Quinolone resistance genes *qnr*, *aac(6')-Ib-cr*, *oqxAB*, and *qepA* in environmental *Escherichia coli*: insights into their genetic contexts from comparative genomics

Ryota Gomi[a, *] and Fumie Adachi[b]

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

[b]Department of Environmental Health, Osaka Institute of Public Health, 1-3-3 Nakamichi, Higashinari-ku, Osaka 537-0025, Japan

[*]Corresponding author

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

ABSTRACT

Previous studies have reported the occurrence of transferable quinolone resistance determinants in environmental *Escherichia coli*. However, little is known about their vectors and genetic contexts. To gain insights into these genetic characteristics, we analyzed the complete genomes of 53 environmental *E. coli* isolates containing one or more transferable quinolone resistance determinants, including 20 sequenced in this study and 33 sourced from RefSeq. The studied genomes carried the following transferable quinolone resistance determinants alone or in combination: *aac(6')-Ib-cr*, *oqxAB*, *qepA1*, *qnrA1*, *qnrB4*, *qnrB7*, *qnrB19*, *qnrD1*, *qnrS1*, and *qnrS2*, with *qnrS1* being predominant. These resistance genes were detected on plasmids of diverse replicon types; however, *aac(6')-Ib-cr*, *qnrS1*, and *qnrS2* were also detected on the chromosome. The genetic contexts surrounding these genes included not only those found in clinical isolates but also novel contexts, such as *qnrD1* embedded within a composite transposon-like structure bounded by Tn*3*-derived inverted-repeat miniature elements (TIMEs). This study provides deep insights into mobile genetic elements associated with transferable quinolone resistance determinants, highlighting the importance of genomic surveillance of antimicrobial resistant bacteria in the environment.

Keywords: *Escherichia coli*, Antimicrobial resistance, Transferable mechanisms of quinolone resistance (TMQR), Plasmid-mediated quinolone resistance (PMQR), Genetic context

35 **1. INTRODUCTION**

36 *Escherichia coli* is a commensal member of the gut microbiota of humans and animals but can
37 also cause intestinal and extraintestinal infections.[1] Another concern besides pathogenicity is
38 the rise of antimicrobial resistance (AMR) in *E. coli*.[2] This is due to the accumulation of AMR
39 determinants, including acquired AMR genes and chromosomal point mutations.[3]

40 Quinolones are antibiotics that are widely used for treatment of infections caused by a variety
41 of bacteria, including *E. coli*.[4] Quinolone resistance in *E. coli* is mainly attributed to mutations
42 in the quinolone resistance-determining regions (QRDRs) of the topoisomerase genes *gyrA* and
43 *parC*.[5,6] Although these chromosomal mutations are generally not horizontally transferred,
44 there are quinolone resistance determinants that can be transmitted between bacteria, including
45 target protection (*qnr*), antibiotic efflux (*qepA* and *oqxAB*), and antibiotic modification
46 (*aac(6')-Ib-cr*).[7] These transferable resistance determinants are called plasmid-mediated
47 quinolone resistance (PMQR) genes or transferable mechanisms of quinolone resistance
48 (TMQR) determinants (a recent review recommended the use of TMQR because these genes
49 are sometimes present on the chromosome, so we use the term TMQR in this paper).[7] It is
50 known that the levels of resistance conferred by TMQR are relatively low; however, TMQR
51 can play an important role in the development of quinolone resistance because (i) the effect of
52 TMQR on quinolone minimum inhibitory concentrations (MICs) is additive to that conferred

53 by other transferable or chromosome-mediated quinolone resistance determinants, and (ii)
54 TMQR can facilitate the selection of mutants with higher-level quinolone resistance.[7,8]

55 Information on the vectors and genetic contexts of AMR genes is important for multiple
56 reasons: for example, (i) it allows identification of frequently associated/linked resistance
57 genes and thus can provide information on co-resistance; (ii) it facilitates better understanding
58 of the evolution of multiresistance regions and how resistance genes spread.[9,10] Previous studies
59 reported the occurrence of TMQR determinants in environmental *E. coli*, and some studies also
60 reported the sequences of replicons carrying TMQR determinants.[11,12] However, in-depth
61 analysis of the genetic contexts of TMQR determinants has not been performed in most cases,
62 and whether the environmental isolates carry TMQR determinants in the contexts related to
63 those in clinical isolates or in previously unreported contexts is largely unknown. Here, we
64 analyzed the complete genomes of environmental *E. coli* isolates, sequenced in this study or
65 obtained from the NCBI reference sequence (RefSeq) database, with the aim to acquire
66 genomic insights into TMQR, including their vectors and genetic contexts.

67

68 **2. MATERIALS AND METHODS**

69 **2.1. *E. coli* isolates**

70 Twenty-four environmental *E. coli* isolates carrying TMQR determinants isolated in Japan
71 were characterized in this study. These 24 isolates included 19 isolates from our previous
72 collections: (i) three isolates from river water samples collected in 2010 (1002W03, 1008S13,
73 and 1008S19),[13] (ii) 10 isolates from river water samples collected between 2011–2013
74 (KFu015, KMi012, KMi014, KMi029, KKa014, KFu021, KOr024, KTa005, KTa007,
75 KTa009),[14] and (iii) six extended-spectrum β-lactamase (ESBL)-producing *E. coli* isolates
76 obtained from municipal wastewater (JSWP006, JSWP021) and hospital wastewater
77 (JKHS004, JKHS006, JKHS016, and JKHS019) in 2015.[15] Five isolates were obtained in the
78 present study: three isolates were screened from *E. coli* isolated from municipal wastewater
79 using CHROMagar ECC (Kanto Chemical Co., Tokyo, Japan) supplemented with cefotaxime
80 (1 mg/L) between 2019–2020 (19M19, 19A14, and 20A24), and two isolates were screened
81 from *E. coli* isolated from river water using CHROMagar ECC supplemented with
82 ciprofloxacin (0.03 mg/L) between 2021–2022 (21F61 and 22F46). For the newly obtained
83 isolates, the presence of TMQR genes was confirmed by PCR using primers described
84 previously,[13,16,17] with a slight modification (5′-GTGAAGTCGATCAGTCAGTG-3′ was used
85 instead of oqxAFw).

86 **2.2. Antimicrobial susceptibility testing**

87 Antimicrobial susceptibility testing of the 24 isolates was performed by microdilution using
88 Dry Plate Eiken (Eiken, Tokyo, Japan) according to CLSI specifications (see **Table S1** for

89 antimicrobials used). The MIC of ciprofloxacin was also determined using the Etest
90 (bioMérieux, Marcy-l'Étoile, France). The results were evaluated according to CLSI criteria[18]
91 and EUCAST epidemiological cutoff (ECOFF) values (https://mic.eucast.org/search/).

92 **2.3. Genome sequencing and assembly**

93 The genomes of 24 isolates were sequenced using a combination of short-read (Illumina) and
94 long-read (Oxford Nanopore Technologies) sequencing. Hybrid assembly of Illumina short
95 reads and ONT long reads was performed using both a long-read-first approach and a short-
96 read-first approach. The details of genome sequencing and assembly are described in the
97 Supplementary Materials and methods. Of the 24 isolates, the genomes of 20 isolates could be
98 completed, while the genomes of four isolates (KMi014, KTa007, KTa009, JKHS019) could
99 not be completed despite repeated long-read sequencing. The 20 completed genomes were
100 further analyzed to elucidate the genetic contexts of TMQR determinants.

101 **2.4. Retrieving complete *E. coli* genomes with TMQR determinants from the NCBI**
102 **database**

103 RefSeq *E. coli* genomes with an assembly level of "complete genome" (n = 2815) were
104 downloaded using the ncbi-genome-download tool (v0.3.1, https://github.com/kblin/ncbi-
105 genome-download) in July 2023. Metadata information, including strain name and isolation
106 source, was extracted from the downloaded files, and genomes determined to be of

107 environmental origin (i.e., surface water, sediment, wastewater, and soil) (n = 203) were
108 retained. AMR genes were detected using ABRicate (v1.0.1,
109 https://github.com/tseemann/abricate) with the NCBI database.[19] This identified 48 genomes
110 with at least one TMQR determinant. For each of the 48 genomes, we searched PubMed and
111 Google Scholar for the associated publication. This identified publications for 33 genomes,
112 which were further analyzed in this study. Detailed information on these 33 genomes is
113 summarized in **Table S2**.

114 **2.5. Genomic analysis**

115 The *E. coli* genomes sequenced in this study and those retrieved from RefSeq were subjected
116 to the following genomic analysis. AMR genes were detected using ResFinder 4.1[20] and
117 ABRicate (v1.0.1, https://github.com/tseemann/abricate) with the NCBI database.[19] Multilocus
118 sequence typing (MLST) was performed using mlst (v2.19.0,
119 https://github.com/tseemann/mlst). Plasmid replicons were detected using PlasmidFinder 2.1,
120 and plasmids were typed using pMLST 2.0.[21] Genomes were annotated using the RAST
121 server,[22] ISfinder,[23] and the blastn program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) with the
122 core nucleotide (core_nt) database (last accessed in August 2024).

