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1 **Contribution of the AcrAB-TolC Efflux Pump to High-Level Fluoroquinolone**

2 **Resistance in *Escherichia coli* Isolated from Dogs and Humans**

3

4 **Running title**: FLUOROQUINOLONE RESISTANCE IN *E. COLI*

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ABSTRACT

Fluoroquinolone resistance is mainly caused by mutations in quinolone resistance-determining regions of DNA gyrase and topoisomerase IV in *Escherichia coli*. The AcrAB-TolC efflux pump contributes to resistance against fluoroquinolone and other antimicrobials. In this study, we investigated a high-level mechanism of fluoroquinolone resistance in *E. coli* that was isolated from human clinical samples and canine fecal samples. *E. coli* strains with high levels of fluoroquinolone resistance have been found to be frequently resistant to cephalosporins. Strains with high-level fluoroquinolone resistance exhibited lower intracellular enrofloxacin (ENR) concentrations, higher expression of AcrA, and a greater reduction in the fluoroquinolone minimum inhibitory concentration for treatment with an efflux pump inhibitor. The frequency of strains with enhanced ENR resistance selection and the survival rate of *E. coli* in the presence of ENR *in vitro* were correlated well with AcrA protein expression levels in the parental strains. These results suggest that AcrAB-TolC efflux pump over-expression is related to high-level fluoroquinolone resistance and the selection of strains with enhanced fluoroquinolone resistance.

KEY WORDS: *E. coli*, efflux pump, enrofloxacin, fluoroquinolone resistance

38 **INTRODUCTION**

39 Antimicrobials that are intended for human use are also used on companion
40 animals. Their excessive and inappropriate administration in animals leads to the
41 generation and spread of antimicrobial-resistant bacteria, which in veterinary
42 medicine represent a potential hazard to human health [1, 37, 33]. Companion
43 animals live in close proximity to humans and receive medical treatment more
44 frequently than food-producing animals [23]. Platell et al. reported that some
45 fluoroquinolone-resistant *Escherichia coli* isolates that were derived from
46 companion animals (i.e., dogs and cats) were very similar to the ones found in
47 humans [27].

48 Fluoroquinolones are widely used to treat various bacterial infections in
49 humans and companion animals. *E. coli* infections, especially urinary tract
50 infections, are frequently treated with fluoroquinolones. Thus, fluoroquinolone
51 resistance has increased among clinical isolates of humans and companion animals
52 [7, 24]. Previous studies reported that extended-spectrum *β*-lactamase
53 (ESBL)-producing *E. coli* and *Klebsiella pneumoniae* are frequently resistant to
54 fluoroquinolone [20, 30]. Cephalosporins and fluoroquinolones are among the most
55 important antimicrobials that are used on humans and animals, mainly because of
56 their broad-spectrum effectiveness against both gram-positive and gram-negative
57 bacteria. Therefore, the appearance of fluoroquinolone- and/or
58 cephalosporin-resistant bacteria is a serious problem in clinical medicine.

59 Fluoroquinolone resistance mechanisms are mainly based on chromosomal
60 mutations in the quinolone resistance-determining regions (QRDRs) of *gyrA* and
61 *gyrB*, which encode DNA gyrase subunits, and *parC* and *pare*, which encode
62 topoisomerase IV subunits [2, 8, 11, 37]. Resistance to nalidixic acid (NAL) and
63 slightly decreased susceptibility to fluoroquinolones is attributed to a single
64 mutation in *gyrA*. A secondary mutation in *gyrA* and additional mutations in *parC*

65 and/or *parE* are necessary for high-level fluoroquinolone resistance [8]. Moreover,
66 plasmid-mediated quinolone resistance-mechanism determinants (PMQRs), such as
67 the *qnr*, *aac(6')-Ib-cr*, and *qepA* genes, have been reported in gram-negative bacteria,
68 including *E. coli* [12, 29, 35]. However, the acquisition of PMQRs results in only
69 low-level fluoroquinolone resistance.

