Jpn. J. Infect. Dis., 68, 196–202, 2015

Original Article

Contribution of QnrA, a Plasmid-Mediated Quinolone Resistance Peptide, to Survival of *Escherichia coli* Exposed to a Lethal Ciprofloxacin Concentration

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SUMMARY: We evaluated the effects of *qnrA* on survival of bacteria exposed to a lethal ciprofloxacin (CIP) concentration and development of quinolone resistance through the accumulation of amino acid substitutions in quinolone resistance-determining regions (QRDRs) of GyrA and ParC, targets of quinolones, in *Escherichia coli*. CIP-susceptible *E. coli* strains of different O-serotypes (O1, O6, O18, O25b, O74, and O78) were transformed by a recombinant plasmid harboring *qnrA*, and the parent strains and their transformants were subjected to killing curve assays and adaptation tests. In the killing curve assay at 2 × the minimum inhibitory concentration of CIP, the viable bacterial cell numbers of strains O1, O6, and O25b were maintained at 10^5–10^8 CFU/mL after 24-h incubation, while the remaining strains showed a 10^5-fold reduction in viable cell numbers. In the adaptation test, a Ser83-Leu substitution in the QRDR of GyrA was identified earlier in the parent strains of O25b and O1 than in their transformants, suggesting that the acquisition of *qnrA* did not necessarily accelerate the rate of accumulation of amino acid substitutions in the QRDR. We confirmed that the presence of *qnrA* contributed to increased survival of the *E. coli* strains displaying certain O-serotypes. Further studies are necessary to evaluate the precise effects of *qnrA* on quinolone resistance acquisition by *Enterobacteriaceae*.

INTRODUCTION

Multidrug resistance in *Enterobacteriaceae*, including resistance to fluoroquinolones (FQs), is on the rise worldwide and has become a serious clinical concern (1–3). FQ resistance most commonly occurs as a result of mutation accumulation in the quinolone resistance-determining regions (QRDRs) of target molecules of FQs, DNA gyrase and/or topoisomerase IV (4). In addition, plasmid-mediated quinolone resistance (PMQR) genes, including *qnr*, *aac* (*6′*)-*Ib-cr*, *qepA*, and *oqxAB*, have been recently reported in transmissible resistance mechanisms (5–8). The presence of these PMQR genes alone has been reported to elevate the minimum inhibitory concentrations (MICs) of FQs by 4- to 128-fold, although the final MIC values usually remain below the breakpoint of ciprofloxacin (CIP) for ''susceptible'' ($\leq 1\,\mu$g/mL) adopted by the Clinical and Laboratory Standards Institute (CLSI) (9) and that ($\leq 0.5\,\mu$g/mL) of the European Committee on Antimicrobial Susceptibility Testing (10).

Qnr proteins belong to the pentapeptide repeat family and protect bacteria from the binding of FQs to DNA gyrase and/or topoisomerase IV. To date, 6 groups of Qnr proteins have been identified (QnrA, QnrB, QnrC, QnrD, QnrS, and QnrVC) (11,12). Allou et al. evaluated the impact of the acquisition of genes encoding Qnrs, and found that the low-level resistance to FQs conferred by these genes was associated with decreased bactericidal activity of CIP (13). In a preliminary study, we also found that *qnrA* contributed to improved bacterial survival at lethal CIP concentrations. However, the precise effects of the acquisition of *qnr* on the reduced bactericidal activity of FQs and development of high-level quinolone resistance are not well known. Therefore, the aim of this study was to evaluate the effects of *qnrA* on the survival of bacteria exposed to lethal CIP concentrations using *Escherichia coli* strains displaying various O-serotypes. In addition, the effects of acquisition of *qnrA* on the development of quinolone resistance through accumulation of amino acid substitutions in the QRDRs of GyrA and ParC were also evaluated.