123 **2.6. Data availability**

124 The complete genomes and sequence reads obtained in the present study have been deposited
125 in GenBank and the NCBI SRA under BioProject PRJNA1078256 (also see **Table S1** for the
126 accession number of each genome).

127

128 **3. RESULTS AND DISCUSSION**

129 **3.1. Basic characteristics of *E. coli* isolates sequenced in this study**

130 Twenty-four *E. coli* isolates with TMQR determinants were sequenced in the present study.
131 These *E. coli* isolates belonged to 20 different STs, including clinically important lineages such
132 as ST131 (**Table S1**). These isolates carried the following TMQR determinants alone or in
133 combination: *qnrS1* (n = 12), *qnrD1* (n = 6), *aac(6')-Ib-cr* (n = 4), *qnrB7* (n = 3), *qnrS2* (n =
134 3), *qepA1* (n = 1), *qnrB19* (n = 1), and *oqxAB* (n = 1) (**Table S1**).

135 The isolates were non-susceptible to a wide variety of antibiotics (**Table S1**). Nonsusceptibility
136 rates for quinolones were 29.2% for nalidixic acid, 37.5% for ciprofloxacin (evaluated by Etest),
137 and 37.5% for levofloxacin. All isolates were non-susceptible to nalidixic acid, ciprofloxacin,
138 and levofloxacin when they carried a QRDR mutation(s). Isolates with TMQR determinants
139 but without QRDR mutations were susceptible to nalidixic acid but either susceptible or non-
140 susceptible to ciprofloxacin and levofloxacin. This is consistent with previous studies reporting
141 that isolates with TMQR determinants but without QRDR mutations sometimes show an

142 unusual phenotype of nalidixic acid susceptibility and ciprofloxacin resistance.[7] Isolates with
143 TMQR determinants but without QRDR mutations were all classified as non-wild type to
144 ciprofloxacin and levofloxacin according to the ECOFF criteria, indicating that interpreting
145 results with ECOFF values can increase the sensitivity for detection of TMQR determinants.

146 **3.2. Basic characteristics of RefSeq *E. coli* genomes with TMQR determinants**

147 Thirty-three RefSeq *E. coli* genomes with TMQR determinants were retrieved, which were
148 associated with 12 publications. These were from wastewater (n = 18) or environmental water
149 (n = 15), isolated between 2012 and 2022, and from various geographical locations, with
150 Switzerland (n = 14 from two publications) and Japan (n = 10 from three publications) being
151 predominant (**Table S2**). In total, 24 different STs were detected among the genomes. These
152 genomes carried the following transferable quinolone resistance determinants alone or in
153 combination: *qnrS1* (n = 21), *aac(6')-Ib-cr* (n = 9), *qnrB4* (n = 3), *oqxAB* (n = 3), *qnrS2* (n =
154 2), *qnrA1* (n = 1), and *qnrB19* (n = 1) (**Table S2**). Although we extracted RefSeq genomes
155 associated with publications, the genetic contexts of TMQR genes were investigated only for
156 four genomes in four publications (**Table S2**). Even for these genomes, the contexts were often
157 insufficiently or incorrectly annotated. We thus performed in-depth analysis of the genetic
158 contexts in the RefSeq genomes together with our genomes as below.

159 **3.3. Genetic contexts of *qnrA1***

160 A RefSeq genome analyzed in this study carried *qnrA1* on an IncA/C plasmid, p009_A. IS*CR1*

161 is usually present upstream of *qnrA1*, whereas downstream structures of *qnrA1* are known to

162 be divergent.[24] In p009_A, *qnrA1* was embedded in a structure related to class 1 integrons In36

163 and In37 (**Figure 1**).[25] A structure related to another class 1 integron, In4873, was detected

164 upstream of *qnrA1*.[26] The association of In36/In37-like structure and In4873-like structure

165 seems to be rare: blastn analysis found only one closely related structure in an IncHI2/IncHI2A

166 plasmid, pME-1a, from a clinical *Enterobacter hormaechei* isolate from the USA (**Figure 1**).[27]

167 It is assumed that these closely related structures derive from the same ancestral structure,

168 which was (i) separately introduced into an IncA/C plasmid and an IncHI2/IncHI2A or (ii)

169 generated in or introduced into one plasmid, which was then mobilized to the other.

170 **3.4. Genetic contexts of *qnrB4***

171 Three RefSeq genomes carried *qnrB4* on different IncF plasmids. *qnrB4* is known to be

172 globally distributed and often associated with the AmpC type β-lactamase gene $bla_{\text{DHA-1}}$.[24] In

173 all three plasmids, *qnrB4* is linked to $bla_{\text{DHA-1}}$ and also to other AMR genes (**Figure 2a**). The

174 context from *sul1* to the truncated class 1 integron was detected in multiple clinical isolates

175 (e.g., CP058590), but the broader context seen in p142_A-OXA181, which links *qnrB4* and

176 *qnrS1*, was not detected in any other public genomes by blastn analysis. The effect on

177 susceptibility to quinolones by carrying multiple *qnr* genes is not completely understood but

178 seems to be minor, because the products of two genes can compete for binding to gyrase.[24]

179 **3.5. Genetic contexts of *qnrB7***

180 Two isolates sequenced to closure in the present study carried *qnrB7*. *qnrB7* was previously
181 reported to be associated with the ESBL gene bla_{SHV-12}.[24] However, reports of complete plasmid
182 (or chromosome) sequences carrying *qnrB7* are scarce; blastn analysis identified only four
183 complete plasmid sequences carrying *qnrB7*. One of them, the IncX3 plasmid
184 pLHST2018_IncX3 harbored by a clinical *Salmonella enterica* isolate from India, carried both
185 *qnrB7* and bla_{SHV-12}.[28] In our isolates, *qnrB7* was also carried by IncX3 plasmids and associated
186 with bla_{SHV-12} (**Figure 2b**). The genetic contexts in our isolates and pLHST2018_IncX3 were
187 similar, but a region next to bla_{SHV-12} was deleted in pLHST2018_IncX3. Detection of closely
188 related AMR plasmids in environmental and clinical bacteria from different countries
189 highlights the importance of global One Health surveillance to track the spread of AMR genes.

190 **3.6. Genetic contexts of *qnrB19***

191 Two genomes, one sequenced by us and the other from RefSeq, carried *qnrB19*. *qnrB19* is
192 usually mobilized by transposition units (TPU) mediated by IS*Ecp1*, such as Tn*2012* and
193 Tn*5387*, or small ColE1-like plasmids containing fragments of these transposons.[29] The most
194 common *qnrB19*-carrying ColE1-like plasmid is a 2699 bp plasmid named pPAB19-1 (or
195 pSGI15/pECY6-7/pMK100), with others being minor variants of this plasmid.[29] The two
196 *qnrB19*-positive genomes carried this gene on small ColE1-like plasmids, p20A24_1 and p32-

197 4_E. p20A24_1 is related to the common plasmid pPAB19-1, with modifications in two regions

198 (**Figure 2c**). p32-4_E was identified as a cointegrate comprising pPAB19-1 and a 1718 bp

199 plasmid almost identical (single nucleotide difference) to pMB9272_7 (CP103528), with

200 putative *oriT* regions being the cointegration points. No close hits were found for this plasmid

201 by blastn analysis (all hits were below 80% query coverage). pMB9272_7 carries only two

202 genes encoding hypothetical proteins, and no plasmid replicon was identified by PlasmidFinder.

203 Cointegrates comprising ColE1-like plasmids and large plasmids were previously shown to

204 play an important role in the spread of AMR.[30] The example of p32-4_E indicates cointegrates

205 of ColE1-like plasmids and other small plasmids may also contribute to the spread of AMR.

