}) were also conserved in draft assemblies of
299 JSRIV003, JSRIV005, JSRIV007, JSRIV008 and JSRIV009. Although bla_{SFH} and bla_{SFC} seem
300 to be mobilized together within the same GIs, strain AU-AP2C carried only bla_{SFC-3} and lacked

301 *bla*$_{SFH}$. A contig carrying *bla*$_{SFC-3}$ in the draft genome assembly of AU-AP2C was long enough
302 for the comparative analysis. In AU-AP2C, a putative GI was inserted at the tRNA-Phe gene,
303 and *bla*$_{SFC-3}$ was also carried on this putative GI (**Figure S6A**). However, the region containing
304 the integrase gene and *bla*$_{SFH-1}$ was replaced by another segment as shown in **Figure S6B**.
305 Three *S. ureilytica* isolates from the lake, JBIWA004, JBIWA011 and JBIWA012, carried
306 *bla*$_{IMI-22}$ and *bla*$_{IMI-23}$. Among the three isolates, the genome of JBIWA004 was sequenced to
307 closure. The two *bla*$_{IMI}$ genes in JBIWA004 were located on a 106,702 bp plasmid, named
308 pJBIWA004_1, with IncFII(pRSB107) and pSM22 replicons. Online BLASTN analysis
309 revealed the plasmid p2-125 from *Serratia liquefaciens* showed the highest coverage (55%)
310 and also showed the high percent identity (96%) to pJBIWA004_1, while other plasmids
311 showed <30% coverage. Alignment of pJBIWA004_1 and p2-125 revealed that the two
312 plasmids shared conjugation regions, but the region containing the *bla*$_{IMI}$ genes was only
313 present in pJBIWA004_1 (**Figure 6A**). Comparison of the genetic contexts of the two *bla*$_{IMI}$
314 genes and that of *bla*$_{IMI-1}$ in *E. cloacae* complex strain N11-1168 showed that the regulator *imiR*
315 gene and a gene encoding a hypothetical protein, which are usually present upstream and
316 downstream of *bla*$_{IMI}$ genes, were truncated or deleted in pJBIWA004_1 (**Figure 6B**). *bla*$_{IMI-22}$
317 and *bla*$_{IMI-23}$ were surrounded by remnants of ISs, which might have contributed to their
318 mobilization. Although only draft genome assemblies were available for JBIWA011 and
319 JBIWA012, the *bla*$_{IMI-22}$ and *bla*$_{IMI-23}$ genes were located on a single contig in each assembly.

320 Moreover, those contigs showed >94% coverage and >99.9% identity to pJBIWA004_1,
321 indicating that JBIWA011 and JBIWA012 also carried $bla_{\text{IMI-22}}$ and $bla_{\text{IMI-23}}$ on pJBIWA004_1-
322 like plasmids.

323 **Conclusions**

324 Here, we identified the prevalence of minor carbapenemases among environmental CPE
325 isolates in Japan. Of note, bla_{SFH} and bla_{SFC}, which have been described only in one *S. fonticola*
326 isolate in Portugal, were prevalent among river *S. fonticola* isolates obtained from different
327 sampling sites. The results of this study indicate aquatic environments as potential reservoirs
328 of minor carbapenemases and highlight the need to continuously monitor antibiotic resistance
329 in the environment.

330

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338 **Transparency declarations**

339 None to declare.

340

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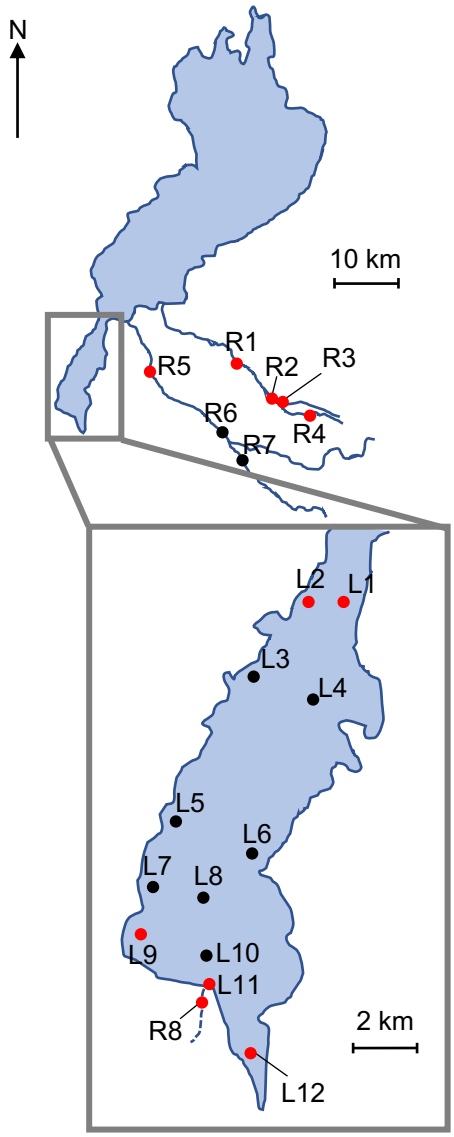
482