MATERIALS AND METHODS

Bacterial strains: Twelve FQ-susceptible *E. coli* strains of 6 different serotypes (O1, O6, O18, O25, O74, and O78; 2 strains each) were used in this study. Serotypes O1, O6, O18, and O25 are frequently detected in *E. coli* isolates obtained from patients with urinary tract infections (14–16). Strains O74 and O78 were frequent serotypes detected among *E. coli* isolates obtained from stool specimens of 2,563 healthy adult volunteers of our investigation conducted at a Public Health Center in 2010. For the 2 strains determined as serotype O25 using *E. coli* antisera, genetic serotyping was also performed (17), which resulted in identification of both strains as O25b. In addition, for all strains, multilocus sequence typing was performed by analyzing 7 housekeeping

Received April 11, 2014. Accepted September 1, 2014. J-STAGE Advance Publication December 24, 2014.
DOI: 10.7883/yoken.JJID.2014.153

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genes, according to the protocol of the *E. coli* website (http://mlst.ucc.ie/mlst/dbs/Ecoli). Sequence types were compared with the species' population structure using eBurst (http://eburst.mlst.net/). Cloning of *qnrA* was performed using the positive strain *E. coli* E15, and quality control for susceptibility testing was performed using the reference strain *E. coli* ATCC 25922.

Cloning of *qnrA*: The *qnrA* sequence and its surrounding regions were amplified with the specific primers ORF513-QnrA.f (5′-CCGGAATTCCGGCGAAGATGACTATGGCAAGCAA-3′) and ampR-QnrA.r (5′-CCCGGATCCGGGGCAGCAGGGTAAAGCGGTGAAT-3′). The product was digested with *Eco*RI and *Bam*HI (Takara Bio Inc., Tokyo, Japan), and the resultant fragments were ligated into the cloning vector pSTV28, which contained a reconstructed chloramphenicol-resistant gene composed of the *Tn9* and *β*-galactosidase genes (TaKaRa). *E. coli* strains displaying various O-serotypes were transformed with the recombinant plasmid pSTVqnrA by electroporation, and the transformants were then selected on X-gal agar containing 20 μg/mL chloramphenicol (Sigma-Aldrich Co. LLC., Tokyo, Japan). In addition, the cloning vector pSTV28 was introduced into each *E. coli* strain and the resultant transformants were used as the parent strain of each transformant harboring *qnrA*.

Antimicrobial susceptibility test: The MIC of CIP was determined using Etest (SYSMEX bioMérieux, Co. Ltd., Tokyo, Japan) performed on Mueller-Hinton agar plates (Becton Dickinson Diagnostic System, Sparks, MD, USA), according to the manufacturer's guidelines. Susceptibility was determined according to CLSI criteria (9).

Minimum bactericidal concentration (MBC) and mutant prevention concentration (MPC): MBCs of CIP for the parent strains and transformants were determined according to the protocol recommended by the CLSI (18). In the present study, MBCs for each strain were determined using 7 different CIP concentrations (1 × MIC, 1.5 × MIC, 2 × MIC, 2.5 × MIC, 3 × MIC, 3.5 × MIC, and 4 × MIC). MBC was defined as the lowest antibiotic concentration that produced a $\geq$99.9% decrease in survival (CFU/mL) relative to that of the starting inoculum.

MPCs of CIP for the parent strains and transformants were also determined, as described previously (19). MPCs for each strain were determined using 10 different concentrations (step range for the parent strains: 0.024, 0.032, 0.047, 0.064, 0.094, 0.19, 0.25, 0.5, 0.75, and 1 μg/mL; step range for transformants: 0.38, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, and 8 μg/mL). MPC was defined as the lowest antibiotic concentration at which no colonies grew on a plate, and the mean MPC for each strain was determined in 3 independent experiments that were repeated 3 times on different days. In addition, DNA sequencing of the QRDRs of the gene products of *gyrA* and *parC* were performed as described previously (20). DNA sequences were determined for resistant colonies recovered on Mueller-Hinton agar plates one step below the MPC value. The nucleotide and deduced amino acid sequences were analyzed using the BLAST program (http://www.ncbi.nlm.nih.gov/blast).