70 An alternative mechanism of fluoroquinolone resistance is decreased
71 intracellular concentrations of antimicrobials. The over-expression of AcrAB-TolC,
72 which is a major multidrug efflux pump in *E. coli*, and the down-regulation of the
73 porin of the outer membrane protein F (OmpF) are caused by mutations or
74 insertions in their regulatory genes [5]. AcrA is a component of AcrAB-TolC that
75 belongs to the resistance-nodulation-division family of exporters, has a tripartite
76 structure, and exhibits broad-substrate specificity [28]. Efflux pump- and/or
77 porin-based resistance results in slightly decreased susceptibility to fluoroquinolone.
78 These resistance mechanisms combined with QRDR mutations lead to high levels of
79 fluoroquinolone-resistance [13]. In addition, efflux pump over-expression and a
80 deficiency in porins also induce resistance to various other classes of antimicrobials,
81 including cephalosporins [9]. Most of the effects of the AcrAB-TolC on multidrug
82 resistance have been studied using laboratory strains but not clinical isolates [13,
83 28]. In laboratory strains, AcrAB over-expression increases the MICs of several
84 antimicrobials. However, in most of them, AcrAB over-expression alone does not
85 modulate the phenotypic switch from susceptibility to resistance. Therefore, other
86 resistance mechanisms are most likely to be responsible for the switch.

87 In this study, we investigated the mechanism of fluoroquinolone resistance by
88 using human and canine *E. coli* isolates, and estimated the risk of transmitting
89 fluoroquinolone-resistant *E. coli* from dogs to humans. We also examined the
90 potential effect of AcrAB-TolC on the survival mechanism of *E. coli* in the presence
91 of fluoroquinolone *in vitro*.

92

93 **MATERIALS AND METHODS**

94 Bacterial strains

95 *E. coli* strains were isolated from humans and dogs. Human strains were
96 isolated and stocked at Sapporo Clinical Laboratories Inc. (Sapporo, Japan) and
97 Hokkaido University Hospital (Sapporo, Japan) from 2008 to 2009. Human clinical
98 samples were collected from the urine, pharynx, phlegm, feces, aspirate fluids,
99 intestinal juice, wounds, vaginal fluid, pus, and blood of outpatients and inpatients.
100 Canine fecal samples were collected using cotton swabs from Rakuno Gakuen
101 University Hospital (Ebetsu, Japan) and 8 animal clinics in Ebetsu City (Japan).
102 The case histories of the dogs varied, including urinary tract infections, cystitis,
103 chronic diarrhea, dermatitis, and septicemia.

104 The 50 *E. coli* clinical isolates contained 12 enrofloxacin (ENR) susceptible
105 strains (FQSECs; ENR minimum inhibitory concentration (MIC) ranged from 0.03
106 to 0.125 μg/mL), 18 intermediate-level ENR-resistant strains (iFQRECs; ENR MIC
107 ranged from 16 to 64 μg/mL), and 20 high-level ENR-resistant strains (hFQRECs;
108 ENR MIC was more than 128 μg/mL). *E. coli* strain AG100, which was used as a
109 reference strain for determining AcrA protein expression levels, was gifted by Dr.
110 Helen I. Zgurskaya (University of Oklahoma, Norman, OK, U.S.A).

111

112 Chemicals and media

113 Phe-Arg-*β*-naphthylamide (PA*β*N) was purchased from Sigma-Aldrich (St.
114 Louis, MO, U.S.A), and 1-(1-naphthylmethyl)-piperazine (NMP) was obtained from
115 Chess (Mannheim, Germany). Luria-Bertani (LB) broth and Mueller-Hinton (MH)
116 agar were obtained from Oxoid (Basingsoke, UK). ENR and ciprofloxacin (CIP) were
117 obtained from Bayer (Osaka, Japan), levofloxacin (LVX) was obtained from
118 Daiichi-Sankyo (Tokyo, Japan), and NM394 [the active form of prulifloxacin (PUR)]