483

484 **Figures**



485

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

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

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

489

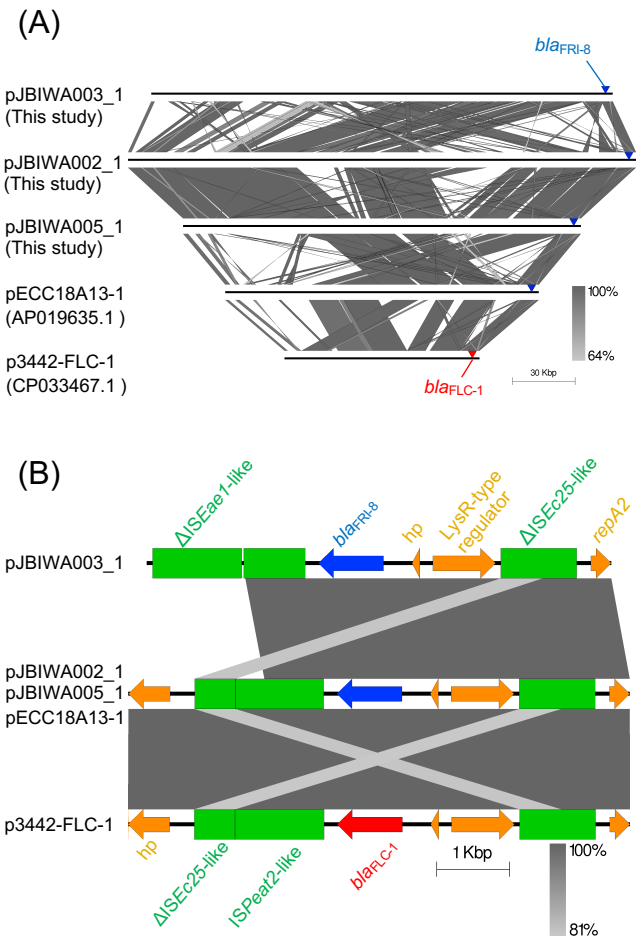


Figure 2. (A) Alignment of plasmids pJBIWA003_1, pJBIWA002_1, pJBIWA005_1, pECC18A13-1, and p3442-FLC-1. Positions of *bla*$_{\text{FRI-8}}$ and *bla*$_{\text{FLC-1}}$ are indicated by blue and red triangles, respectively. (B) Genetic contexts of *bla*$_{\text{FRI-8}}$ and *bla*$_{\text{FLC-1}}$. Blue arrows indicate *bla*$_{\text{FRI-8}}$, a red arrow indicates *bla*$_{\text{FLC-1}}$, green boxes indicate insertion sequences, and orange arrows indicate other coding sequences. hp: hypothetical protein. The alignments were generated with Easyfig.[54]

