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MECHANISMS OF RESISTANCE

Tigecycline Nonsusceptibility Occurs Exclusively in Fluoroquinolone-Resistant *Escherichia coli* Clinical Isolates, Including the Major Multidrug-Resistant Lineages O25b:H4-ST131-H*30*R and O1-ST648

Toyotaka Sato,[a] Yuuki Suzuki,[a] Tsukasa Shiraishi,[a] Hiroyuki Honda,[a,b] Masaaki Shinagawa,[c] Soh Yamamoto,[a] Noriko Ogasawara,[a] Hiroki Takahashi,[b] Satoshi Takahashi,[c,d] Yutaka Tamura,[e] Shin-ichi Yokota[a]

Department of Microbiology, Sapporo Medical University School of Medicine, Sapporo, Japan[a]; Department of Respiratory Medicine and Allergology, Sapporo Medical University School of Medicine, Sapporo, Japan[b]; Division of Laboratory Medicine, Sapporo Medical University Hospital, Sapporo, Japan[c]; Department of Infection Control and Laboratory Medicine, Sapporo Medical University School of Medicine, Sapporo, Japan[d]; Laboratory of Food Microbiology and Food Safety, Department of Health and Environmental Sciences, School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu, Japan[e]

ABSTRACT Tigecycline (TGC) is a last-line drug for multidrug-resistant *Enterobacteriaceae*. We investigated the mechanism(s) underlying TGC nonsusceptibility (TGC resistant/intermediate) in *Escherichia coli* clinical isolates. The MIC of TGC was determined for 277 fluoroquinolone-susceptible isolates (ciprofloxacin [CIP] MIC, <0.125 mg/liter) and 194 fluoroquinolone-resistant isolates (CIP MIC, >2 mg/liter). The MIC_{50} and MIC_{90} for TGC in fluoroquinolone-resistant isolates were 2-fold higher than those in fluoroquinolone-susceptible isolates (MIC_{50}, 0.5 mg/liter versus 0.25 mg/liter; MIC_{90}, 1 mg/liter versus 0.5 mg/liter, respectively). Two fluoroquinolone-resistant isolates (O25b:H4-ST131-*H*30R and O125:H37-ST48) were TGC resistant (MICs of 4 and 16 mg/liter, respectively), and four other isolates of O25b:H4-ST131-*H*30R and an isolate of O1-ST648 showed an intermediate interpretation (MIC, 2 mg/liter). No TGC-resistant/intermediate strains were found among the fluoroquinolone-susceptible isolates. The TGC-resistant/intermediate isolates expressed higher levels of *acrA* and *acrB* and had lower intracellular TGC concentrations than susceptible isolates, and they possessed mutations in *acrR* and/or *marR*. The MICs of *acrAB*-deficient mutants were markedly lower (0.25 mg/liter) than those of the parental strain. After continuous stepwise exposure to CIP *in vitro*, six of eight TGC-susceptible isolates had reduced TGC susceptibility. Two of them acquired TGC resistance (TGC MIC, 4 mg/liter) and exhibited expression of *acrA* and *acrB* and mutations in *acrR* and/or *marR*. In conclusion, a population of fluoroquinolone-resistant *E. coli* isolates, including major extraintestinal pathogenic lineages O25b:H4-ST131-*H*30R and O1-ST648, showed reduced susceptibility to TGC due to overexpression of the efflux pump AcrAB-TolC, leading to decreased intracellular concentrations of the antibiotics that may be associated with the development of fluoroquinolone resistance.

KEYWORDS antimicrobial resistance, bacterial infection, efflux pump

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Address correspondence to Toyotaka Sato, sato.t@sapmed.ac.jp.

The emergence of multidrug-resistant bacteria is a major concern for clinicians. A high prevalence of fluoroquinolone-resistant *Escherichia coli* has been reported in cases of extraintestinal *E. coli* infection (e.g., urinary tract infections and septicemia) worldwide (1, 2). One particular lineage, O25b:H4-ST131 (3, 4) (especially subclone

*H*30R) (5), shows fluoroquinolone resistance in the clinical setting. Strains belonging to this lineage not only show fluoroquinolone resistance but also harbor extended-spectrum β-lactamases (ESBLs), e.g., CTX-M type, and aminoglycoside resistance genes (6, 7). Thus, numerous O25b:H4-ST131-*H*30R strains are frequently multidrug resistant, leading to difficulties for clinicians trying to select appropriate antimicrobial therapy.

Tigecycline (TGC) is a glycylcycline antimicrobial that is effective against a variety of Gram-positive and -negative bacteria (8, 9). Previous large-scale surveillance studies show that more than 90% of *Enterobacteriaceae* isolates are susceptible to TGC (10, 11). Especially, >99.7% of *E. coli* isolates are susceptible to TGC (10, 11). TGC has a new and unique backbone, meaning that it does not exhibit cross-resistance to any antimicrobials, including tetracycline and minocycline (12, 13); thus, it is currently a last-line drug for treating multidrug-resistant bacteria, particularly those resistant to carbapenem, such as New Delhi metallo-β-lactamase (NDM)-producing *Enterobacteriaceae* (14–16).

The mechanism responsible at least in part for the TGC resistance in *Enterobacteriaceae*, such as *Enterobacter* spp. and *Klebsiella pneumoniae*, has been suggested to be due to overexpression of chromosomal multidrug efflux pumps, such as AcrAB-TolC and OqxAB-TolC (17, 18). It has been suggested that overexpression of AcrAB-TolC underlies reduced TGC susceptibility in *E. coli* (19, 20). Linkevicius et al. reported that spontaneous mutants with reduced susceptibility to TGC (the highest TGC MIC was 0.75 mg/liter) were selected, and they identified mutations in genes contributing overexpression of the AcrAB efflux pump, such as *marR* and *acrR* (19). However, the *E. coli* strains used in that study did not exceed the TGC resistance breakpoint (TGC MIC of >2 mg/liter), and it was not concluded whether overexpression of AcrAB was sufficient for the TGC-resistant phenotype in *E. coli*. Keeney et al. reported that a series of isogenic *E. coli* clinical strains with decreased TGC susceptibility (the highest TGC MIC was 2 mg/liter) were isolated from a single patient given TGC treatment (20). In that study, *marA* and *acrB* were identified as the genes responsible for the decrease of TGC susceptibility as determined by transposon mutagenesis. This study suggested that it was possible for the TGC-nonsusceptible phenotype to be acquired by overexpression of AcrAB during TGC treatment. However, the functional role of AcrAB against TGC has not been determined, and the mechanism related to AcrAB overexpression being behind the emergence of TGC-nonsusceptible *E. coli* is still unclear. AcrAB is a multidrug efflux pump, and other antibiotics excreted by AcrAB should influence the acquisition of the TGC-nonsusceptible phenotype of *E. coli* without the use of TGC in the clinical fields.