119 was obtained from Meiji Seika Pharma (Tokyo, Japan). NAL was obtained from
120 Wako Junyaku (Osaka, Japan). Cefazolin (CEZ), cefalexin (CEX), and cefpodoxime
121 (CPD) were obtained from Sigma-Aldrich.
122
123 Susceptibility testing
124 Susceptibility to a panel of antimicrobials was studied using the agar dilution
125 method in the presence or absence of efflux pump inhibitor (EPI), PAβN, and NMP,
126 in accordance with the Clinical and Laboratory Standards Institute (CLSI) [6].
127 *Staphylococcus aureus* ATCC29213, *Enterococcus faecalis* ATCC29212, *E. coli*
128 ATCC25922, and *Pseudomonas aeruginosa* ATCC27853 were used as quality
129 control strains. CLSI breakpoints were used when available and antimicrobial
130 breakpoints that were not included in the CLSI were defined by a population of the
131 isolates that were tested in this study. The concentrations of PAβN (80 μg/mL) and
132 NMP (160 μg/mL) were based on their available ranges, as previously described [14,
133 31]
134
135 DNA amplification and sequencing of QRDRs and PMQR
136 Mutations in the QRDRs of the *gyrA*, *parC*, *parE,* and *gyrB* genes were detected
137 by direct DNA sequencing of their PCR products, as described in previous studies
138 [27, 31]. Nucleotide sequences were determined using a BigDye Terminator v3.1
139 Cycle Sequencing Kit with a 3130 Genetic Analyzer (Applied Biosystems, Foster
140 City, CA). The PMQR genes *qnrA*, *qnrB*, *qnrS*, *aac(6')Ib-cr,* and *qepA* were detected
141 as previously reported [3, 22, 16].
142
143 Detection of β-lactamase genes
144 β-Lactamase genes were detected by PCR and direct DNA sequencing according
145 to a method described in previous studies [17,25,38].

146

147 Accumulation assay

148 The intracellular ENR concentration was assayed using a fluorometric uptake
149 assay [34] with ENR at a final concentration of 15 μg/mL and carbonyl cyanide
150 m-chlorophenylhydrazone (CCCP, Sigma-Aldrich) at a final concentration of 150
151 μM. The data represent the mean value ± standard errors that were calculated from
152 3 independent experiments.

153

154 Western blot analysis

155 Bacteria were cultured in LB broth until the logarithmic phase at 37°C.
156 Harvested cells were lysed with 1% sodium dodecyl sulfate (SDS) at 100°C for 10
157 min. Protein concentrations were determined by the bicinchoninic acid assay
158 (Thermo Scientific, Waltham, MA, U.S.A) using horse immunoglobulin G as a
159 standard. Protein (1 μg) from the total cell lysate was used for SDS-polyacrylamide
160 gel electrophoresis on a 12.5% polyacrylamide slab gel [18]. The proteins on the gel
161 were transferred to an Immobilon-P polyvinylidene fluoride membrane (Millipore,
162 Billerica, MA, U.S.A), and the membrane was blocked with 5% (w/v) skim milk in a
163 buffer that contained 20 mM Tris-HCl (pH 7.6), 137 mM NaCl, and 0.1% Tween20
164 (TBST) for 1 hr with agitation. Then, the membrane was incubated with rabbit
165 anti-AcrA polyclonal antibody (1:10,000 dilution) for 1 hr at room temperature with
166 agitation. The membrane was washed 3 times with TBST and incubated in an
167 alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin antibody
168 (1:2,000 dilution; Biosource International, Camarillo, CA, U.S.A). After washing
169 with TBST, specific binding was visualized with tetrazolium bromochloroindolyl
170 phosphate and nitro blue tetrazolium. The resulting bands were scanned with a
171 flatbed scanner, and they were analyzed using the public domain ImageJ program
172 (U. S. National Institutes of Health, Bethesda, MD, U.S.A). This method was

173 performed in duplicate.

174

175 Frequency of strains with enhanced fluoroquinolone resistance *in vitro*

176 Bacteria were cultured to the late logarithmic phase at 37°C in LB broth,
177 harvested by centrifugation, and re-suspended in sodium phosphate buffer (pH
178 7.2). Total colony-forming units (CFU) were measured by serial dilution on MH
179 agar plates. The cells (10^9 CFU/mL) were inoculated on MH agar plates that
180 contained ENR MIC of each isolate at 4-fold concentration. The number of strains
181 with enhanced fluoroquinolone resistance colonies was counted after 48 hr of
182 culturing. The frequency of strains with enhanced fluoroquinolone resistance was
183 calculated, whereby the data represent the mean value ± standard deviation of the
184 results from 3 independent experiments.