206 **3.7. Genetic contexts of *qnrD1***

207 Three genomes sequenced to closure in the present study carried *qnrD1*. *qnrD1* was first

208 described on a 4270 bp plasmid, p2007057, in a clinical *Salmonella enterica* strain isolated in

209 China.[31] *qnrD1* has since been detected on multiple plasmids; however, blastn analysis

210 identified that *qnrD1* is rare in *Enterobacteriaceae* and mostly detected on ~2.7 kbp plasmids

211 in *Morganellaceae*. Two of the three genomes carried *qnrD1* on a plasmid identical to

212 p2007057 (**Figure 3**). The remaining genome carried *qnrD1* on a 6657 bp plasmid, named

213 pKFu015_4, with no close blastn hits (all hits were below 50% query coverage). The *qnrD1*

214 region, including *qnrD1* and ORF2, in pKFu015_4 is identical to ~2.7 kbp *Morganellaceae*

215 plasmids such as pRS12-11 (KF364953) and is ~95% identical to the corresponding region in

216 p2007057 (and thus pKMi029_6 and pKTa005_7). Analysis of sequences surrounding the
217 *qnrD1* region revealed 209 bp directly oriented repeats upstream and downstream of the region.
218 This repeat carries imperfect 33 bp inverted repeats (IRs) closely related to some Tn*3*-family
219 transposons (e.g., Tn*Ec4*), though it does not encode a transposase. The blastn analysis revealed
220 that this repeat is identical to a Tn*3*-derived inverted-repeat miniature element (TIME), named
221 $TIME_{IS101}$.[32] Pairs of TIMEs are known to be involved in mobilization of intervening sequences
222 when the corresponding Tn*3* family transposases are provided *in trans*, and these composite
223 transposon-like structures were previously named TIME-COMP.[32,33] Thus, the TIME-COMP
224 structure, $TIME_{IS101}$-*qnrD1*-ORF2-$TIME_{IS101}$, seems to have been mobilized as a single unit
225 into a ColE1-like plasmid, generating pKFu015_4. The presence of 5 bp direct repeats
226 (AGCTA) surrounding this structure supports this idea. Moreover, we also found a ColE1-like
227 plasmid without insertion of this TIME-COMP structure among the genomes sequenced in this
228 study (pKMi029_5, CP147114), further supporting this. *qnrD1* was previously suggested to be
229 mobilized as a mobile insertion cassette (mic) element (a nonautonomous element bracketed
230 by two IRs),[34] but this observation indicates another mode of *qnrD1* mobilization, namely
231 TIME-COMP.

232 **3.8. Genetic contexts of *qnrS1***

233 *qnrS1* was found to be the most prevalent TMQR gene both in our isolates (n = 12) and in the
234 RefSeq genomes (n = 21) and was detected on plasmids of diverse replicon types or on the

235 chromosome (**Table S1** and **Table S2**). Although earlier studies showed low *qnrS1*
236 prevalence,[7] some recent studies identified *qnrS1* as the most prevalent TMQR gene in the One
237 Health context.[35,36] The genetic contexts of *qnrS1* were largely classified into 13 types (types
238 A to M in **Figure 4**). Types B, E, and I are found only in the genomes analyzed in this study
239 and no other hits were found by blastn analysis. On the other hand, the other genetic contexts
240 were found in at least one public genome, and some were described in previous studies (e.g.,
241 types A, F and G previously described in clinical or animal isolates).[37-41] *qnrS1* is frequently
242 linked to other resistance genes in the contexts, including an ESBL gene $bla_{\text{CTX-M-15}}$ in types B–
243 D. The potential evolutionary pathways of these context types are described in **Figure S1**.
244 Briefly, IS*26*-mediated rearrangements and IS*Ecp1*-mediated transposition seem to have
245 played major roles in diversification of the contexts and linkage of *qnrS1* to other AMR genes.

246 **3.9. Genetic contexts of *qnrS2***

247 Two genomes sequenced to closure in this study and two RefSeq genomes carried *qnrS2*. *qnrS2*
248 was previously reported to be part of a mic element bracketed by 22 bp imperfect IRs.[42] In all
249 four genomes, the mic elements containing *qnrS2* were truncated (**Figure 5**). Two RefSeq
250 genomes carried *qnrS2* in the same genetic context on IncX1 plasmids. The *qnrS2* region in
251 these plasmids was followed by the *fosA3* genetic context type K,[43] linking *qnrS2* to *fosA3*,
252 Δbla_{TEM}, and $bla_{\text{CTX-M-55}}$. This linkage was not detected in any other public genomes by blastn
253 analysis. Two ST2179 genomes sequenced in this study carried *qnrS2* on the chromosome. In

254 19M19, the *qnrS2* region was duplicated. The blastn search identified five *E. coli* genomes

255 carrying *qnrS2* on the chromosome, three of which belong to ST2179. The genetic contexts of

256 *qnrS2* in these three genomes were closely related to that in 19M19, but the *qnrS2* region was

257 not duplicated (**Figure 5**). It is assumed that a resistance region containing *qnrS2* was inserted

258 into an ancestral ST2179 genome, which was followed by IS*26*-mediated rearrangements,

259 yielding the variation of the contexts seen in **Figure 5**.

260 **3.10. Genetic contexts of *aac(6')-Ib-cr***

261 Three genomes sequenced to closure in this study and nine RefSeq genomes carried *aac(6')-*

262 *Ib-cr*. *aac(6')-Ib-cr* was detected on the chromosome (n = 6) or on IncF plasmids (n = 6)

263 (**Figure 6**). The isolates with chromosomal *aac(6')-Ib-cr* belonged to five different STs (ST10,

264 ST410, ST648, ST1266, and ST2179). A chromosomal location for *aac(6')-Ib-cr* was also

265 reported previously in clinical ST131 and ST648 isolates from Spain.[44] These examples

266 indicate chromosomal integration of *aac(6')-Ib-cr* has occurred multiple times in different STs.

267 *aac(6')-Ib-cr* has been identified in a gene cassette as a segment of class 1 integrons.[45] The

268 IS*26*-*aac(6')-Ib-cr*-bla_{OXA-1}-*ΔcatB3*-IS*26* structure was prevalent and detected in seven

269 genomes (58%), while the upstream and downstream sequences were divergent (**Figure 6**).

270 This structure is also common in public genomes (more than 1000 hits by blastn). However,

271 the IS*26* sequences are inversely oriented and thus this structure is neither a transposon nor a

272 pseudo-compound transposon.[46] The prevalence of this structure among various
273 plasmids/chromosomes can partially be explained by the presence of other mobile genetic
274 elements surrounding it, which might have aided the movement of multiresistance regions
275 containing this structure.

276 **3.11. Genetic contexts of *oqxAB***

277 Mobile *oqxAB* genes are commonly found within an IS*26*-*oqxA*-*oqxB*-*oqxR*-IS*26* pseudo-
278 compound transposon, named Tn*6010*.[47] All four *oqxAB* genes identified in the present study
279 are situated within Tn*6010* and carried by plasmids of different Inc types (**Figure 7**). The
280 sequences upstream of Tn*6010* (in reference to the orientation of *oqxAB* genes) in three RefSeq
281 plasmids share some common regions, which were also detected upstream of Tn*6010* in
282 multiple public genomes by blastn. On the other hand, the upstream sequence of Tn*6010* was
283 replaced by a truncated Tn*1721* and a truncated Tn*2* in pJKHS004_1, linking *tetA* and a
284 truncated bla_{TEM} to *oqxAB*. No genomes were found by blastn analysis to carry this
285 multiresistance region.

286 **3.12. Genetic contexts of *qepA1***

287 *qepA* genes are typically associated with IS*CR3* and embedded within complex integrons.[48]
288 One isolate sequenced in this study carried *qepA1*, and the gene was located between a
289 truncated *intI1* and IS*CR3* on an IncF plasmid (**Figure 8**). Other AMR genes, *dfrB4*, *sul1*, and

290 *mph(A)*, were found to be linked to *qepA1* in this context. The blastn analysis of the entire
291 resistance region bounded by IS*26* revealed closely related sequences in three *E. coli* plasmids,
292 one from a clinical isolate in the USA and two from clinical isolates in Myanmar,[49,50] indicating
293 intercontinental occurrence of this multiresistance region and highlighting the need for a One
294 Health approach to AMR surveillance.

295 **3.13. Study limitations**

296 This study has some limitations. First, a limited number of complete genomes (n = 20) were
297 determined in this study. We also included RefSeq genomes in our analysis to extend our
298 dataset, which might partially mitigate this limitation. Second, there are potential biases in the
299 analyzed isolates. Even though we supplemented our dataset with RefSeq genomes, most
300 RefSeq genomes were from Japan and Switzerland. This seems to reflect the situation where
301 long-read sequencing is still not widely used to study antibiotic resistance in environmental
302 bacteria. Another potential bias lies in the isolation procedure. For example, our nine
303 wastewater isolates and some RefSeq isolates were obtained using selective media for ESBL-
304 producers. This could have influenced the relative abundance of certain genetic contexts (e.g.,
305 *qnrS1* genetic context types B–D, which link $bla_{\text{CTX-M-15}}$ with *qnrS1*). Third, the numbers of
306 complete genomes were small for *qnrA1* (n = 1), *qnrB7* (n = 2), *qnrB19* (n = 2), and *qepA* (n =
307 1). This might reflect the scarcity of these genes in environmental *E. coli*. However, we also
308 included genomes of clinical isolates in our analysis, which allowed comparative genomic

309 analysis for these genomes. Notably, and while not a limitation of our own study, we
310 unexpectedly found that even though the sequences of these clinical isolates were previously
311 reported, the genetic contexts of TMQR genes were not analyzed in many of the original studies
312 and thus were elucidated for the first time in the present study.