We consider that TGC may not work well against fluoroquinolone-resistant isolates because they frequently exhibit overexpression of AcrAB-TolC concurrent with the major fluoroquinolone resistance mechanism, point mutation(s) in the quinolone resistance-determining regions (QRDRs) of *gyrA*, *gyrB*, *parC*, and *parE*, in *E. coli* (21). However, no studies have confirmed this. Here, we examined the association between fluoroquinolone resistance and nonsusceptibility to TGC in *E. coli* isolates derived from clinical specimens in Japan.

RESULTS AND DISCUSSION

Susceptibility of fluoroquinolone-resistant and fluoroquinolone-susceptible *E. coli* isolates to TGC. The MICs for TGC were higher in fluoroquinolone-resistant *E. coli* isolates than in fluoroquinolone-susceptible isolates (Fig. 1). The MIC_{50} and MIC_{90} for fluoroquinolone-resistant isolates (0.5 and 1 mg/liter, respectively) were 2-fold higher than those for fluoroquinolone-susceptible isolates (0.25 and 0.5 mg/liter, receptively). The MIC distribution and MIC_{50} and MIC_{90} for fluoroquinolone-susceptible *E. coli* isolates were quite similar to those for *E. coli* isolates deposited in the EUCAST database (International MIC Distribution, Reference Database 2015-06-11) (22). All fluoroquinolone-susceptible isolates were susceptible to TGC. In contrast, seven (3.6%) of the fluoroquinolone-resistant isolates were not susceptible to TGC. Of these, five had a TGC MIC of 2 mg/liter, which is defined as intermediate by EUCAST, whereas the other two isolates (MICs of 4 mg/liter and 16 mg/liter) were defined as resistant. This result

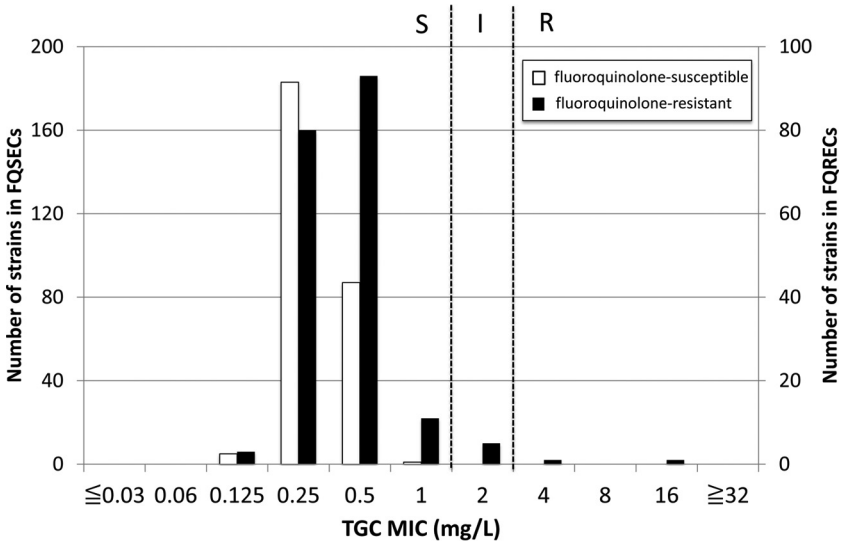

FIG 1 MICs for TGC in fluoroquinolone-susceptible and -resistant *E. coli* clinical isolates. Susceptible and resistant, as defined by EUCAST, are indicated by dashed lines. S, susceptible; I, intermediate; R, resistant; FQSECs, fluoroquinolone-susceptible *E. coli* isolates; EQRECs, fluoroquinolone-resistant *E. coli* clinical isolates.

indicated that TGC nonsusceptibility was exclusive to fluoroquinolone-resistant *E. coli*. All of the TGC-nonsusceptible isolates except HUE1 (23) had four amino acid substitutions in the QRDRs of GyrA and ParC as described below.

Characterization of TGC-nonsusceptible *E. coli* isolates. The seven TGC-nonsusceptible isolates were derived from various clinical specimens and patient backgrounds (Table 1). Five of the seven isolates belonged to a specific lineage, O25b:H4-ST131-*H*30R, which is a known fluoroquinolone-resistant lineage identified frequently in clinical settings worldwide (Table 1) (5, 7). In addition, one TGC-nonsusceptible strain belonged to another major fluoroquinolone-resistant linage, O1-ST648, that is also distributed worldwide and which frequently expresses β-lactamases, CTX-M type ESBLs or CMY-2, and as reported in one study, NDM-5 (24–26). Thus, these clones are often resistant to multiple drugs (e.g., penicillins, cephalosporins, and aminoglycosides) (5, 7, 24–26). Similarly, most TGC-nonsusceptible isolates belonging to O25b:H4-ST131-*H*30R and O1-ST648 were resistant to 2 to 4 of the five classes of antimicrobials (cephalosporins, carbapenems, aminoglycosides, fluoroquinolones, and fosfomycin). Genes encoding β-lactamases, including CTX-M type ESBLs, were the most common. On the other hand, none of the strains were resistant to imipenem and amikacin (Table 1). Thus, these TGC-nonsusceptible *E. coli* isolates could still be treated with other antibiotics; however, identifying suitable antimicrobials may be difficult if these strains acquire additional resistance mechanisms in the future.