185

186 Time-killing assay

187 Bacteria were cultured overnight at 37°C in 2 mL of LB broth, harvested,
188 re-suspended in 2 mL of fresh LB broth, and incubated for 1.5 hr at 37°C without
189 agitating to reach the early stationary phase (approximate growth from 5×10^7 to 2
190 $\times 10^8$ CFU/mL). Then, 4-fold concentrated ENR MIC was added at 3 hr, 6 hr, and 9
191 hr. Following this, the number of CFU was determined at different time points by
192 plating serial dilutions on MH agar plates, which were incubated for 18 hr at 37°C.
193 This assay was performed for 3 independent experiments.

194

195 Statistical analysis

196 Statistical significance was determined by the Student's *t*-test and Fisher's exact
197 test ($p < 0.05$).

198

199 **RESULTS**

200 Relationship between QRDR mutations and fluoroquinolone susceptibility

201 We investigated fluoroquinolone MICs and QRDR mutations (Tables 1 and 2).
202 Similar to ENR, all the iFQRECs and hFQRECs were resistant to the other 3
203 fluoroquinolones (CIP, LVX, and PUR), and the FQSECs were susceptible to them.
204 All the iFQRECs and hFQRECs were also resistant to NAL, with the MIC being
205 greater than 128 μg/mL (data not shown).

206 All 38 iFQRECs and hFQRECs, with the exception of one strain, carried 3 or 4
207 mutations in *gyrA* (S83 and D87) and *parC* (S80 and/or E84). A few strains that had
208 these 3 mutations also carried a mutation in *parC*; A108T and in *parE*; E460A, and
209 S458T, respectively. One strain had 2 mutations in the QRDR: S80I and E84V in
210 *parC* (Table 1). For the strains that carried 2 or 3 mutations in the QRDR, the ENR
211 MIC ranged from 16 to 64 μg/mL, and for those that carried 4 mutations, the ENR
212 MIC ranged from 32 to >256 μg/mL. Among the strains that shared the same
213 mutations in the QRDRs, a 2- to >4-fold variation was observed for ENR MIC (Table
214 1). These phenomena were also observed for the other 3 fluoroquinolones.

215

216 Relationship between ENR resistance and CPD resistance

217 Sixteen isolates were resistant to CEZ, CEX, and CPD. Among them, 15
218 possessed $bla_{\text{CTX-M-27}}$ (n = 3), $bla_{\text{CTX-M-14}}$ (n = 4), and $bla_{\text{CMY-2}}$ (n = 8; Table 1). Another
219 strain (CE4) had mutations in the chromosomal *ampC* promoter region (T-32A; data
220 not shown).

221 The appearance of CPD resistance tended to be higher in the hFQRECs (55.0%,
222 11 of the 20 strains) than in the iFQRECs (27.8%, 5 of the 18 strains), and was
223 significantly higher than that in the FQSECs (0%; Table 1). Regarding
224 CPD-susceptible and CPD-resistant classifications, high-level fluoroquinolone
225 resistance was seen at a higher frequency in the CPD-resistant strains (68.8%, 11 of
226 16 strains) than in the CPD-susceptible isolates (26.5%, 9 of 34 strains; $p < 0.05$,

227 data not shown), whereas intermediate-level fluoroquinolone resistance was seen at
228 a similar frequency in the CPD-resistant isolates (31.3%, 5 of 16 strains) and in the
229 CPD-susceptible isolates (35.2%, 12 of 34 strains).
230
231
232 Effects of efflux pump inhibitors on the fluoroquinolone MICs of clinical isolates
233 PAβN (80 μg/mL) significantly influenced the fluoroquinolone MICs of the
234 clinical isolates (2- to 64-fold reduction in ENR and CIP; 1- to 32-fold reduction in
235 LVX; 1- to 16-fold reduction in PUR; Table 1). In the fluoroquinolone-resistant *E.*
236 *coli* isolates, the reductions in the MIC tended to increase with higher MIC for all
237 the fluoroquinolones. The degree of ENR MIC reduction by PAβN and NMP was
238 significantly greater in the hFQREC strains than in the iFQREC and FQSEC
239 strains (Fig. 1A-1 and 1A-2). Other fluoroquinolone MICs were also reduced by 2- to
240 32-fold in the hFQRECs more than for the FQSEC and iFQREC strains with lower
241 MICs. In the FQSECs, the reductions in fluoroquinolone MICs were greater in the
242 strains that had higher fluoroquinolone MICs (Table. 2).
243
244 Determination of intracellular ENR concentrations
245 The intracellular ENR concentrations were significantly lower ($p < 0.05$) in the
246 hFQRECs compared with the FQSECs, and they tended to be lower compared with
247 the iFQRECs (Fig. 1C-1). In the presence of CCCP, all the hFQRECs showed a
248 significant increase in their intracellular ENR concentration compared with the
249 FQSECs. However, there were no differences in the iFQRECs (Fig. 1C-2). We
250 calculated the excretion of ENR by subtracting the intracellular ENR concentration
251 in the absence of CCCP from that in the presence of CCCP (Fig. 1C-3). The
252 hFQRECs and iFQRECs showed significantly higher efflux activity than the
253 FQSECs, and the efflux activity of the hFQRECs tended to be higher than that of