Killing curve assay: Killing curve assays were performed for the parent strains and transformants harboring *qnrA*. Each strain was cultivated in Luria-Bertani (LB) broth containing 100 μM isopropyl-*β*-D-1-thiogalactopyranoside (IPTG) (Nacalai Tesque Inc., Kyoto, Japan) for 24 h, and the bacterial test suspension was then adjusted to an optical density of approximately 0.1 at 660 nm (ca.10^8 CFU/mL). The test suspensions were diluted 100-fold in LB broth (ca.10^6 CFU/mL), and 2 × or 3 × the MIC of CIP for each parent strain (or transformant) was added to the test suspension of the parent strain (or transformant). After 0, 4, 6, 8, 24, or 48 h incubation at 37°C, aliquots (100 μL) of the mixture were spread onto nutrient agar (Eiken, Chemical Co. Ltd. Tokyo, Japan) after serial 10-fold dilutions, and the plates were incubated at 37°C for 18–24 h. The total number of bacterial colonies that grew on each plate was counted, and the viability (CFU/mL) of the original bacterial culture was calculated. To avoid distortion of the results owing to experimental error, all experiments were performed in triplicate and repeated 3 times on different days.

Adaptation to CIP and sequencing of the QRDRs of *gyrA* and *parC*: The parent strains and transformants were subjected to an adaptation test to CIP for 20 passages. Aliquots (50 μL) of overnight culture were added to 5 mL LB broth containing 100 μM IPTG and incubated at 37°C with shaking at 120 rpm. When the optical density at 660 nm reached approximately 0.1 (ca.10^8 CFU/mL), 1/2 MIC of CIP for each strain was added to the bacterial culture, and incubation was continued at 37°C with shaking at 120 rpm for 5 days. The 1/2 MIC culture (50 μL) was then incubated on LB agar plates containing the same concentration of CIP for 24 h at 37°C. Ten colonies grown on each agar plate were randomly selected, and the MICs of CIP were determined using Etest. Of the colonies selected, 1 exhibiting the highest MIC value was named passage 1 (P1) and subjected to the next passage. This procedure was repeated 19 times after the selection of P1.

In addition, DNA sequences of the QRDRs of *gyrA* and *parC* were determined as described previously (20). The nucleotide and deduced amino acid sequences were analyzed using the BLAST program (http://www.ncbi.nlm.nih.gov/blast).

RESULTS AND DISCUSSION

Among the 12 FQ-susceptible *E. coli* strains analyzed in this study, 7 were identified as ST95 and 2 as O25b-ST131. Acquisition of *qnrA* increased the MICs of CIP by 20.8- to 31.7-fold (Table 1), but the MICs remained below the susceptibility breakpoint to CIP according to the CLSI criteria ($\leq$1 μg/mL). Acquisition of *qnrA* also increased the MBC and MPC values of CIP by 15.8- to 79.1-fold (0.285–1.14 μg/mL) and by 2.6- to 21.1-fold (0.5–4 μg/mL), respectively, relative to those of the parent strains (Table 1). These results were consistent with those of previous reports (21,22). The MBCs and MPCs of CIP for the transformants of O25b strains were highest among the compared O-serotypes at 0.75–1.14 and 3–4 μg/mL, respectively. These results suggested that the acquisition of *qnrA* confers enhanced survivability of the bacteria in the presence of FQs, indicating that O25b strains harboring *qnrA* might easily

Table 1. MIC, MBC, and MPC of ciprofloxacin for the parent strains and transformants

Strain	Serotype	MLST	MIC (μg/mL)		MBC (μg/mL)		MPC (μg/mL)		MSW[1] (MPC/MIC [μg/mL])	
			Parent	Transformant	Parent	Transformant	Parent	Transformant	Parent	Transformant
No. 3	O1:H12	ST95	0.008	0.19	0.016	0.285	0.19	0.75	23.8	3.9
No. 16	O1:HUT	ST95	0.012	0.25	0.018	0.50	0.19	0.75	15.8	3.0
No. 155	O6:HUT	ST92	0.006	0.19	0.006	0.475	0.19	1.0	31.7	5.3
No. 252	O6:HUT	ST95	0.008	0.19	0.012	0.38	0.19	0.5	23.8	2.6
No. 92	O18:H7	ST95	0.006	0.19	0.006	0.38	0.19	1.0	31.7	5.3
No. 286	O18:H7	ST95	0.008	0.25	0.016	0.375	0.094	0.75	11.8	3.0
No. 23	O25b:H4	ST131	0.012	0.38	0.024	1.14	0.19	4.0	15.8	10.5
No. 41	O25b:H4	ST131	0.012	0.25	0.012	0.75	0.38	3.0	31.7	12.0
No. 27	O74:H7	ST95	0.012	0.38	0.018	0.38	0.38	1.5	31.7	3.9
No. 205	O74:H7	ST95	0.008	0.25	0.012	0.375	0.19	0.75	23.8	3.0
No. 189	O78:H6	ST3200	0.008	0.25	0.016	0.375	0.094	1.0	11.8	4.0
No. 485	O78:HUT	ST23	0.008	0.19	0.012	0.38	0.094	0.75	11.8	3.9

[1]: MSW, mutant selection window (i.e., the antibiotic concentration found between the MIC and MPC).
MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; MPC, mutant prevention concentration; HUT, H-antigen untypable; Parent, parent strain.

acquire resistance to FQs.