TGC nonsusceptibility mechanism in fluoroquinolone-resistant *E. coli*. Previous studies have examined TGC resistance mechanism in Enterobacteriaceae (17, 18). Reductions in the TGC susceptibility of *E. coli* laboratory strains and clinical isolates are accompanied by overexpression of AcrAB-TolC; however, these *E. coli* strains have never exceeded the breakpoint for TGC (>2 mg/liter) (19, 20, 27).

Here, we used quantitative RT-PCR to measure the expression of *acrA* and *acrB*. All TGC-nonsusceptible isolates showed higher expression of *acrA* and *acrB* than TGC-susceptible isolates (Fig. 2A and B, left panels). Also, there was a significant correlation between the expression of *acrA* and *acrB* and the MIC for TGC (*acrA*, $r^2 = 0.758$, $P < 0.001$ [Fig. 2A, right panel]; *acrB*, $r^2 = 0.543$, $P = 0.01$ [Fig. 2B, right panel]). In addition, we measured the intracellular TGC concentration fluorometrically. The results showed that all TGC-nonsusceptible isolates had significantly lower intracellular concentrations of TGC (Fig. 2C, left panels); there was also a significant correlation between intracellular

TABLE 1 Characteristics and antibiogram of TGC-resistant/nonsusceptible *E. coli* clinical isolates

Strain	Specimen	Patient age (yr)	Patient gender	Yr	Phylogenetic group	Serotype	Sequence type	MIC (mg/liter)[a]									β-Lactamase(s)
								CAZ (>16)[b]	CPD (>1)	FEP (>4)	IPM (>8)	GEN (>4)	AMK (>16)	CIP (>1)	TGC (>2)	FOF (>32)	
HUE1	Urine catheter	77	Female	2008	A	O125:H37	48	<4 (S)[b]	4 (R)	0.5 (S)	0.125 (S)	64 (R)	0.5 (S)	4 (R)	16 (R)	0.5 (S)	NT[c]
SRE54	Urine	78	Female	2008	B2	O25b:H4	131-*H*30R	4 (S)	>128 (R)	8 (R)	0.125 (S)	64 (R)	2 (S)	256 (R)	4 (R)	64 (R)	TEM-1b, CTX-M-14
SME37	Vaginal fluids	39	Female	2008	B2	O25b:H4	131-*H30*R	16 (I)	>128 (R)	4 (I)	0.06 (S)	0.5 (S)	1 (S)	128 (R)	2 (I)	0.5 (S)	CTX-M14
SRE58	Scrotum	60	Male	2008	B2	O25b:H4	131-*H*30R	64 (R)	>128 (R)	128 (R)	0.25 (S)	1 (S)	4 (S)	256 (R)	2 (I)	0.5 (S)	TEM-1b, CTX-M-2, CMY-8
SME108	Urine	41	Male	2015	D	O1	648	1 (S)	0.5 (S)	0.125 (S)	0.06 (S)	64 (R)	1 (S)	128 (R)	2 (I)	0.5 (S)	NT
SRE82	Feces	69	Female	2015	B2	O25b:H4	131-*H*30R	8 (S)	32 (R)	0.125 (S)	0.125 (S)	1 (S)	1 (S)	128 (R)	2 (I)	1 (S)	NT
SRE48	Urine	48	Male	2015	B2	O25b:H4	131-*H*30R	16 (I)	>128 (R)	4 (I)	0.125 (S)	0.5 (S)	1 (S)	128 (R)	2 (I)	1 (S)	CTX-M-27

[a]CAZ, ceftazidime; CPD, cefpodoxime; FEP, cefepime; IPM, imipenem; GEN, gentamicin; AMK, amikacin; CIP, ciprofloxacin; TGC, tigecycline; FOF, fosfomycin; S, susceptible; I, intermediate; R, resistant.
[b]MIC breakpoint (mg/liter) for resistance interpretation.
[c]NT, not tested.