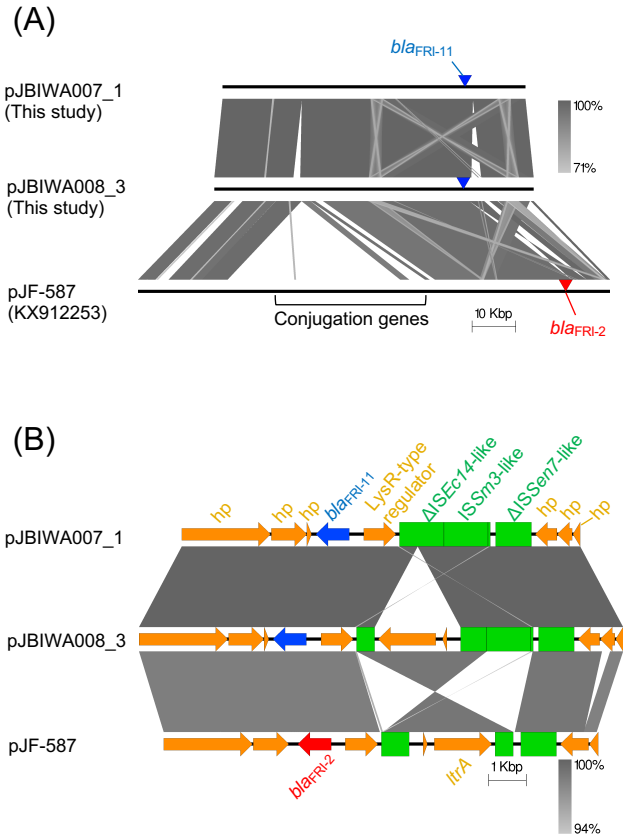


Figure 3. (A) Alignment of pJBIWA007_1, pJBIWA008_3, and pJF-587. Positions of *bla*$_{\text{FRI-11}}$ and *bla*$_{\text{FRI-2}}$ are indicated by blue and red triangles, respectively. (B) Genetic contexts of *bla*$_{\text{FRI-11}}$ and *bla*$_{\text{FRI-2}}$. Blue arrows indicate *bla*$_{\text{FRI-11}}$, a red arrow indicates *bla*$_{\text{FRI-2}}$, green boxes indicate insertion sequences, and orange arrows indicate other coding sequences.