254 the iFQRECs.

255

256 Analysis of AcrA expression

257 AcrA expression in the hFQRECs was significantly higher than that in the
258 iFQRECs and FQSECs (Table 1 and Fig. 1B). The ENR MIC was correlated with
259 the AcrA expression level for each QRDR genotype (Table 1). AcrA expression in the
260 iFQRECs showed no significant difference from that in the FQSECs (Fig. 1B).

261 HUE6, which is a human hFQREC strain, showed lower AcrA expression,
262 despite the low ENR concentration in the absence of CCCP compared with other
263 isolates with the same QRDR genotype (Table 1). In contrast, RE63, which is an
264 iFQREC that was isolated from dogs, showed higher AcrA expression compared
265 with other isolates with the same ENR MIC and QRDR genotypes.

266

267 Frequency of strains with enhanced fluoroquinolone resistance *in vitro*

268 The frequency of strains with enhanced fluoroquinolone resistance was
269 determined by *in vitro* exposure to ENR by using the FQSECs (Table 2). The
270 frequency of strains with enhanced fluoroquinolone resistance among the strains
271 with low AcrA expression (relative values to strain AG100; less than unity) ranged
272 from 0.2×10^{-9} to 3.7×10^{-9}, and those in strains with moderate AcrA expression
273 (relative values to strain AG100; more than unity) ranged from 1.5×10^{-8} to $5.8 \times$
274 10^{-8}. The frequency of selection of strains with enhanced ENR resistance was
275 significantly correlated with the level of AcrA expression ($r^2 = 0.41$, $p < 0.05$),
276 whereas the frequency was not correlated with the QRDR mutations in *gyrA* and
277 *gyrB* (Table 2).

278

279

280 Time-killing assay

281 We investigated the survival rate for susceptible isolates that were exposed to
282 ENR and CPD (Table 2). The survival rate for those that were exposed to ENR at 6
283 hr was significantly correlated with the frequency of strains with enhanced ENR
284 resistance ($r^2 = 0.67$, $p < 0.01$) and the level of AcrA expression ($r^2 = 0.66$, $p < 0.01$).
285 The survival rate for those that were exposed to CPD at 9 hr was also correlated
286 with the level of AcrA expression ($r^2 = 0.52$, $p < 0.05$).
287
288
289 **DISCUSSION**
290 This study has revealed the mechanism of fluoroquinolone resistance in *E. coli*
291 that was isolated from humans and dogs, and the contribution of AcrAB-TolC to its
292 survival in the presence of fluoroquinolone *in vitro*. We found no apparent
293 differences in the fluoroquinolone-resistance mechanisms when we compared *E. coli*
294 that was isolated from dogs and humans. Further work is necessary to clarify the
295 risk of transmitting fluoroquinolone-resistant *E. coli* from dogs to humans.
296 The degree of fluoroquinolone resistance in *E. coli* that was isolated from dogs
297 and humans was primarily determined by the number of QRDR mutations that
298 each bacterium had (Table 1). However, the hFQRECs had a wider range of ENR
299 MIC than the iFQRECs regardless of the same QRDR genotypes, which could not be
300 explained solely by the QRDR mutations. EPIs dramatically reduced
301 fluoroquinolone MICs for almost all the clinical isolates, and the intracellular ENR
302 concentration increased in the presence of CCCP (Table 1 and Fig. 1). The influence
303 of fluoroquinolone MIC by EPIs and AcrA expression was clearly higher in the
304 hFQRECs than in the iFQRECs and FQSECs. These data suggest that efflux pump
305 over-expression in combination with QRDR mutations leads to extremely high-level
306 fluoroquinolone resistance. These results are consistent with the previously
307 described in *E. coli* and *Salmonella enterica* serovar Typhimurium [9, 10]. However,

308 high-level fluoroquinolone resistance could not be completely explained by the
309 QRDR mutations and AcrAB-TolC expression in the clinical isolates.