Killing curve analyses revealed a selective advantage for survival of the transformants harboring *qnrA*. Following exposure to 2 × MIC of CIP, the parent strains showed a 10^5-fold reduction in survival after incubation for 24 h, and no viable bacteria were recovered after 48 h (Fig. 1A). The transformants of strains O1, O6, and O25b maintained levels of 10^5–10^8 CFU/mL viable bacteria after incubation for 24 h at 2 × MIC of CIP, while the remaining transformants (O18, O74, and O78) showed a 10^5-fold reduction after 24 h and no viable bacteria were recovered after incubation for 48 h (Fig. 1B). In addition, at 3 × MIC of CIP, only viable bacteria cells of the transformants of O25b strains were recovered up to 10^5 CFU/mL after incubation for 48 h (Fig. 2), and the effect of *qnrA* on bacterial survival was greatest in the O25b:H4-ST131 strains. These results suggested that the contribution of *qnrA* to bacterial survival is not equivalent in all serotypes of *E. coli* strains. To date, some investigators have reported that in the present of CIP, *E. coli* isolates with *qnr* could survive longer than those without *qnr* (13,21). In the present study, we also confirmed that the acquisition of *qnrA* contributed to increased bacterial survival under exposure to lethal CIP concentrations in *E. coli* strains displaying certain O-serotypes (O1, O6, and O25b). Therefore, the presence of *qnr* might well confer an appreciable effect on bacterial viability under conditions of lethal CIP concentrations.

Although *E. coli* strains of certain O-serotypes harboring *qnrA* survived when exposed to lethal CIP concentrations within the mutant selection window (MSW), in which single-step mutants will be enriched, no amino acid substitutions in the QRDRs of GyrA and/or ParC were found in the transformants recovered after incubation for 48 h in the killing curve assays. Although the presence of *qnrA* extended the range of the MSW, exposure to lethal CIP concentrations within the MSW may nonetheless block the growth of susceptible and single-step mutants. Furthermore, in the MPC assays, relatively more amino acid substitutions were found in the QRDRs of GyrA in the parent strains than in their transformants (Table 2). This observation led us to speculate that the presence of *qnrA* might have the same effect as acquisition of resistant mutations on the survival of the transformants.

We next evaluated the effects of the acquisition of *qnrA* on the development of FQ resistance through the accumulation of amino acid substitutions in the QRDRs of GyrA and/or ParC by continuous exposure to 1/2 MIC of CIP. As shown in Fig. 3, the MIC values of CIP were gradually elevated after adaptation of the O1-ST95 and O25b-ST131 strains by 20 passages, and those of the transformants harboring *qnrA* increased up to 32 μg/mL. In addition, amino acid substitutions, which were consistent with the hot spots of substitutions observed in clinical FQ-resistant strains (23,24), were found in the QRDRs of GyrA and/or ParC in the parent strains and/or transformants. A Ser83-Leu substitution in GyrA was identified after passage 4 in the parent strains of O25b-ST131, after passage 13 in the transformants of O25b-ST131, and after passage 15 in the transformants of O1-ST95 (Table 3). On the other hand, a Ser80-Arg substitution in ParC was identified only in the transformants after passage 17 (O1T-17 and O25T-17) (Table 3). Some investigators have suggested that the presence of *qnrA* might increase MPC values, which would facilitate selection for one-step FQ-resistant mutants with any substitutions in *gyrA* and/or *parC* (21,22). However, Cesaro et al. reported that topoisomerase mutations were rarely selected by CIP and moxifloxacin in *qnr*-harboring strains (25). Although the conditions of each experiment were different (e.g., bacterial origin, CIP concentration, and methodology), our results are more similar to those of Cesaro et al. The increased levels of MICs were similar between strains harboring *qnrA* and those that acquired a single Ser83-Leu substitution in GyrA (Table 1 and Table 3). This suggests that QnrA might completely protect DNA gyrase and/or topoisomerase IV from CIP; therefore, the transformants harboring *qnrA* would survive in a lethal concentration of CIP, even without QRDR mutations. Indeed, the fact that more amino acid substitutions were observed in the QRDRs of GyrA