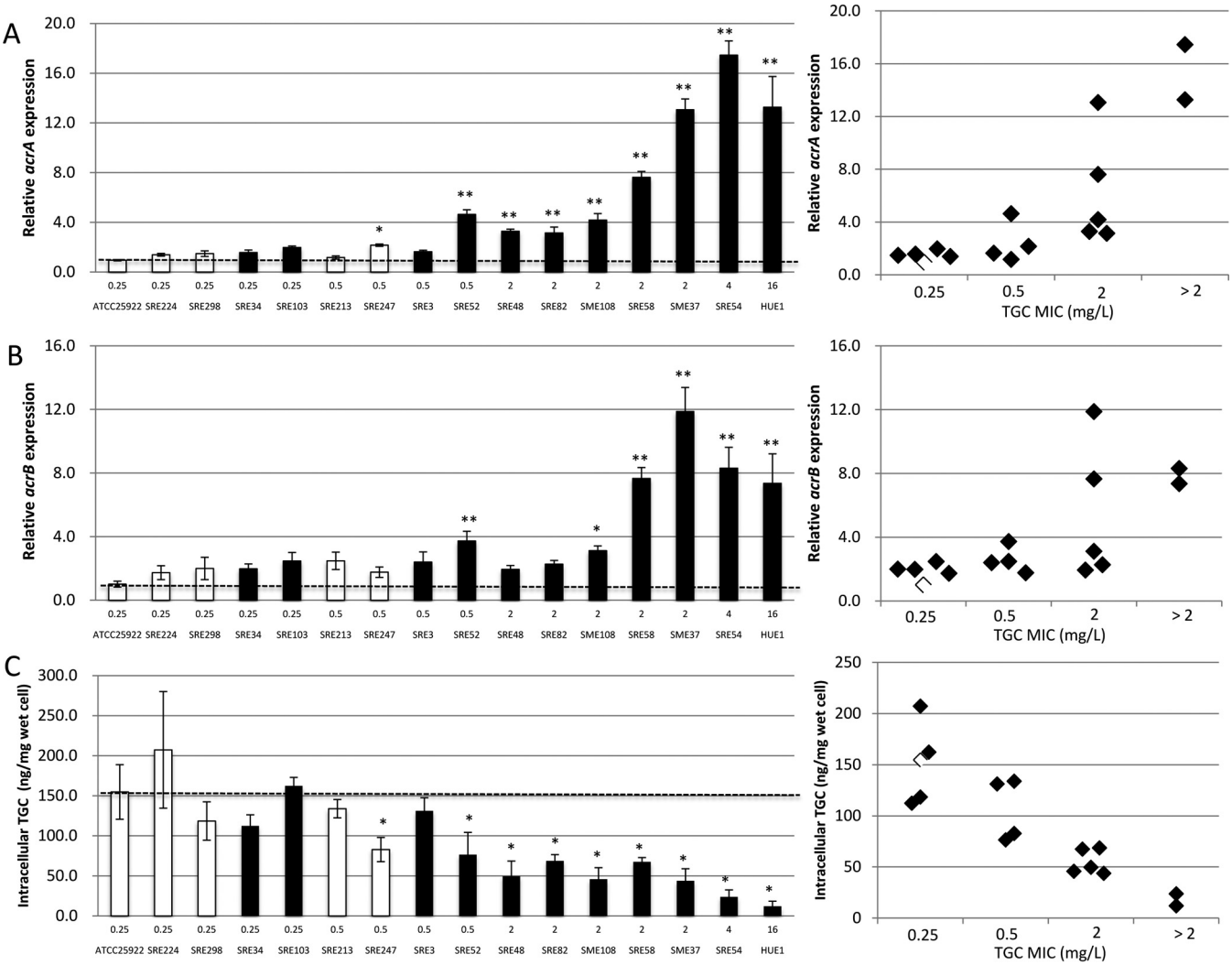

FIG 2 Expression levels of mRNA for *acrA* (A) and *acrB* (B), and intracellular TGC concentrations (C) in *E. coli* clinical isolates. (A and B) Expression of *acrA* (A) and *acrB* (B) mRNAs. Values on the *y* axis are shown as relative expression normalized against a reference strain, ATCC 25922 as 1. (C) Intracellular TGC concentrations. Values on the *x* axis represent the MIC for TGC (mg/liter). Left panels, results for individual strains. White bars represent fluoroquinolone-susceptible *E. coli* isolates (CIP MIC, <0.125 mg/liter), and black bars represent fluoroquinolone-resistant *E. coli* isolates (CIP MIC, >2 mg/liter). Dashed lines show the level for the reference strain, ATCC 25922. *, $P < 0.05$; **, $P < 0.01$ (compared with ATCC 25922). Error bars represent the standard deviation. Right panels, relationship between the measurements and the MIC for TGC (mg/liter). Empty diamond, value for the reference strain, ATCC 25922.

TGC concentrations and the MIC ($r^2 = 0.815$, $P < 0.001$ [Fig. 2C, right panel]). TGC-resistant isolates SRE54 and HUE1 had the lowest intracellular concentrations of TGC (23.8 ± 8.7 and 12.0 ± 6.4 ng/mg wet cells, respectively); however, addition of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (a protonophore that inhibits proton motive force-driven efflux pumps) increased the intracellular TGC concentration in these isolates (to 239.2 ± 25.2 and 265.7 ± 40.9 ng/mg wet cells, respectively). This level was similar to that in CCCP-treated ATCC 25922 (237.2 ± 20.0 ng/mg wet cells), indicating that the reduced intracellular TGC concentration was due to a proton-dependent mechanism, probably mediated by efflux pumps.

Overexpression of AcrAB in *E. coli* is typically caused by functional disruptions in the repressor genes, *acrR* or *marR*, due to mutations that cause amino acid substitutions or gene disruptions (28, 29). Dysfunction of MarR activates the *marRAB* operon and leads to overexpression of MarA, which activates the promoter of *acrAB* and leads to AcrAB overexpression (29, 30).

In this study, we identified several mutations (point mutations generating amino acid substitutions, nucleotides deletions, and insertions of insertion sequence element)

TABLE 2 Mutations in genes associated with CIP or TGC resistance in TGC-resistant/intermediate *E. coli* clinical isolates[a]

Strain	QRDR mutations	PMQR	Mutation(s)		*marA* expression (mean ± SD)[b]	MIC (mg/liter)	
			AcrR	MarR		CIP	TGC
HUE1	WT	***qnrS***[c]	**Disrupted**[c] **(219_220InsIS3-IS629)**	**Disrupted**[c] **(ΔC at 223)**	142.2 ± 9.0	4[c]	16
SRE54	**GyrA S83L, D87N; ParC S80I, E84V**	ND[f]	**Disrupted (273_274insIS1)**	**L71P (CTG→CCG)**[d], G103S, Y137H	506.7 ± 52.2	256	4
SME37	**GyrA S83L, D87N; ParC S80I, E84V**	ND	**Disrupted (570_571insIS1)**	**Disrupted (110_111insTATTTCCGCTGGA)**	57.5 ± 3.2	128	2
SRE58	**GyrA S83L, D87N; ParC S80I, E84V**	ND	**Disrupted (273_274insIS1)**	**K44Y(AAG→TAC)**[d], G103S, Y137H	50.8 ± 3.4	256	2
SME108	**GyrA S83L, D87N; ParC S80I, E84G**	ND	**H115Y**[e] **(CAC→TAC)**	A53E (GCG→GAG)[d], G103S, Y137H	0.4 ± 0.1	128	2
SRE82	**GyrA S83L, D87N; ParC; S80I, E84V**	ND	**Disrupted (Δ126_135)**	G103S, Y137H	2.0 ± 0.6	64	2
SRE48	**GyrA S83L, D87N, ParC S80I, E84V**	ND	**R13S (CGC→AGC)**[d]	**M1G (GTG→GGG)**[d], G103S, Y137H	20.8 ± 3.9	128	2

[a]Abbreviations: QRDR, quinolone resistance-determining region; PMQR, plasmid-mediated quinolone resistance gene; CIP, ciprofloxacin; TGC, tigecycline. Boldface indicates a contribution to the overexpression of *acrAB*.
[b]*marA* expression is shown as relative expression level normalized against reference strain ATCC 25922 as 1.
[c]Data were reported previously (31).
[d]Novel mutation found in this study.
[e]Mutation associated with overexpression of *acrA* or *acrB* in *E. coli* previously reported (28).
[f]ND, not detected.

in *acrR* and *marR* in TGC-nonsusceptible isolates (Table 2). AcrR was disrupted in five of the seven TGC-nonsusceptible isolates (HUE1, SRE54, SME37, SRE58, and SRE82) by insertion of an insertion sequence element(s) or nucleotide deletions. In HUE1 and SME37, MarR were also disrupted by the deletion of a nucleotide (HUE1) (31) and a nucleotides insertion (SME37). Expression levels of *marA* were extremely high in these strains. These results suggested that the TGC nonsusceptibility of HUE1 and SME37 was contributed by overexpression of AcrAB due to the double disruption of the repressors AcrR and MarR.