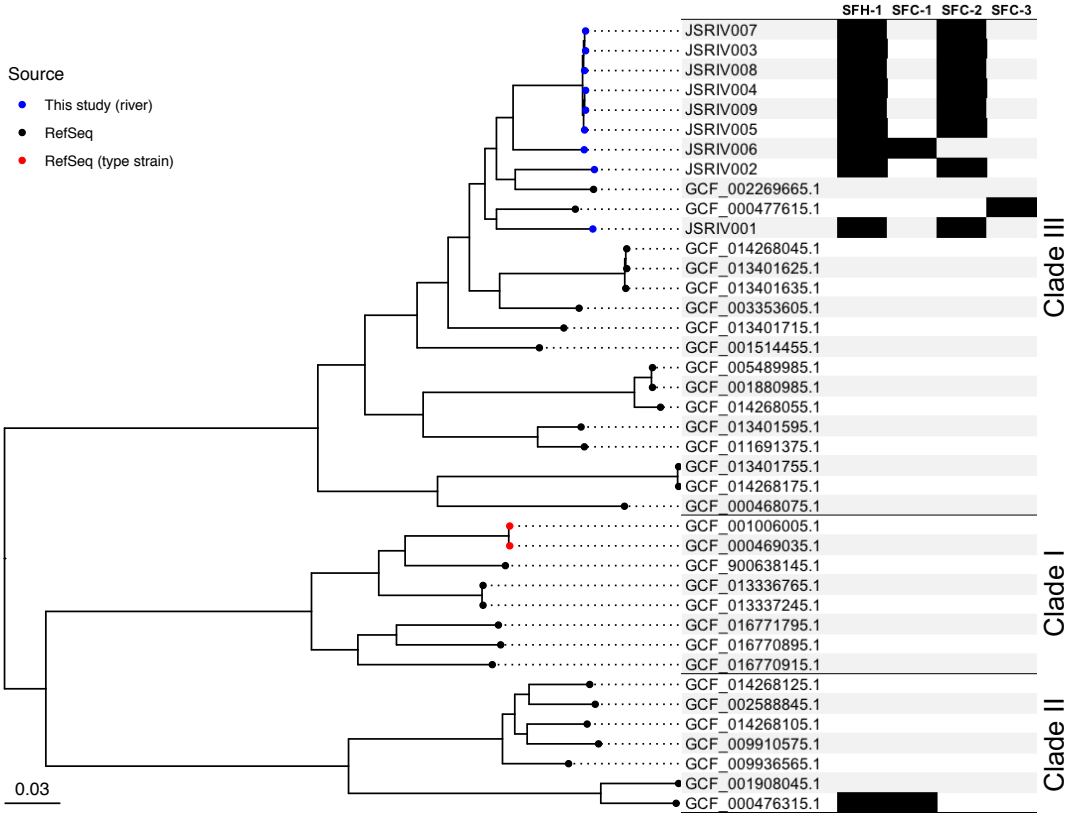
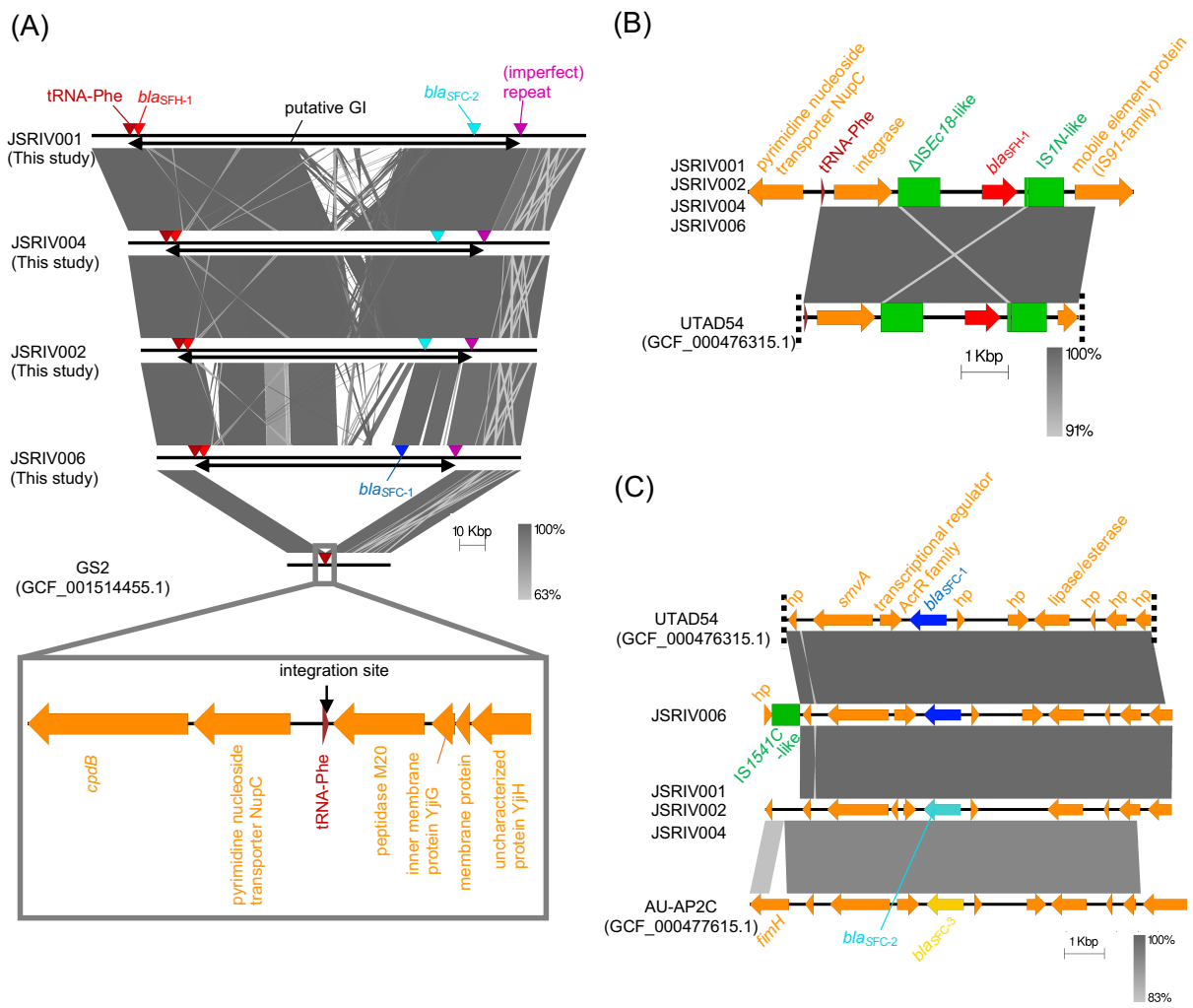


Figure 4. Phylogenetic tree constructed using RefSeq assemblies labelled *S. fonticola* and assemblies of nine river *Serratia* isolates. The RefSeq assemblies were downloaded in March 2021. The tree was constructed based on 177,349 SNPs occurring in at least 80% of the genomes. Four RefSeq assemblies (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.[55] The black blocks represent the presence of a carbapenemase gene.