310 Efflux pump over-expression should enhance the frequency of strains with
311 enhanced fluoroquinolone resistance, because it decreases the intracellular
312 fluoroquinolone concentration. Consistent with this, the frequency of strains with
313 enhanced fluoroquinolone resistance was higher for susceptible isolates of
314 *Salmonella enterica* serovar Choleraesuis with low intracellular ENR
315 concentrations [34]. In this study, strains that exhibited higher AcrA expression
316 showed higher ENR MICs, and we observed a correlation between the levels of AcrA
317 expression, survival in ENR, and the frequency of QRDR mutations (Table 2). In
318 addition, among the strains that had enhanced ENR resistance and contained the
319 same QRDR mutation, the FQSECs with moderate AcrA expression had higher
320 fluoroquinolone MICs compared with the FQSECs with low AcrA expression (Table
321 2). These results suggest that the over-expression of AcrAB-TolC decreases the
322 intracellular ENR concentration largely compared to the low expression of
323 AcrAB-TolC, and thus provides a strong absolute survival advantage in the
324 presence of fluoroquinolone. Although we could not explain this relationship directly,
325 this advantage can be used to select for strains with enhanced ENR resistance and
326 make the first-step QRDR mutation easier to occur in FQSECs with moderate AcrA
327 expression than FQSECs with low AcrA expression. Therefore, AcrAB-TolC efflux
328 pump over-expression may not be limited to generating high-level fluoroquinolone
329 resistance, but it may be related to the appearance of first-step
330 fluoroquinolone-resistant mutants.

331 In addition, the hFQREC strains were frequently CPD-resistant (Table 1). A
332 suggested mechanism for the appearance of fluoroquinolone and cephalosporin
333 concomitant resistance is the co-localization of ESBL and PMQR determinants in
334 the same genetic platform [15, 19]. However, in this study, we did not find any

335 strains that harbored such co-localizations, which suggests that there may be other
336 concomitant fluoroquinolone and cephalosporin-resistance mechanisms.

337 Efflux pump over-expression is responsible for resistance to fluoroquinolones
338 and other antimicrobials [28]. Our data show that over-expression of the efflux
339 pump that is mediated by AcrA is associated with the multidrug-resistant
340 phenotype (cephalosporins, CHL, NAL, and fluoroquinolones) in *E. coli* clinical
341 isolates (data not shown). In CPD-susceptible strains, CPD MIC was higher with
342 higher AcrA expression (Tables 1 and 2). Previous studies showed that a decrease in
343 OmpF expression enhances cephalosporin and fluoroquinolone MICs [5, 21], and
344 OmpF has been shown to be regulated by MarA, which also regulates AcrAB
345 expression [4, 5, 13]. These facts also indicate that AcrAB-TolC may influence
346 cephalosporin susceptibility in clinical isolates. More number of definitive studies is
347 needed to elucidate the relationship between AcrAB-TolC and cephalosporin
348 resistance.

349 In conclusion, high levels of fluoroquinolone resistance are caused by the
350 upregulation of efflux mechanisms in addition to QRDR mutations in both human
351 and canine *E. coli* isolates. In addition, AcrAB-TolC over-expression confers QRDR
352 mutations and survival advantages with respect to ENR exposure.

353

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Disclosure Statement

The authors have no competing interests relating to this study.