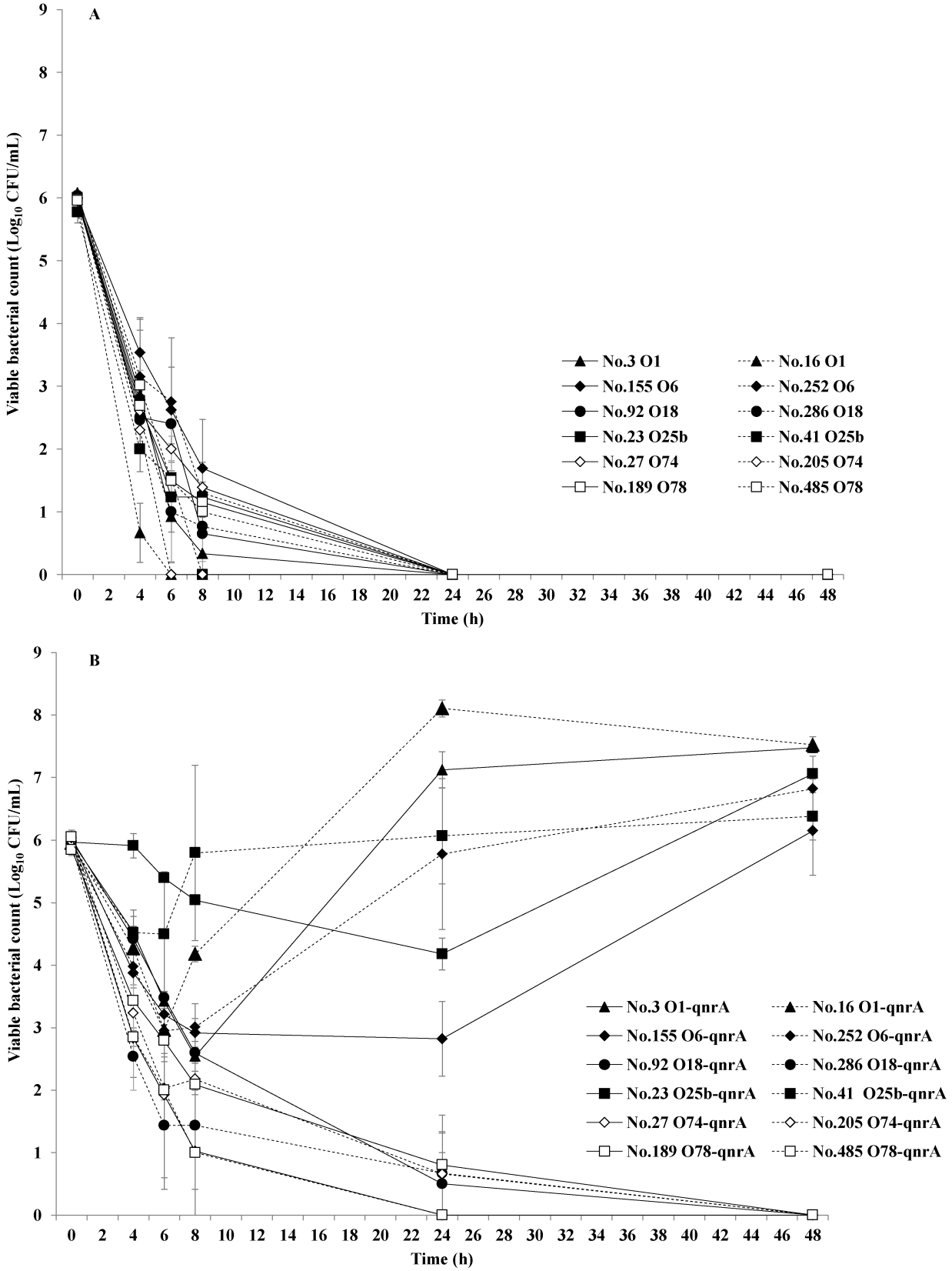

Fig. 1. Killing curve assay at 2 × MIC of CIP. (A) Parent strains, (B) transformants. In the killing curve assay, 2 × MIC of CIP for the parent strains or their transformants was added to each test suspension, and the number of viable bacterial cells was counted after 0, 4, 6, 8, 24, or 48 h incubation at 37°C. The actual concentrations of CIP used in killing curve assays were as follows: parent strain Nos. 92 and 155: 0.012 μg/mL; parent strain Nos. 3, 189, 205, 252, 286, and 485: 0.016 μg/mL; parent strain Nos. 16, 23, 27, and 41: 0.024 μg/mL; transformant Nos. 3, 92, 155, 252, and 485: 0.38 μg/mL; transformant Nos. 16, 41,189, 205, and 286: 0.50 μg/mL; transformant Nos. 23 and 27: 0.76 μg/mL. All experiments were performed in triplicate, and the values reported represent the mean values. Error bars represent standard deviations (SD) of results from 3 experiments.