The other five TGC-nonsusceptible isolates, SRE54, SRE58, SME108, SRE82, and SRE48, possessed two or three amino acid substitutions in MarR (Table 2). Among them, G103S and Y137H do not influence the activation of the *marRAB* operon that leads to *acrAB* overexpression (29, 30). We identified four other novel amino acid substitutions (L71P, K44Y, A53E, and M1G) in MarR of these strains. Among them, *marA* levels were overexpressed in SRE54, SRE58, and SRE48, suggesting that L71P, K44Y, and M1G substitutions disrupt MarR function, which suppresses the transcription of the *marRAB* operon. In the previous study, K44A, which is a different substitution occurring at the same position in MarR of SRE58, activated the transcription of the *marRAB* operon and increased tetracycline and chloramphenicol MICs compared with those of wild-type (WT) MarR (32). Taking the results together, L71P, K44Y, and M1G substitutions in MarR were identified to play a role in enhancing AcrAB expression and lead to TGC nonsusceptibility in collaboration with the disruption of AcrR in SRE54 and SRE58 and perhaps also with the novel substitution R13S in AcrR of SRE48. In contrast, SME108, whose MarR contained A53E, expressed *marA* at a low level (Table 2). Thus, the A53E substitution in MarR of SME108 should not be associated with overexpression of AcrAB, whereas H115Y in AcrR contributed it, as previous described (28).

To address the contribution of AcrAB-TolC to TGC resistance, we next determined the MIC for TGC in *acrAB*-deficient mutants derived by Red/ET homologous recombination. These gene-deficient mutants showed a markedly lower MIC for TGC (Table 3), which was defined as TGC susceptible according to EUCAST. In addition, complementation of *acrAB* into the *acrAB*-deficient mutants recovered the TGC MICs of the parent strains (Table 3). Therefore, the TGC nonsusceptibility in *E. coli* is mainly due to the low intracellular concentration of TGC resulting from overexpression of AcrAB-TolC.

TABLE 3 TGC and CIP susceptibilities of mutants with defective efflux pump genes derived from TGC-resistant/intermediate *E. coli* clinical isolates

Strain	MIC (mg/liter)	
	CIP	TGC
SRE54	256	4
SRE54 Δ*acrAB*	4	0.25
SRE54-Δ*acrAB*/pHSG576	4	0.25
SRE54 Δ*acrAB*/pHSGacrAB	256	4
SME37	128	2
SME37 Δ*acrAB*	8	0.25
SME37 Δ*acrAB*/pHSG576	8	0.25
SME37Δ *acrAB*/pHSGacrAB	128	2
SRE58	256	2
SRE58 Δ*acrAB*	16	0.25
SRE58 Δ*acrAB*/pHSG576	16	0.25
SRE58 Δ*acrAB*/pHSGacrAB	256	2
SME108	128	2
SME108 Δ*acrAB*	8	0.25
SME108 Δ*acrAB*/pHSG576	8	0.25
SME108 Δ*acrAB*/pHSGacrAB	128	2
SRE82	128	2
SRE82 Δ*acrAB*	8	0.25
SRE82 Δ*acrAB*/pHSG576	8	0.25
SRE82 Δ*acrAB*/pHSGacrAB	128	2
SRE48	128	2
SRE48 Δ*acrAB*	8	0.25
SRE48 Δ*acrAB*/pHSG576	8	0.25
SRE48 Δ*acrAB*/pHSGacrAB	128	2
HUE1	4	16
HUE1 Δ*acrAB*	1	0.25
HUE1 Δ*acrAB*/pMW219	1	0.25
HUE1 Δ*acrAB*/pMWacrAB	4	8
HUE1 Δ*acrF*	2	2

Two TGC-resistant *E. coli* isolates, SRE54 and HUE1, showed similar levels of *acrA* and *acrB* expression but quite different MICs for TGC (4 mg/liter and 16 mg/liter), despite the finding that HUE1 had lower intracellular TGC concentrations. We previously reported that HUE1 (determined as O125:H37-ST48) (31) is a fluoroquinolone-resistant *E. coli* strain that does not have any mutations in the QRDRs of *gyrA*, *gyrB*, *parC*, and *parE* (23). We previously showed that HUE1 exhibited fluoroquinolone resistance by acquiring plasmid-mediated quinolone resistance genes, *qnrS and oqxAB*, and overexpressing chromosomal efflux pump genes (including *acrAB*, *acrEF*, and *tolC*) (31). Thus, we anticipated that the high MIC for TGC exhibited by HUE1 was caused by overexpression of additional efflux pumps. A defect in *acrF* reduced the MIC for TGC (by 8-fold compared with that in HUE1 [Table 3]), suggesting that the high-level TGC resistance of HUE1 was caused by cooperation between overexpressed efflux pumps AcrAB-TolC and AcrEF(-TolC).

The TGC nonsusceptibility of several strains cannot be fully explained by overexpression of *acrAB*. SRE52 exhibited a lower TGC MIC (0.5 mg/liter) than the breakpoint; however, the *acrA* or *acrB* expression and the intracellular TGC concentration were at levels similar to those for some TGC-nonsusceptible isolates (SRE48, SRE82, and SME108, whose MICs were 2 mg/liter) (Fig. 2). Among five TGC-nonsusceptible isolates (TGC MIC, 2 mg/liter), SRE48, SRE82, and SME108 exhibited lower *acrA* and *acrB* expression levels than SRE58 and SRE37. These strains had similar intracellular TGC concentrations (Fig. 2). A recent study revealed that the reduced TGC MICs of *E. coli* mutants selected on TGC-supplemented agar were likely because of mutations in genes participating in the lipopolysaccharide core biosynthesis pathway, especially *lpcA*, *rfaE*, *rfaD*, *rfaC*, and *rfaF* (19). Thus, these mutations may alter outer membrane composition and physiology and affect the uptake of TGC. Other, unknown mechanisms could also be involved. Whether mechanisms other than *acrAB* overexpression contribute to TGC nonsusceptibility in these clinical isolates is a subject for further study.