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543 **FIGURE LEGENDS**

544 FIG. 1. The effect of the efflux pump at different ENR MICs (μg/mL) in *E. coli* clinical isolates

545

546 A total of 50 isolates were used in these 3 experiments: 12 were FQSECs, 18 were iFQRECs, and 20 were hFQRECs. A, the
547 effects of PA*β*N (80 μg/mL, A-1) and NMP (160 μg/mL, A-2). B, Western blot analysis of AcrA. The AcrA expression level is shown
548 as the relative value compared to that obtained for strain AG100 (wild-type expression of AcrA) that was used to assess the
549 relative volume of AcrA above the wild-type levels. C, Intracellular concentration (ng/mg wet cells) of ENR in the absence of CCCP
550 (C-1) and in the presence of CCCP (C-2). The excretion of ENR was calculated by subtracting the intracellular ENR
551 concentration from that in the presence of CCCP (C-3; ng/mg wet cells). The data represent the mean value ± standard error. *, p
552 < 0.05

553

621 TABLE 2. Appearance frequency of strains with enhanced ENR-resistance, expression levels of AcrA protein, and survival rate in

622 ENR containing media of FQSECs

Strains	MIC (μg/ml)							AcrA expression level(s)[b]	Appearance frequency of ENR-resistant mutant[c]	QRDRs mutation of ENR-resistant mutant	ENR MIC of ENR-resistant mutant (μg/ml)	Survival rate of ENR for 6h (× 10^{-2})[d]
	ENR	CIP	LVX	PUR	CEZ	CEX	CPD					
RE15	0.03 (×2[a])	0.01 (×2)	0.01 (×2)	0.03 (×1)	1	4	0.25	0.18	1.4 ± 0.6	S83L(GyrA)	0.25	5.4
CE52	0.03 (×2)	0.01 (×2)	0.03 (×4)	0.03 (×1)	2	8	0.5	0.61	3.5 ± 1.7	S83L(GyrA)	0.25	6.4
CE51	0.03 (×4)	0.03 (×4)	0.03 (×4)	0.03 (×1)	2	8	0.5	0.79	0.2 ± 0.04	D87G(GyrA)	0.25	1.1
CE55	0.03 (×4)	0.03 (×2)	0.03 (×4)	0.03 (×1)	2	8	0.5	0.87	3.7 ± 3.1	S83L(GyrA)	0.5	31.3
RE6	0.03(×4)	0.03 (×2)	0.06 (×4)	0.03 (×1)	1	8	0.5	1.22	12.7 ± 6.2	S83L(GyrA)	0.5	32.5
RE13	0.03 (×4)	0.03 (×2)	0.06 (×4)	0.03 (×2)	1	8	0.5	1.26	10.9 ± 3.8	S83L(GyrA)	0.5	22.2
RE10	0.03 (×4)	0.03 (×2)	0.03 (×4)	0.03 (×2)	2	8	0.5	1.29	15.0 ± 3.5	D87Y(GyrA)	0.25	17.4
CE58	0.03 (×4)	0.03 (×4)	0.06 (×8)	0.03 (×1)	2	8	0.5	1.32	24.0 ± 3.0	S83L(GyrA)	0.5	24.7
RE14	0.03 (×4)	0.03 (×4)	0.06 (×8)	0.03 (×2)	2	16	0.5	1.59	25.8 ± 8.8	D87Y(GyrA)	0.25	28.2
RE12	0.06 (×8)	0.03 (×4)	0.06 (×4)	0.03 (×1)	2	8	0.5	1.79	25.0 ± 4.7	S83L(GyrA)	0.5	54.4
CE54	0.06 (×8)	0.06 (×8)	0.125 (×8)	0.06 (×2)	2	16	1	1.85	40.0 ± 19.3	D426N(GyrB)	0.25	52.3
RE1	0.125 (×64)	0.125 (×16)	0.125 (×16)	0.125 (×4)	4	32	2	4.98	58.8 ± 9.5	S83L(GyrA)	1	54.9

623

624

625 [a]) Reduction of ENR MIC in the presence of 80 μg/ml of PAβN (-fold). [b]) AcrA expression levels (relative amount of AG100)

626 determined by western blot analysis. [c]) Emerging frequency of strains with enhanced ENR resistance (× 10^{-9}). Data represent the