313

314 **4. CONCLUSIONS**

315 Here, we performed in-depth analysis of the genetic contexts of TMQR determinants in
316 environmental *E. coli*. To our knowledge, this is the most comprehensive study analyzing the
317 genetic contexts of TMQR determinants in environmental bacteria. The genetic contexts
318 described in this study included those closely related to the contexts found in clinical isolates.
319 We also detected novel contexts in our genomes (e.g., TIME-COMP containing *qnrD1*) and
320 previously uncharacterized contexts in RefSeq genomes (e.g., a small cointegrate plasmid
321 carrying *qnrB19*). Some TMQR genes were frequently linked to other AMR genes in the
322 described contexts, and this information will contribute to our understanding of how multidrug
323 resistance emerges and spreads in bacteria. We also found multiple examples of TMQR
324 determinants located on the chromosome. Chromosomal location of TMQR genes may
325 contribute to maintenance of these genes, but further studies are needed to confirm this. Overall,
326 this study provides valuable insights into mobile genetic elements associated with TMQR

327 determinants and highlights the importance of genomic surveillance of antimicrobial resistant
328 bacteria in the environment.

329

330 **CRediT authorship contribution statement**

331 Conceptualization: R.G.; methodology: R.G.; validation: R.G.; formal analysis: R.G.;
332 investigation: R.G. and F.A; resources: R.G. and F.A; data curation: R.G.; visualization: R.G.;
333 funding acquisition: R.G.; writing – original draft: R.G.; writing – review & editing: R.G. and
334 F.A.

335

336 **Acknowledgments**

337 This work was supported by JSPS KAKENHI (grant number JP22K18038), the Kurita Water
338 and Environment Foundation (22B006), the Sumitomo Foundation (2230113), and the
339 Environment Research and Technology Development Fund (JPMEERF20235R01) of the
340 Environmental Restoration and Conservation Agency, provided by the Ministry of the
341 Environment of Japan. Computations were partially performed on the NIG supercomputer at
342 ROIS National Institute of Genetics. We acknowledge the NGS core facility of the Genome
343 Information Research Center at the Research Institute for Microbial Diseases of Osaka

University for the support in DNA sequencing. We also thank Isao Nakamoto and Yasufumi Matsumura for their support in antimicrobial susceptibility testing.

References

1 Kaper, J. B., Nataro, J. P. & Mobley, H. L. Pathogenic *Escherichia coli*. *Nat Rev Microbiol* **2**, 123-140 (2004). https://doi.org/10.1038/nrmicro818

2 Denamur, E., Clermont, O., Bonacorsi, S. & Gordon, D. The population genetics of pathogenic *Escherichia coli*. *Nat Rev Microbiol* **19**, 37-54 (2021). https://doi.org/10.1038/s41579-020-0416-x

3 Poirel, L. *et al*. Antimicrobial Resistance in *Escherichia coli*. *Microbiol Spectr* **6**, ARBA-0026-2017 (2018). https://doi.org/10.1128/microbiolspec.ARBA-0026-2017

4 Aldred, K. J., Kerns, R. J. & Osheroff, N. Mechanism of quinolone action and resistance. *Biochemistry* **53**, 1565-1574 (2014). https://doi.org/10.1021/bi5000564

5 Aoike, N. *et al*. Molecular characterization of extraintestinal *Escherichia coli* isolates in Japan: relationship between sequence types and mutation patterns of quinolone resistance-determining regions analyzed by pyrosequencing. *J Clin Microbiol* **51**, 1692-1698 (2013). https://doi.org/10.1128/JCM.03049-12

6 van der Putten, B. C. L. *et al*. Quantifying the contribution of four resistance mechanisms to ciprofloxacin MIC in *Escherichia coli*: a systematic review. *J Antimicrob Chemother* **74**, 298-310 (2019). https://doi.org/10.1093/jac/dky417

7 Ruiz, J. Transferable Mechanisms of Quinolone Resistance from 1998 Onward. *Clin Microbiol Rev* **32**, e00007-00019 (2019). https://doi.org/10.1128/CMR.00007-19

8 Hooper, D. C. & Jacoby, G. A. Mechanisms of drug resistance: quinolone resistance. *Ann N Y Acad Sci* **1354**, 12-31 (2015). https://doi.org/10.1111/nyas.12830

9 Partridge, S. R. Analysis of antibiotic resistance regions in Gram-negative bacteria. *FEMS Microbiol Rev* **35**, 820-855 (2011). https://doi.org/10.1111/j.1574-6976.2011.00277.x

10 Partridge, S. R. & Tsafnat, G. Automated annotation of mobile antibiotic resistance in Gram-negative bacteria: the Multiple Antibiotic Resistance Annotator (MARA) and database. *J Antimicrob Chemother* **73**, 883-890 (2018). https://doi.org/10.1093/jac/dkx513

11 Amin, M. B. *et al*. High prevalence of plasmid-mediated quinolone resistance (PMQR) among *E. coli* from aquatic environments in Bangladesh. *PLoS One* **16**, e0261970 (2021). https://doi.org/10.1371/journal.pone.0261970

12 Che, Y. *et al*. High-resolution genomic surveillance elucidates a multilayered hierarchical transfer of resistance between WWTP- and human/animal-associated bacteria. *Microbiome* **10**, 16 (2022). https://doi.org/10.1186/s40168-021-01192-w

13 Adachi, F., Yamamoto, A., Takakura, K. & Kawahara, R. Occurrence of fluoroquinolones and fluoroquinolone-resistance genes in the aquatic environment. *Sci Total Environ* **444**, 508-514 (2013). https://doi.org/10.1016/j.scitotenv.2012.11.077

14 Gomi, R. *et al*. Whole-Genome Analysis of Antimicrobial-Resistant and Extraintestinal Pathogenic *Escherichia coli* in River Water. *Appl Environ Microbiol* **83**, e02703-02716 (2017). https://doi.org/10.1128/AEM.02703-16

15 Gomi, R. *et al*. Occurrence of Clinically Important Lineages, Including the Sequence Type 131 C1-M27 Subclone, among Extended-Spectrum-beta-Lactamase-Producing *Escherichia coli* in Wastewater. *Antimicrob Agents Chemother* **61**, e00564-00517 (2017). https://doi.org/10.1128/AAC.00564-17

16 Yamane, K., Wachino, J., Suzuki, S. & Arakawa, Y. Plasmid-mediated *qepA* gene among *Escherichia coli* clinical isolates from Japan. *Antimicrob Agents Chemother* **52**, 1564-1566 (2008). https://doi.org/10.1128/AAC.01137-07

17 Hansen, L. H., Sorensen, S. J., Jorgensen, H. S. & Jensen, L. B. The prevalence of the OqxAB multidrug efflux pump amongst olaquindox-resistant *Escherichia coli* in pigs. *Microb Drug Resist* **11**, 378-382 (2005). https://doi.org/10.1089/mdr.2005.11.378

18 CLSI. Performance Standards for Antimicrobial Susceptibility Testing—Thirtieth Edition: M100. (2020).

19 Feldgarden, M. *et al*. AMRFinderPlus and the Reference Gene Catalog facilitate examination of the genomic links among antimicrobial resistance, stress response, and virulence. *Sci Rep* **11**, 12728 (2021). https://doi.org/10.1038/s41598-021-91456-0

20 Bortolaia, V. *et al*. ResFinder 4.0 for predictions of phenotypes from genotypes. *J Antimicrob Chemother* **75**, 3491-3500 (2020). https://doi.org/10.1093/jac/dkaa345

21 Carattoli, A. *et al*. *In silico* detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. *Antimicrob Agents Chemother* **58**, 3895-3903 (2014). https://doi.org/10.1128/AAC.02412-14

22 Aziz, R. K. *et al*. The RAST Server: rapid annotations using subsystems technology. *BMC Genomics* **9**, 75 (2008). https://doi.org/10.1186/1471-2164-9-75

23 Siguier, P., Perochon, J., Lestrade, L., Mahillon, J. & Chandler, M. ISfinder: the reference centre for bacterial insertion sequences. *Nucleic Acids Res* **34**, D32-36 (2006). https://doi.org/10.1093/nar/gkj014

24 Strahilevitz, J., Jacoby, G. A., Hooper, D. C. & Robicsek, A. Plasmid-mediated quinolone resistance: a multifaceted threat. *Clin Microbiol Rev* **22**, 664-689 (2009). https://doi.org/10.1128/CMR.00016-09

25 Wang, M. *et al*. Plasmid-mediated quinolone resistance in clinical isolates of *Escherichia coli* from Shanghai, China. *Antimicrob Agents Chemother* **47**, 2242-2248 (2003). https://doi.org/10.1128/AAC.47.7.2242-2248.2003

26 Papagiannitsis, C. C. *et al*. Survey of metallo-beta-lactamase-producing Enterobacteriaceae colonizing patients in European ICUs and rehabilitation units, 2008-11. *J Antimicrob Chemother* **70**, 1981-1988 (2015). https://doi.org/10.1093/jac/dkv055

27 Chavda, K. D. *et al*. First Report of $bla_{\text{VIM-4}}$- and *mcr-9*-Coharboring *Enterobacter* Species Isolated from a Pediatric Patient. *mSphere* **4** (2019). https://doi.org/10.1128/mSphere.00629-19