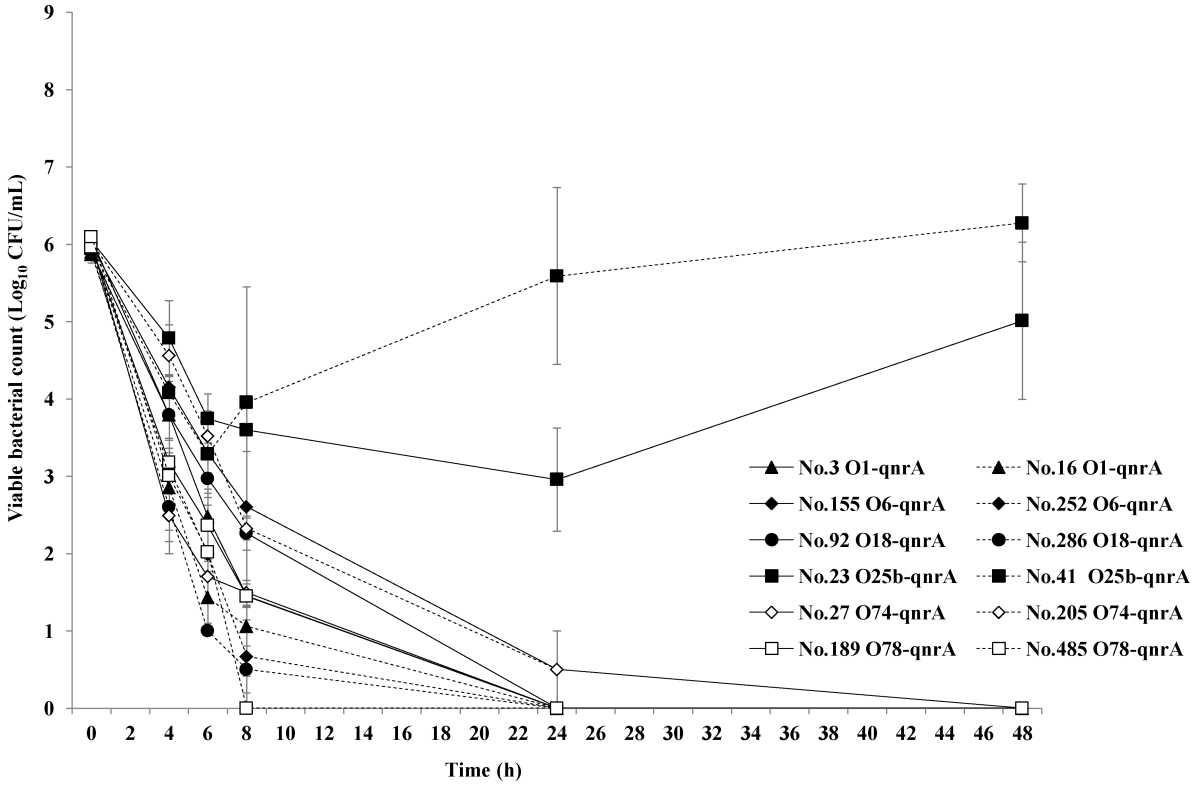

Fig. 2. Killing curve assay at 3 × MIC of CIP for transformants. In the killing curve assay, 3 × MIC of CIP for the transformants was added to each test suspension, and the number of viable bacterial cells was counted after 0, 4, 6, 8, 24, or 48 h incubation at 37°C. The actual concentrations of CIP used in killing curve assays were as follows: transformant Nos. 3, 92, 155, 252, and 485: 0.57 μg/mL; Nos. 16, 41,189, 205, and 286: 0.75 μg/mL; Nos. 23 and 27: 1.14 μg/mL. All experiments were performed in triplicate, and the values reported represent the mean values. Error bars represent standard deviations (SD) of results from 3 experiments.