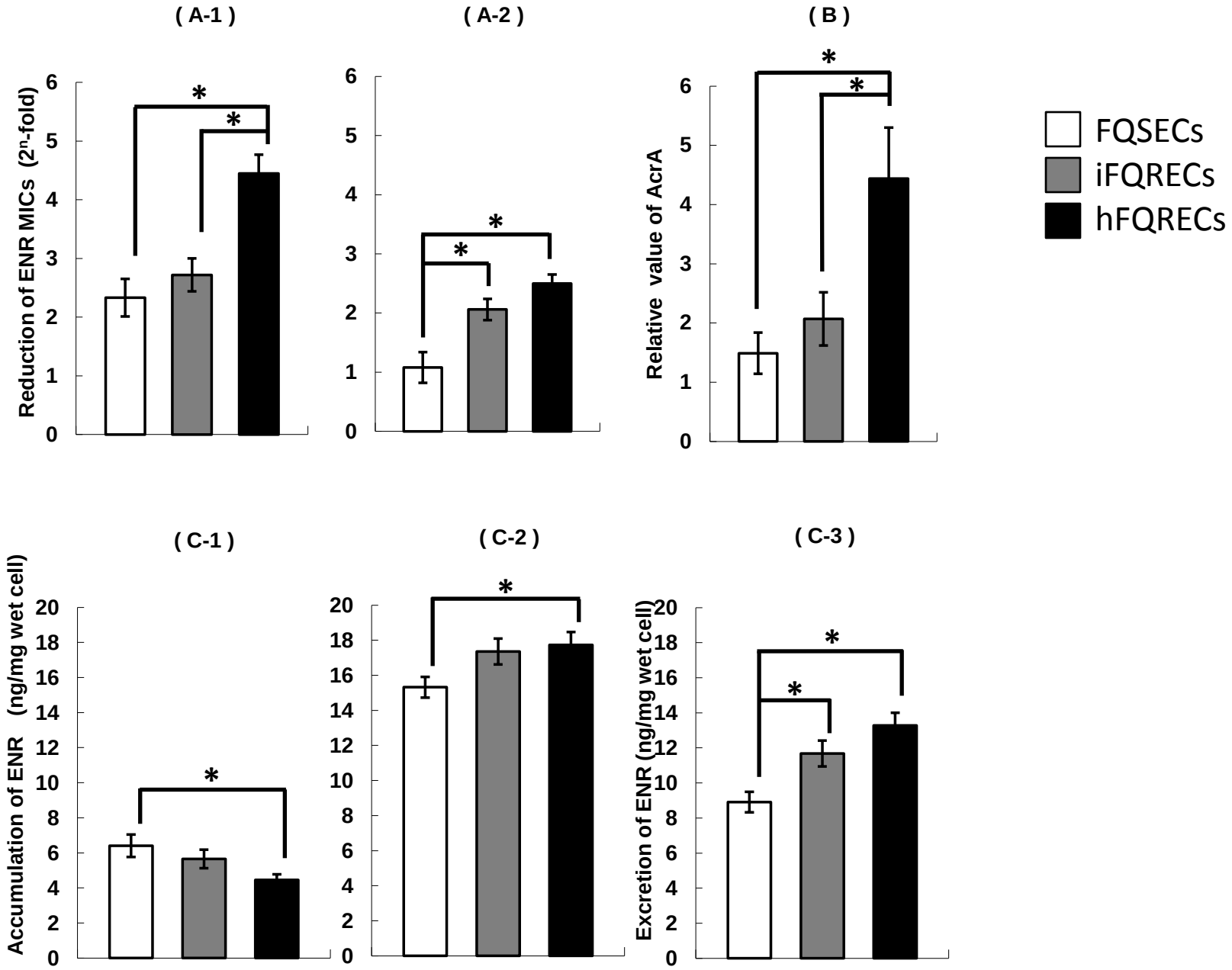
627 mean value ± standard deviation of the results from 3 independent experiments.

628 [d]) Time-killing assay following exposure to ENR for 6h. The value represents the relative number of CFU at 0 h.

629 ENR, enrofloxacin: CIP, ciprofloxacin: LVX, levofloxacin: PUR, prurifloxacin: CEZ, cefazoline: CEX, cefalexine: CPD,

630 cefpodoxime

FIG. 1


(A-1)
(A-2)
(B)
Reduction of ENR MICs (2n-fold)
Relative value of AcrA
FQSECs
iFQRECs
hFQRECs
(C-1)
(C-2)
(C-3)
Accumulation of ENR (ng/mg wet cell)
Excretion of ENR (ng/mg wet cell)
*