28 Jacob, J. J. *et al*. *Salmonella* Typhi acquires diverse plasmids from other Enterobacteriaceae to develop cephalosporin resistance. *Genomics* **113**, 2171-2176 (2021). https://doi.org/10.1016/j.ygeno.2021.05.003

29 Tran, T. *et al*. Small plasmids harboring *qnrB19*: a model for plasmid evolution mediated by site-specific recombination at *oriT* and Xer sites. *Antimicrob Agents Chemother* **56**, 1821-1827 (2012). https://doi.org/10.1128/AAC.06036-11

30 Ares-Arroyo, M., Rocha, E. P. C. & Gonzalez-Zorn, B. Evolution of ColE1-like plasmids across gamma-Proteobacteria: From bacteriocin production to antimicrobial resistance. *PLoS Genet* **17**, e1009919 (2021). https://doi.org/10.1371/journal.pgen.1009919

31 Cavaco, L. M., Hasman, H., Xia, S. & Aarestrup, F. M. *qnrD*, a novel gene conferring transferable quinolone resistance in *Salmonella enterica* serovar Kentucky and Bovismorbificans strains of human origin. *Antimicrob Agents Chemother* **53**, 603-608 (2009). https://doi.org/10.1128/AAC.00997-08

32 Szuplewska, M., Ludwiczak, M., Lyzwa, K., Czarnecki, J. & Bartosik, D. Mobility and generation of mosaic non-autonomous transposons by Tn*3*-derived inverted-repeat miniature elements (TIMEs). *PLoS One* **9**, e105010 (2014). https://doi.org/10.1371/journal.pone.0105010

33 Partridge, S. R., Kwong, S. M., Firth, N. & Jensen, S. O. Mobile Genetic Elements Associated with Antimicrobial Resistance. *Clin Microbiol Rev* **31**, e00088-00017 (2018). https://doi.org/10.1128/CMR.00088-17

34 Guillard, T. *et al*. Mobile insertion cassette elements found in small non-transmissible plasmids in *Proteeae* may explain *qnrD* mobilization. *PLoS One* **9**, e87801 (2014). https://doi.org/10.1371/journal.pone.0087801

35 Aworh, M. K. *et al*. Quinolone-resistant *Escherichia coli* at the interface between humans, poultry and their shared environment- a potential public health risk. *One Health Outlook* **5**, 2 (2023). https://doi.org/10.1186/s42522-023-00079-0

36 Muloi, D. M. *et al*. Genomic epidemiology of *Escherichia coli*: antimicrobial resistance through a One Health lens in sympatric humans, livestock and peri-domestic wildlife in Nairobi, Kenya. *BMC Med* **20**, 471 (2022). https://doi.org/10.1186/s12916-022-02677-7

37 Karah, N. *et al*. Plasmid-mediated quinolone resistance determinants *qnr* and *aac(6')-Ib-cr* in *Escherichia coli* and *Klebsiella* spp. from Norway and Sweden. *Diagn Microbiol Infect Dis* **66**, 425-431 (2010). https://doi.org/10.1016/j.diagmicrobio.2009.12.004

38 Dolejska, M., Villa, L., Hasman, H., Hansen, L. & Carattoli, A. Characterization of IncN plasmids carrying $bla_{\text{CTX-M-1}}$ and *qnr* genes in *Escherichia coli* and *Salmonella* from animals, the environment and humans. *J Antimicrob Chemother* **68**, 333-339 (2013). https://doi.org/10.1093/jac/dks387

39 Kehrenberg, C., Friederichs, S., de Jong, A., Michael, G. B. & Schwarz, S. Identification of the plasmid-borne quinolone resistance gene *qnrS* in *Salmonella enterica* serovar Infantis. *J Antimicrob Chemother* **58**, 18-22 (2006). https://doi.org/10.1093/jac/dkl213

40 He, W. *et al*. Persistence and molecular epidemiology of bla_{NDM}-positive Gram-negative bacteria in three broiler farms: A longitudinal study (2015-2021). *J Hazard Mater* **446**, 130725 (2023). https://doi.org/10.1016/j.jhazmat.2023.130725

41 Xie, M., Li, R., Liu, Z., Chan, E. W. C. & Chen, S. Recombination of plasmids in a carbapenem-resistant NDM-5-producing clinical *Escherichia coli* isolate. *J Antimicrob Chemother* **73**, 1230-1234 (2018). https://doi.org/10.1093/jac/dkx540

42 Cattoir, V., Poirel, L., Aubert, C., Soussy, C. J. & Nordmann, P. Unexpected occurrence of plasmid-mediated quinolone resistance determinants in environmental *Aeromonas* spp. *Emerg Infect Dis* **14**, 231-237 (2008). https://doi.org/10.3201/eid1402.070677

43 Lv, L. *et al*. Multiple Plasmid Vectors Mediate the Spread of *fosA3* in Extended-Spectrum-beta-Lactamase-Producing *Enterobacterales* Isolates from Retail Vegetables in China. *mSphere* **5**, e00507-00520 (2020). https://doi.org/10.1128/mSphere.00507-20

44 Ruiz, E. *et al*. *qnr*, *aac(6')-Ib-cr* and *qepA* genes in *Escherichia coli* and *Klebsiella* spp.: genetic environments and plasmid and chromosomal location. *J Antimicrob Chemother* **67**, 886-897 (2012). https://doi.org/10.1093/jac/dkr548

45 Rodriguez-Martinez, J. M. *et al*. Plasmid-mediated quinolone resistance: Two decades on. *Drug Resist Updat* **29**, 13-29 (2016). https://doi.org/10.1016/j.drup.2016.09.001

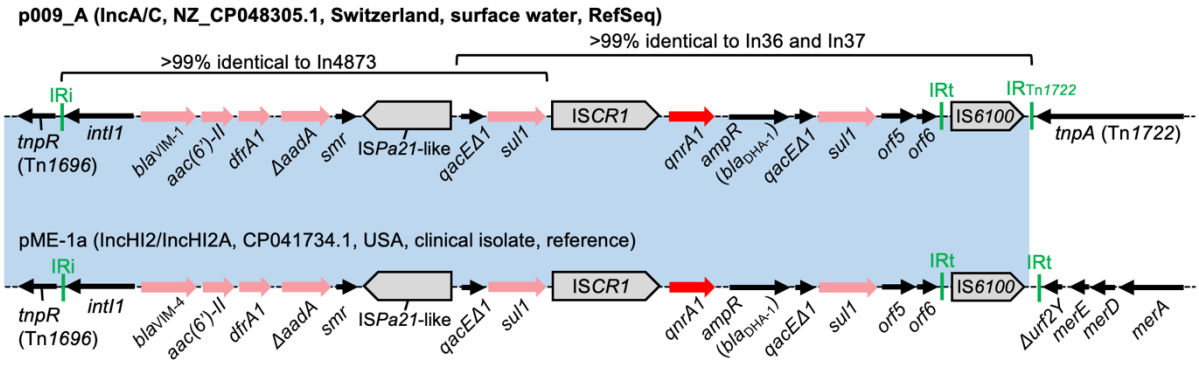
486 46 Harmer, C. J., Pong, C. H. & Hall, R. M. Structures bounded by directly-oriented
487 members of the IS*26* family are pseudo-compound transposons. *Plasmid* **111**, 102530
488 (2020). https://doi.org/10.1016/j.plasmid.2020.102530
489 47 Norman, A., Hansen, L. H., She, Q. & Sorensen, S. J. Nucleotide sequence of pOLA52:
490 a conjugative IncX1 plasmid from *Escherichia coli* which enables biofilm formation
491 and multidrug efflux. *Plasmid* **60**, 59-74 (2008).
492 https://doi.org/10.1016/j.plasmid.2008.03.003
493 48 Cattoir, V., Poirel, L. & Nordmann, P. Plasmid-mediated quinolone resistance pump
494 QepA2 in an *Escherichia coli* isolate from France. *Antimicrob Agents Chemother* **52**,
495 3801-3804 (2008). https://doi.org/10.1128/AAC.00638-08
496 49 Johnson, T. J. *et al*. Complete Genome Sequence of a CTX-M-15-Producing
497 *Escherichia coli* Strain from the *H*30Rx Subclone of Sequence Type 131 from a Patient
498 with Recurrent Urinary Tract Infections, Closely Related to a Lethal Urosepsis Isolate
499 from the Patient's Sister. *Genome Announc* **4**, e00334-00316 (2016).
500 https://doi.org/10.1128/genomeA.00334-16
501 50 Sugawara, Y. *et al*. Genetic characterization of bla_{NDM}-harboring plasmids in
502 carbapenem-resistant *Escherichia coli* from Myanmar. *PLoS One* **12**, e0184720 (2017).
503 https://doi.org/10.1371/journal.pone.0184720
504 51 Chan, P. T., Ohmori, H., Tomizawa, J. & Lebowitz, J. Nucleotide sequence and gene
505 organization of ColE1 DNA. *J Biol Chem* **260**, 8925-8935 (1985).