514

515 **Figure 5.** (A) Alignment of the putative GIs and the surrounding regions of river *Serratia*

516 isolates sequenced to closure and the corresponding chromosomal region of *S. fonticola* clade

517 III strain GS2. Positions of the bla_{SFC-1}, bla_{SFC-2}, bla_{SFH-1}, and tRNA-Phe genes, and (imperfect)

518 repeats are indicated by blue, light blue, red, brown, and purple triangles, respectively. Double-

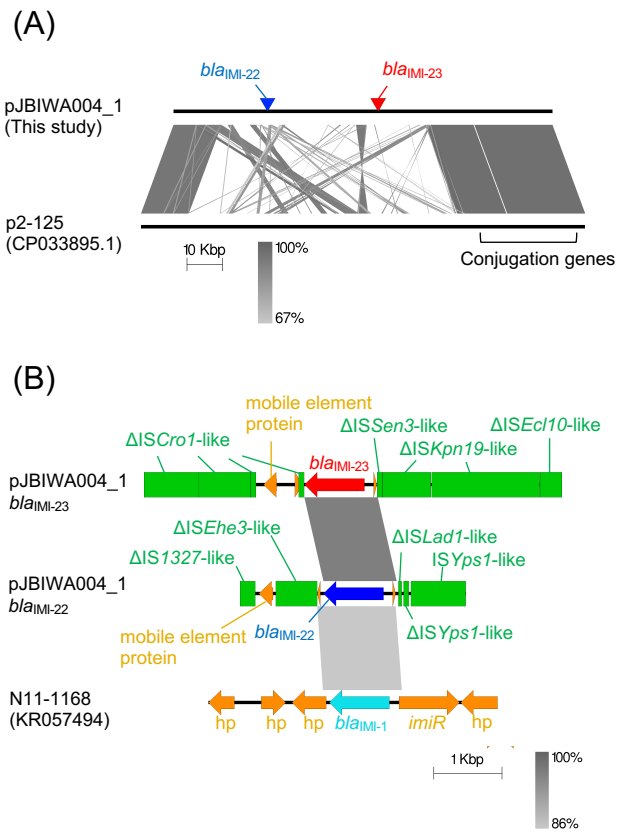
519 headed arrows indicate genomic regions corresponding to GIs. Genetic contexts of (B) bla_{SFH}

520 and (C) bla_{SFC}. Red arrows indicate bla_{SFH-1}, blue arrows indicate bla_{SFC-1}, a light blue arrow

521 indicates bla_{SFC-2}, a yellow arrow indicates bla_{SFC-3}, green boxes indicate insertion sequences,

522 and orange arrows indicate other coding sequences. Dashed lines indicate contig breaks.

523



524

525 **Figure 6**. (A) Alignment of pJBIWA004_1 and p2-125. Positions of *bla*$_{\text{IMI-22}}$ and *bla*$_{\text{IMI-23}}$ are

526 indicated by blue and red triangles, respectively. (B) Genetic contexts of *bla*$_{\text{IMI}}$ genes. A blue

527 arrow indicates *bla*$_{\text{IMI-22}}$, a red arrow indicates *bla*$_{\text{IMI-23}}$, a light blue arrow indicates *bla*$_{\text{IMI-1}}$, green

528 boxes indicate insertion sequences, and orange arrows indicate other coding sequences.

529

530 **Tables**

531 **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

532 [a]Genomes of isolates in bold were sequenced by both DNBSEQ and ONT sequencers. Among
533 the 12 isolates in bold, all the genomes except JBIWA006 were completed. Also see **Table S2**
534 for the accession numbers of the closed replicons and **Table S3** for the SRA accession numbers
535 of the incomplete genomes.

536 [b]STs/clades are shown in parentheses.

537 [c]UD: undetermined.