***In vitro* development of TGC resistance by CIP exposure.** We examined whether the acquisition of the TGC-nonsusceptible phenotype was caused by the development of fluoroquinolone resistance. Eight CIP-susceptible *E. coli* isolates were exposed to CIP at successively increasing concentrations, and their CIP and TGC MICs were determined. All eight *E. coli* clinical isolates increased the CIP MIC during CIP exposure, accompanied a by mutation(s) in *acrR*, *marR*, and/or the QRDR of *gyrA* (no mutations were observed in other QRDRs of *gyrB*, *parC*, and *parE*). Finally, two isolates (SME207 and SME212) acquired mutations in both *acrR*/*marR* and *gyrA*, four isolates (ATCC 25922, SME21, SME183, and SME179) acquired mutations in *acrR*/*marR*, and two isolates (SME19 and SME65) acquired mutations only in *gyrA* (Table 4). The increase of AcrAB expression caused by the amino acid substitutions in AcrR and MarR described above enhanced the fluoroquinolone MIC (32–34), concurrent with the amino acid substitution(s) at S83 and/or D87 in GyrA (21). These results confirmed that these mutations were required for development of fluoroquinolone resistance.

The TGC MIC increased in six (ATCC 25922, SME21, SME207, SME183, SME179, and SME212) of the eight *E. coli* isolates (Table 4). These six isolates acquired a mutation(s) in *acrR* and/or *marR*. Other two isolates (SME19 and SME65) that had no mutation in *acrR* and *marR* did not have an altered TGC MIC, while the CIP MIC was increased because of a QRDR mutation(s) in GyrA. The results indicated that the increase of the TGC MIC induced by CIP was caused by mutations in *acrR* and *marR*. SME179 acquired the TGC-resistant phenotype (MIC, 4 mg/liter) in four steps of CIP exposure with increasing the concentrations, and a T5N substitution in AcrR and an R86W substitution in MarR occurred (SME179CIP1). SME212 conferred TGC resistance in an eighth step, and 4 nucleotides (nucleotide positions 211 to 214 were deleted in *marR* [SME212CIP2]). During these steps, the two strains exhibited a marked increase of *acrA* and *acrB* expression (Fig. 3). These results revealed that CIP exposure caused reduction of CIP susceptibility in two ways, namely, by QRDR mutations and AcrAB overexpression, and TGC-resistant *E. coli* were generated from clinical isolates by the latter mechanism. Notably, acquisition of the TGC-resistant phenotype of SME179 and SME212 was achieved when they exceeded the CIP breakpoint (the CIP MIC was >1 mg/liter in SME179CIP1 and SME212CIP2 [Table 4]). The finding that CIP exposure selects TGC-resistant strains with AcrAB overexpression mediated by AcrR and/or MarR mutations, accompanying the acquisition of CIP resistance, agrees with our observation that TGC-nonsusceptible *E. coli* clinical isolates were found exclusively in fluoroquinolone-resistant populations (Fig. 1). Thus, we conclude that the emergence of TGC-nonsusceptible *E. coli* isolates was associated with the development of fluoroquinolone resistance. We identified that one of the strains that acquired TGC resistance *in vitro*, SME179, belonged to ST58. Some ST58 strains produce CTX-M and are found in healthy individuals, clinical patients, and animals worldwide (35–37). It should be taken into account that pathogenic and multidrug linages other than O25b:H4-ST131-*H*30R and O1-ST648 also have an ability to develop TGC resistance.

During the development of TGC resistance, a mutant derived from SME212 (SME212CIP0.03) exhibited slightly (2-fold) increased TGC MICs with no mutations in *acrR* or *marR*. The mechanisms underlying these slight reductions of TGC susceptibility are unclear. The genes *soxSR*, *rob*, and *acrS* are also regulatory genes of *acrAB* (29, 38, 39), and mutations in genes participating in the lipopolysaccharide core biosynthesis pathway (19), as described above, might be involved in the slight reductions of TGC susceptibility we observed.

A mutant derived from *E. coli* ATCC 25922 (ATCC 25922 CIP0.03) exhibited a 4-fold increase in TGC MIC after three steps of CIP exposure. This mutant spontaneously acquired a point mutation (ACC→AAC) that substituted threonine for asparagine at amino acid position 5 in *acrR* and a frameshift (a nucleotide deletion of guanine at nucleotide position 311) in *marR* (Table 4). It has been known that fluoroquinolone exposure induces the SOS response and upregulates a low-fidelity replication polymerase that increases the rate of spontaneous mutations from 10^{-9} to 10^{-5} mutations per

TABLE 4 CIP and TGC MICs and mutations in *acrR*, *marR*, and QRDR of mutants with reduced susceptibility to TGC and CIP selected by *in vitro* CIP exposure[a]