506

507

508

509 **FIGURES**



510

511 **Figure 1**. Genetic contexts of *qnrA1*. Red arrows indicate TMQR determinants, pink arrows

512 indicate other AMR genes, gray pointed boxes indicate insertion sequences (ISs), and black

513 arrows indicate other genes. Diagrams are not exactly to scale (this also applies to the other

514 figures). Inc type, nucleotide accession number, country of origin, isolation source, and

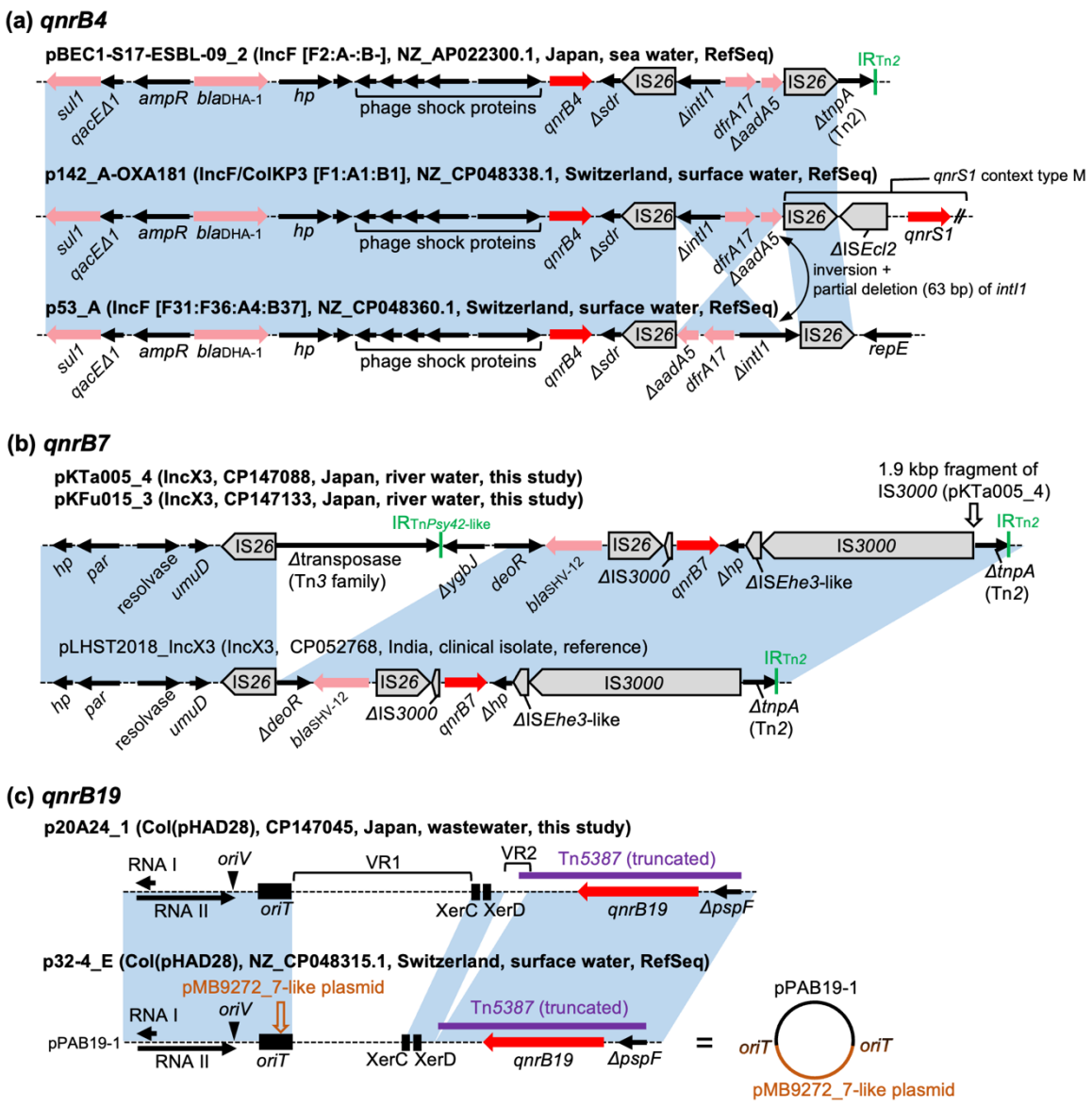
515 sequence note are shown in parentheses next to the plasmid name. The context detected in

516 pME-1a is shown for reference purposes. pME-1a carries $bla_{\text{VIM-4}}$ instead of $bla_{\text{VIM-1}}$. The blue

517 shaded regions indicate nucleotide sequences with >99% identity. IR: inverted repeat. IRi:

518 inverted repeat at *intI1* end. IRt: inverted repeat at *tni* end.

519



520

521 **Figure 2**. Genetic contexts of *qnrB* genes. Genes and elements are shown as in **Figure 1**. IncF

522 replicon sequence types are indicated in square brackets. Nucleotide sequences with >99%

523 identity are indicated by the blue shaded regions. (a) Genetic contexts of *qnrB4*. In p53_A, the

524 *ΔintI1-dfrA17-ΔaadA5* structure is inverted and the *ΔintI1* sequence is 63-bp longer compared

525 with the other two plasmids. (b) Genetic contexts of *qnrB7*. The contexts are almost identical

526 in pKTa005_4 and pKFu015_3 except that a 1.9 kbp fragment of IS*3000* is inserted next to

527 another IS*3000* in pKTa005_4. The context in pLHST2018_IncX3 is shown for reference

528 purposes. (c) Genetic contexts of *qnrB19*. Panel (c) is enlarged compared with panels (a) and

529 (b), and describes the whole plasmid structures. p20A24_1 has undergone modifications in

530 variable regions called VR1 and VR2 in comparison to the common plasmid pPAB19-1.[29] A

531 circular structure of p32-4_E is also shown to describe the relationship to pPAB19-1 and the

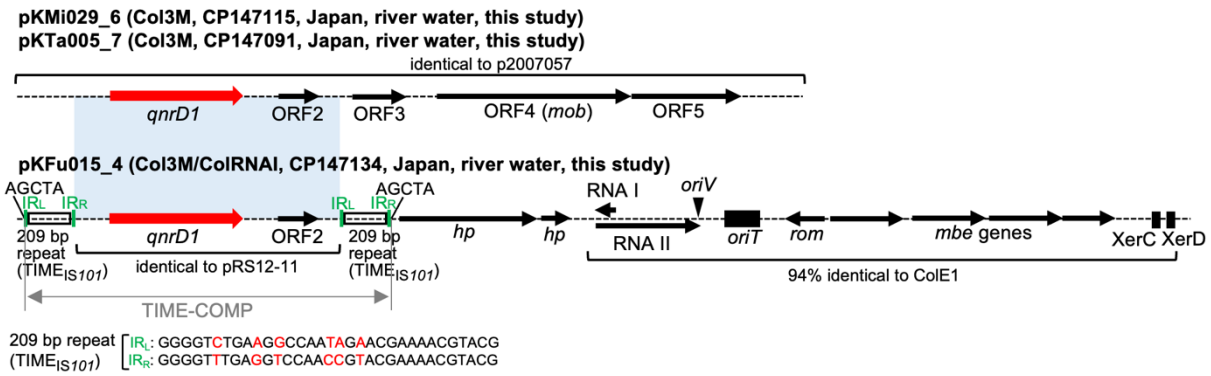
532 pMB9272_7-like plasmid. The putative ColE1-like *ori* (RNAI, RNAII, and *oriV*), *oriT*, and

533 Xer sites (sites used for converting plasmid multimers into monomers) are indicated. The *oriT*

534 sequence in the pMB9272_7-like plasmid was predicted by comparison with well-

535 characterized plasmids such as ColE1. hp: hypothetical protein.

536



537

538 **Figure 3**. Structures of plasmids carrying *qnrD1*. Genes and elements are shown as in earlier

539 figures. The light blue shaded regions indicate nucleotide sequences with ~95% identity.