Table 2. MPC of ciprofloxacin for the parent strains and transformants and acquisition of amino acid substitutions in the QRDRs

Strain	Serotype	MLST	MPC (μg/mL)[1]		Amino acid substitutions in the QRDRs[2]			
			Parent strain	Transformant	Parent strain		Transformant	
					GyrA	ParC	GyrA	ParC
No. 3	O1:H12	ST95	0.19	0.75	S83L	wt	wt	wt
No. 16	O1:HUT	ST95	0.19	0.75	S83L	wt	wt	wt
No. 155	O6:HUT	ST92	0.19	1.0	wt	wt	wt	wt
No. 252	O6:HUT	ST95	0.19	0.5	S83L	wt	wt	wt
No. 92	O18:H7	ST95	0.19	1.0	S83L	wt	S83L	wt
No. 286	O18:H7	ST95	0.094	0.75	S83L	wt	wt	wt
No. 23	O25b:H4	ST131	0.19	4.0	S83L	wt	wt	wt
No. 41	O25b:H4	ST131	0.38	3.0	D87Y	wt	wt	wt
No. 27	O74:H7	ST95	0.38	1.5	wt	wt	S83L	wt
No. 205	O74:H7	ST95	0.19	0.75	S83L	wt	wt	wt
No. 189	O78:H6	ST3200	0.094	1.0	S83L	wt	wt	wt
No. 485	O78:HUT	ST23	0.094	0.75	wt	wt	wt	wt

[1]: The MPC was defined as the lowest antibiotic concentration at which no colonies grew on a plate.
[2]: DNA sequences of the QRDRs of GyrA and ParC were performed for resistant colonies recovered on Mueller-Hinton plates one step below the MPC value.
MPC, mutant prevention concentration; HUT, H-antigen untypable; wt, wild type; S83L, amino acid substitution of Serine to Leucine at position 83; D87Y, amino acid substitution of Aspartic acid to Tyrosine at position 87.

in the parent strains than in their transformants also supports this hypothesis. Therefore, further studies considering the effects of different experimental conditions, including bacterial origin, CIP concentration, and methodology, are warranted to confirm whether *qnrA* facilitates the selection of higher-level quinolone-resistant mutants.

In conclusion, we confirmed that the acquisition of *qnrA* alone contributed to increased survival of *E. coli* exposed to lethal CIP concentrations but did not necessarily accelerate QRDR mutations resulting in the accumulation of amino acid substitutions. The effects of