Strain	CIP concn in LB broth (mg/liter)	MIC (mg/liter)		Mutations[b]		
		CIP	TGC	AcrR	MarR	QRDR[a] mutations
ATCC 25922	0	0.015	0.25	WT	WT	WT
ATCC 25922CIP0.008	0.008	0.015	0.25	NT	NT	NT
ATCC 25922CIP0.01	0.015	0.015	0.25	WT	WT	WT
ATCC 25922CIP0.03	0.03	0.125	1	**T5N[c] (ACC→AAC)**	**Disrupted (ΔG at 311)**	WT
SME21	0	0.008	0.25	WT	WT	WT
SME21CIP0.008	0.008	0.008	0.25	NT	NT	NT
SME21CIP0.01	0.01	0.01	0.5	NT	NT	NT
SME21CIP0.03	0.03	0.03	0.5	WT	**Disrupted (Q117stop, CAG→TAG)**	WT
SME21CIP0.06	0.125	0.125	1	**Disrupted (339_340ins)[d]**	**Disrupted (Q117stop, CAG→TAG)**	WT
SME207	0	0.01	0.25	WT	V79I, G103S, Y137H	WT
SME207CIP0.01	0.008	0.01	0.25	NT	NT	NT
SME207CIP0.03	0.01	0.03	0.25	NT	NT	NT
SME207CIP0.06	0.03	0.03	0.25	NT	NT	NT
SME207CIP0.125	0.06	0.06	0.25	NT	NT	NT
SME207CIP0.25	0.125	0.25	0.25	NT	NT	NT
SME207CIP0.5	0.25	0.5	0.25	NT	NT	NT
SME207CIP1	0.5	0.5	0.5	**I62L[c] (ATC→CTC)**	**A70T[c] (GCA→ACA)**, V79I, G103S, Y137H	GyrA ΔS83 (Δ247_249)
SME183[e]	0	1	0.5	WT	G103S, Y137H	WT
SME183CIP0.5	0.5	1	0.5	NT	NT	NT
SME183CIP1	1	2	1	**Disrupted (488_489insTCGGC)**	G103S, Y137H	WT
SME19	0	0.01	0.25	WT	S3N, G103S, Y137H	WT
SME19CIP0.01	0.01	0.01	0.25	NT	NT	NT
SME19CIP0.03	0.03	0.03	0.25	NT	NT	NT
SME19CIP0.06	0.06	0.06	0.25	NT	NT	NT
SME19CIP0.125	0.125	0.5	0.25	NT	NT	NT
SME19CIP1	1	1	0.25	NT	NT	NT
SME19CIP2	2	4	0.25	NT	NT	NT
SME19CIP4	4	4	0.25	WT	S3N, G103S, Y137H	GyrA S83L-D87Y
SME65	0	0.06	1	WT	**G69E (GGG→GAG)[c]**, G103S	WT
SME65CIP0.03	0.03	1	1	NT	NT	NT
SME65CIP0.06	0.06	1	1	NT	NT	NT
SME65CIP0.125	0.125	1	1	NT	NT	NT
SME65CIP0.25	0.25	1	1	NT	NT	NT
SME65CIP0.5	0.5	1	1	NT	NT	NT
SME65CIP1	1	1	1	WT	**G69E (GGG→GAG)[c]**, G103S	GyrA S83L
SME179[e]	0	0.25	0.5	WT	G103S, Y137H	WT
SME179CIP0.125	0.125	0.25	0.5	NT	NT	NT
SME179CIP0.25	0.25	0.25	0.5	NT	NT	NT
SME179CIP0.5	0.5	1	1	WT	**R86W (AGG→TGG)[c]**, G103S, Y137H	WT
SME179CIP1	1	2	4	**T5N[c] (ACC→AAC)**	**R86W (AGG→TGG)[c]**, G103S, Y137H	WT
SME212	0	0.03	0.5	WT	G103S	WT
SME212CIP0.01	0.01	0.03	0.5	NT	NT	NT
SME212CIP0.03	0.03	0.06	1	NT	NT	NT
SME212CIP0.06	0.06	0.125	1	WT	G103S	GyrA S83P
SME212CIP0.125	0.125	0.125	1	NT	NT	NT
SME212CIP0.25	0.25	1	1	NT	NT	NT
SME212CIP0.5	0.5	2	1	NT	NT	NT
SME212CIP1	1	2	1	WT	G103S	GyrA S83P-D87G
SME212CIP2	2	4	4	WT	**Disrupted (Δ211_214)**	GyrA S83P-D87G

[a]QRDR, quinolone resistance-determining region; NT, not tested.
[b]Boldface indicates a contribution to the overexpression of *acrAB*.
[c]Mutation associated with overexpression of *acrA* or *acrB* previously reported (32–34). The S3N, V79I, G103S, and Y137H mutations in MarR do not contribute to overexpression of *acrA* or *acrB* (29, 30 33).
[d]Insertion of transposase and part of a mobile element protein gene (nucleotide positions 154 to 504) found in *Citrobacter freundii* strain P10159 (accession number CP012554.1).
[e]Positive for *qnrS*.

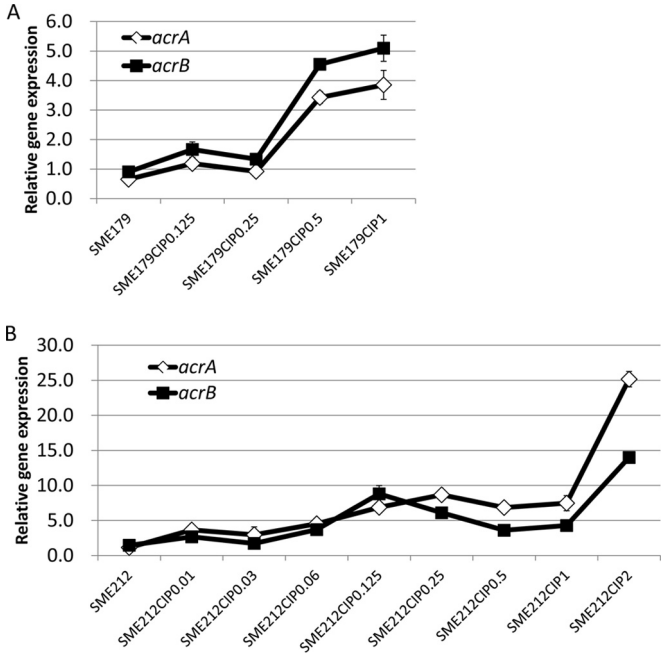

FIG 3 Expression levels of *acrA* and *acrB* mRNAs in mutants derived from strains SE179 (A) and SME212 (B) with reduced TGC susceptibility caused by exposure to CIP. The expression of *acrA* and *acrB* mRNA in mutants derived from SME179 and SME212 after continuous stepwise CIP exposure in LB broth is shown. Values on the *y* axis are shown as relative expression normalized against a reference strain, ATCC 25922 as 1. The CIP and TGC MICs and genetic analyses for these mutants are shown in Table 4.

base pair (40). This suggests that multiple spontaneous mutations may take place in an *E. coli* cell exposed to fluoroquinolone. Although we need to confirm that this phenomenon actually occurs *in vivo*, it deserves attention because this may contribute to the rapid development of TGC nonsusceptibility during fluoroquinolone treatment.