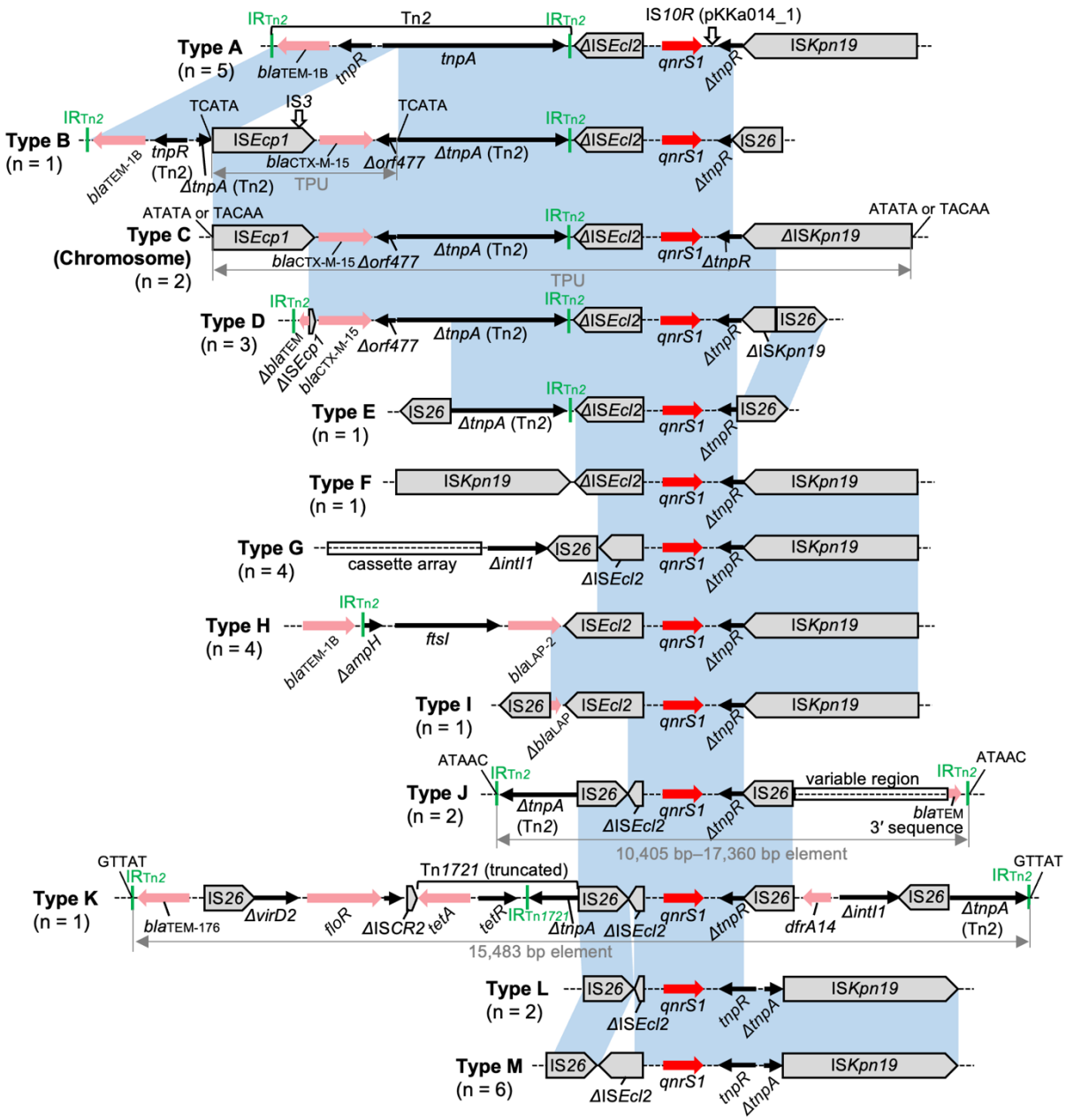
540 pKFu015_4 carries regions corresponding to the *ori*, *bom* (basis of mobility), *rom* (RNA one

541 inhibition modulator), *mob*, and *cer* (ColE1 resolution sequence) regions in plasmid ColE1.[51]

542 IR_L: inverted repeat left. IR_R: inverted repeat right. IR_L and IR_R of $TIME_{IS101}$ are shown at the

543 bottom of the figure, and different nucleotides are marked in red.

544



545

546 **Figure 4**. Genetic contexts of *qnrS1*. Genes and elements are shown as in earlier figures. The

547 blue shaded regions indicate nucleotide sequences with >99% identity. Note that for simplicity,

548 only *qnrS1* and directly flanking regions without complex rearrangements (such as inversions)

549 are shaded in blue (e.g., the left IS*Kpn19* in the type F context shares >99% nucleotide identity

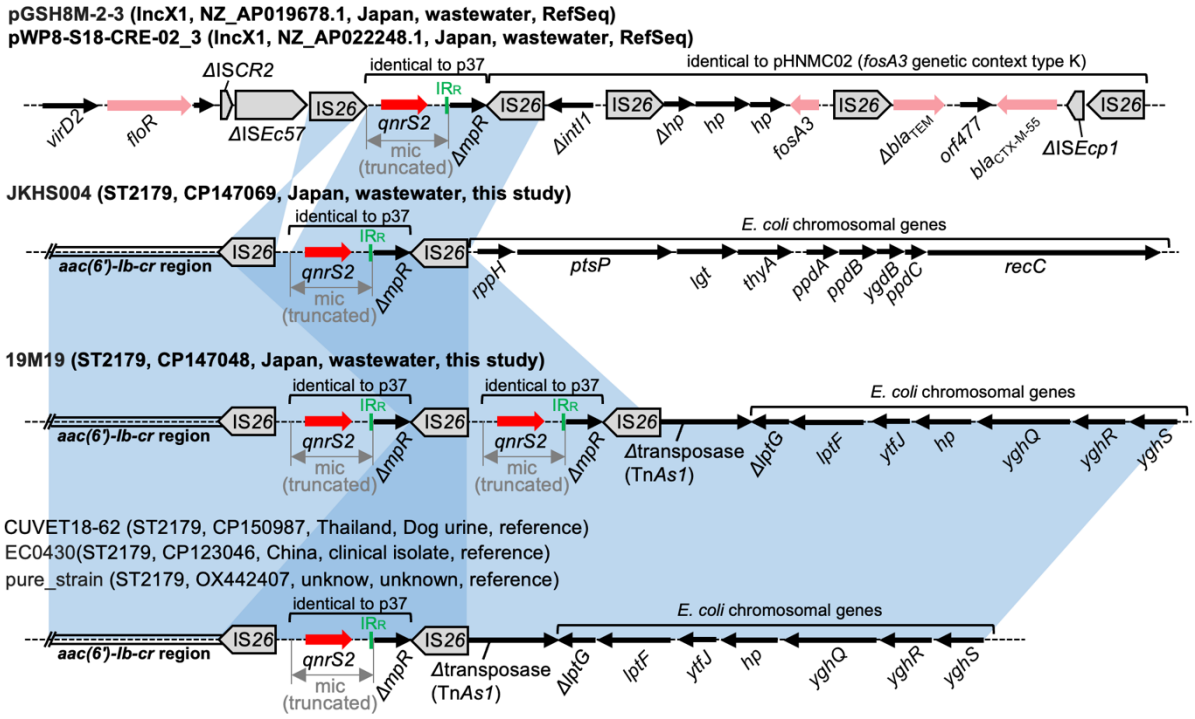
550 with IS*Kpn19* in the type G context, but this region is not shaded). The number of genomes

551 carrying each context type is shown in parentheses. See **Table S1** and **Table S2** for information

552 on genomes carrying each context type, and **Figure S1** for possible evolutionary pathways of

553 these contexts.

554



555

556 **Figure 5**. Genetic contexts of *qnrS2*. Genes and elements are shown as in earlier figures.

557 JKHS004 and 19M19 carry *qnrS2* on the chromosome, and chromosomal STs are indicated

558 instead of plasmid types. The blue shaded regions indicate nucleotide sequences with >99%