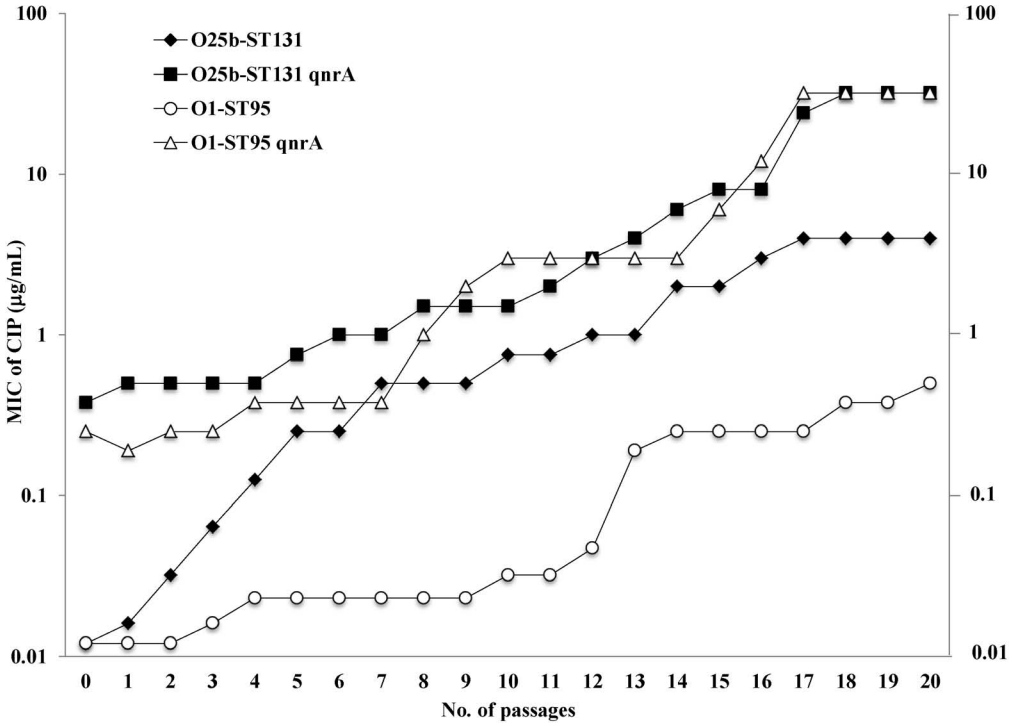

Fig. 3. MICs of CIP after persistent passages. Adaptation to CIP was generated by repeated subculture in LB broth containing CIP. The bacterial culture (ca.10^8 CFU/mL) was added to LB broth containing 1/2 MIC of CIP for each strain, and incubation was continued at 37°C with shaking at 120 rpm for 5 days. The 1/2 MIC culture (50 μL) was spread onto LB agar plates containing the same concentration of CIP, and 10 colonies grown on each agar plate were randomly selected. MICs of CIP were determined using Etest, and that showing the highest MIC value was named passage 1 (P1). This procedure was repeated 19 times after the selection of P1. The MIC values of CIP were gradually elevated after the adaptation of 20 passages in the O1-ST95 and O25b-ST131 strains, and the MICs of CIP for the transformants harboring the *qnrA* increased up to 32 μg/mL (solid squares).

Table 3. MIC and amino acid substitutions in the QRDRs of O1-ST95 strains and O25b-ST131 strains

Strain		ID	No. of passage	MIC of CIP (μg/mL)	Amino acid substitution in the QRDRs	
					GyrA	ParC
O1-ST95	Parent strain	O1-0	P 0	0.012	wt	wt
		O1-14	P 14	0.25	S83L	wt
	Transformant	O1T-0	P 0	0.25	wt	wt
		O1T-15	P 15	6	S83L	Wt
		O1T-17	P 17	32	S83L	S80R
O25b-ST131	Parent strain	O25-0	P 0	0.012	wt	Wt
		O25-4	P 4	0.19	S83L	Wt
	Transformant	O25T-0	P 0	0.38	wt	Wt
		O25T-13	P 13	4	S83L	Wt
		O25T-17	P 17	24	S83L	S80R

wt, wild type; S83L, amino acid substitution of Serine to Leucine at position 83; S80R, amino acid substitution of Serine to Arginine at position 80.

qnrA on viability were greatest in O25b strains exposed to a lethal amount of CIP among all O-serotypes compared, which could explain the rapid spread of *E. coli* O25b:H4-ST131. At present, the *qnr* are rapidly spreading among Family *Enterobacteriaceae*. Although no significant difference have been observed in the clinical outcomes of serious infections, such as those of the bloodstream, between *qnr*-positive and *qnr*-negative groups (26), some investigators have reported that the presence of *qnr* determinants reduced the efficacy of CIP in animal models of pneumonia and urinary tract infections (27–29). Therefore, further investigation is necessary to elucidate the impact of low-level resistance to FQs due to acquisition of *qnr*. In particular, *Enterobacteriaceae* isolates exhibiting relatively low MICs around the susceptibility breakpoint of FQs should be evaluated to determine the clinical significance of isolates harboring *qnr*.

Acknowledgments This study was supported by grants from the Japanese Ministry of Health, Labour, and Welfare (H21-Shinkou-

Ippan-008 and H24-Shinkou-Ippan-010). We are grateful to the department of Infection Control and Prevention, The University of Tokyo Hospital for providing *E. coli* E15.

Conflict of interest None to declare.

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