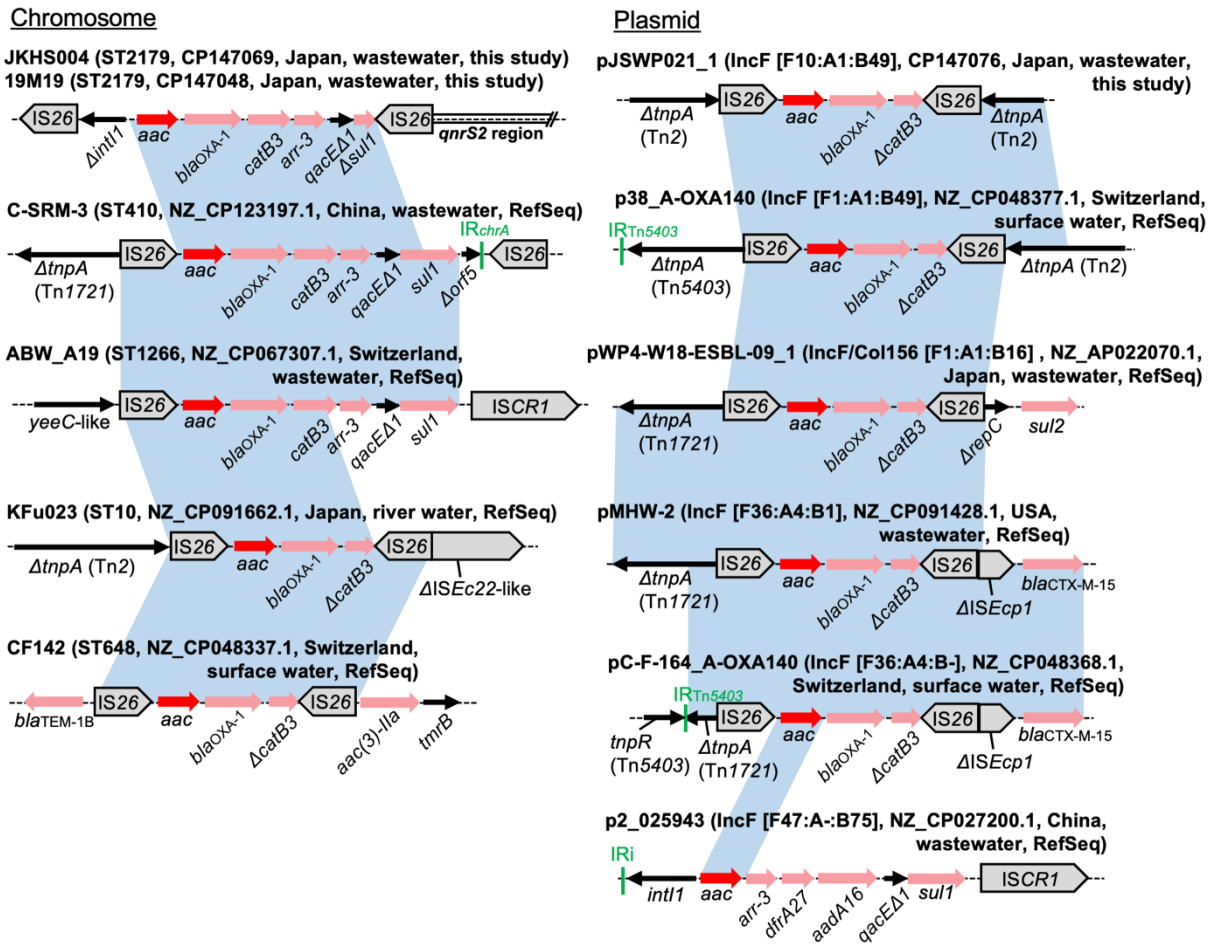
559 identity. The shared context detected in CUVET18-62, EC0430, and pure_strain is shown for

560 reference purposes. The chromosomal IS*26*-*qnrS2*-*ΔmpR*-IS*26* structure in ST2179 genomes

561 is different from that in the IncX1 plasmids, in that the IS*26* sequences are directly-oriented

562 and a ~200 bp fragment is present between the truncated mic and the left IS*26*.

563



564

565 **Figure 6**. Genetic contexts of *aac(6′)-Ib-cr*. The contexts are shown separately for

566 chromosomes and plasmids. *aac(6′)-Ib-cr* is abbreviated as *aac* in the figure. Genes and

567 elements are shown as in earlier figures. The blue shaded regions indicate nucleotide sequences

568 with >99% identity. Note that for simplicity, only *aac(6′)-Ib-cr* and flanking regions are shaded

569 in blue (e.g., IS*26* sequences in JKHS004 and 19M19 share >99% nucleotide identity to IS*26*

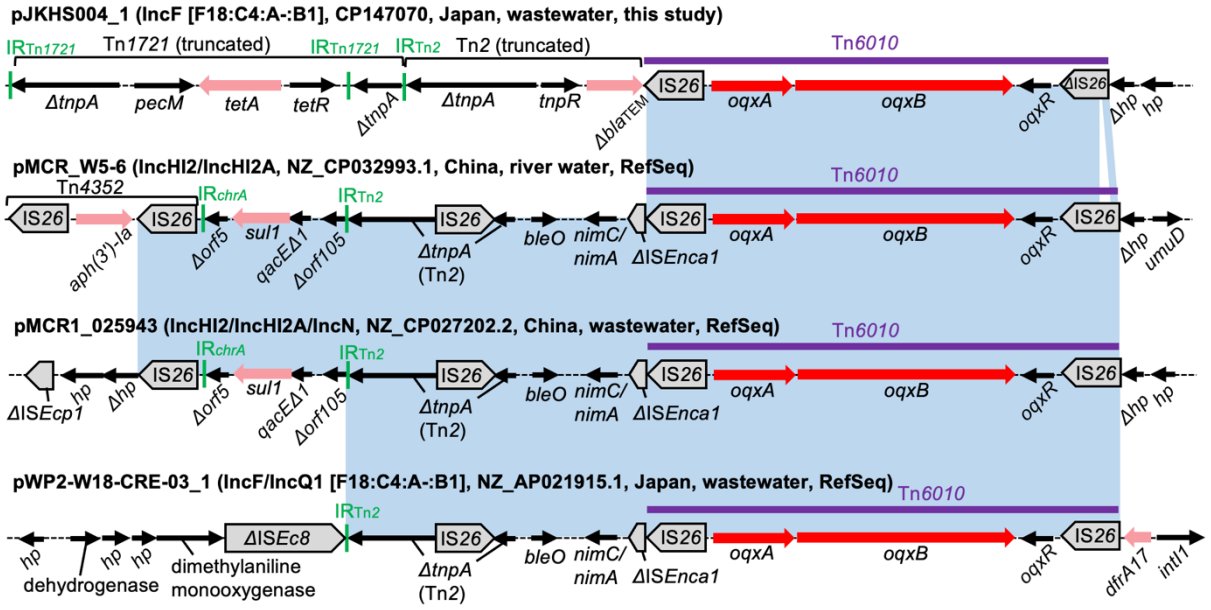
570 sequences in C-SRM-3, but these regions are not shaded). In all replicons except p2_025943,

571 either or both of the 5′-conserved segment and the 3′-conserved segment of class 1 integrons

572 containing *aac(6')-Ib-cr* are interrupted by IS*26*. These truncated integrons are likely to have

573 originated from the class 1 integron *intI1-aac(6')-Ib-cr-bla*$_{OXA-1}$*-catB3-arr-3-qacEΔ1-sul1*.

574



575

576 **Figure 7**. Genetic contexts of *oqxAB*. Genes and elements are shown as in earlier figures. The

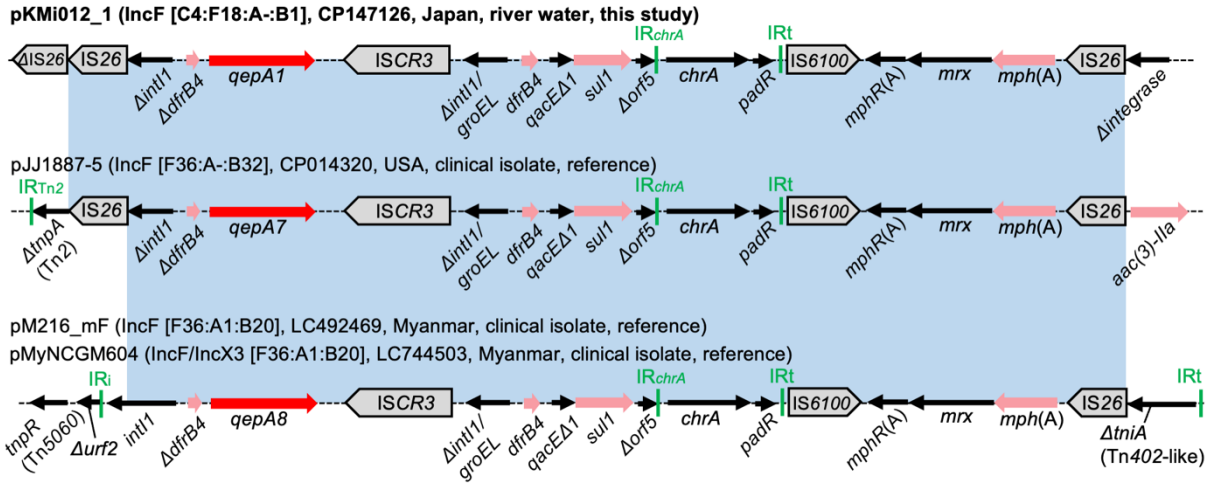
577 blue shaded regions indicate nucleotide sequences with >99% identity. Note that for simplicity,

578 only Tn*6010* and flanking regions are shaded in blue (e.g., the left IS*26* in Tn*4352* shares >99%

579 nucleotide identity to other IS*26* sequences, but these regions are not shaded). Tn*6010* in

580 pJKHS004_1 has an internally deleted copy of IS*26*.

581

582 

583 **Figure 8**. Genetic contexts of *qepA1*. Genes and elements are shown as in earlier figures. The

584 blue shaded regions indicate nucleotide sequences with >99% identity. The contexts detected

585 in three plasmids from clinical isolates are shown for reference purposes. These clinical

586 plasmids carry *qepA7* or *qepA8* instead of *qepA1*. The *ΔintI1/groEL-dfrB4-qacEΔ1-sul1-Δorf5*

587 region, the chromate resistance (*chrA*) region, and the macrolide resistance (*mph*(A)) region

588 are present downstream of *qepA* in all four plasmids.

589