Conclusion. First, we showed that several strains of certain populations of fluoroquinolone-resistant *E. coli*, including major extraintestinal pathogenic lineages O25b:H4-ST131-*H*30R and O1-ST648, show reduced susceptibility to TGC (1.0% and 3.6% of fluoroquinolone-resistant *E. coli* isolates were resistant or nonsusceptible to TGC, respectively). Considering the frequency of fluoroquinolone resistance (for example, the fluoroquinolone resistance rate in *E. coli* was 23.4% in our previous study [4]), approximately 0.8% or fewer of *E. coli* clinical isolates could be nonsusceptible or resistant to TGC. Indeed, previous large-scale surveillance studies indicated that fewer than 0.3% of the *E. coli* clinical isolates were nonsusceptible to TGC (10, 11). Thus, although detailed studies of TGC nonsusceptibility in *E. coli* clinical isolates have not been performed previously, our results are consistent with clinical observations. Importantly, our data suggest that TGC nonsusceptibility is closely associated with the acquisition of fluoroquinolone resistance. The mechanism responsible for the reduced TGC susceptibility involved overexpression of an efflux pump, AcrAB-TolC, which deceases intracellular concentrations of TGC. Indeed, AcrAB-TolC was overexpressed upon exposure to fluoroquinolones. Notably, the wide-spread lineage O25b:H4-ST131-*H*30R, which is resistant to a number of antibiotics, including fluoroquinolones, was found to be resistant or nonsusceptible to TGC. Even as fluoroquinolone-resistant strains sharing QRDR mutations acquire higher fluoroquinolone resistance because of the overexpression of AcrAB-TolC (41) during exposure to insufficient concentrations of fluoroquinolones, TGC-nonsusceptible *E. coli* could be generated without using TGC. Therefore, clinicians should pay careful attention to the possible development of TGC resistance along with fluoroquinolone resistance in order to preserve TGC as an effective last-line-drug for the treatment of multidrug-resistant bacterial infections.

TABLE 5 Primer sequences used for RT-PCR

Target gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Product size (bp)
acrA	CTCTCAGGCAGCTTAGCCCTAA	TGCAGAGGTTCAGTTTTGACTGTT	107
acrB	GGTCGATTCCGTTCTCCGTTA	CTACCTGGAAGTAAACGTCATTGGT	105
marA	AATCGCGCAAAAGCTGAAGG	ATGCGGCGGAACATCAAAGT	121
rrsE	GTACACACCGCCCGTCACAC	GTGATCCAACCGCAGGTTCC	144
gyrB	CGATATTCGCCGCTTTCAGG	CGTCGTATGCTGCGCGTTAC	130

MATERIALS AND METHODS

Bacterial isolates. Two hundred seventy-seven fluoroquinolone-susceptible *E. coli* isolates (ciprofloxacin [CIP] MIC, <0.125 mg/liter) and 194 fluoroquinolone-resistant *E. coli* isolates (CIP MIC, >2 mg/liter) were isolated from human clinical specimens. Of these, 100 fluoroquinolone-susceptible *E. coli* isolates and 118 fluoroquinolone-resistant *E. coli* isolates (six strains were added for this study) were from human specimens collected in 2008 and 2009, as described previously (4). One hundred seventy-seven fluoroquinolone-susceptible *E. coli* isolates and 76 fluoroquinolone-resistant *E. coli* isolates were from human clinical specimens collected in 2015 and 2016 in Japan (Sapporo Medical University Hospital, Sapporo, Japan) and stored in the laboratory. None of the patients from whom the *E. coli* isolates were isolated had a history of TGC treatment.

Susceptibility testing and genetic analysis. TGC was purchased from AdooQ BioScience (Irvine, CA). Susceptibility to TGC was tested using the agar plate dilution method according to guidelines of the Clinical and Laboratory Standards Institute (CLSI) (42). *E. coli* ATCC 25922 was used as a reference. The MIC values for CIP, cefpodoxime, cefepime, and gentamicin were obtained from previous studies of *E. coli* isolates from 2008 and 2009 (4, 6, 43). MICs for ceftazidime (GSK Japan, Tokyo, Japan), imipenem (MSD, Tokyo, Japan), amikacin (Wako Pure Chemical, Osaka, Japan), and fosfomycin (Meiji Seika Pharma, Tokyo, Japan) were also determined using the agar plate dilution method. MIC results were interpreted according to the EUCAST breakpoint table (version 5) (44). O (45) and H (46) serotyping and multilocus sequence typing (47) were done as described previously. For identification of the *H*30Rx subclone of O25b:H4-ST131, *H*30 was determined by PCR using a specific primer set (48), R was determined according to the CIP MIC, and x was determined by detecting single-nucleotide polymorphisms (SNPs) using direct sequencing as previously described (5). Plasmid-mediated quinolone resistance genes *qnrA*, *qnrB*, *qnrS*, *aac*(*6′*)*-Ib-cr*, *qepA*, and *oqxAB* were detected by PCR as described previously (31, 49–51). The nucleotide sequences of *acrR* and *marR* were determined according to published protocols (31, 33) and compared with those of *E. coli* strain K-12 substrain MG1655, (GenBank accession number U00096), as a reference strain.

Real-time reverse transcription-PCR (RT-PCR). Overnight cultures were diluted 1:100 in LB broth and grown to mid-logarithmic phase. RNA was isolated using an RNeasy Plus minikit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The concentration of RNA was measured spectrophotometrically (Infinite M200 Pro; Tecan, Kawasaki, Japan), and 0.5 μg was used to synthesize cDNA using the ReverTra Ace qPCR RT master mix with genomic DNA (gDNA) remover (Toyobo, Tokyo, Japan). The expression of genes *acrA*, *acrB*, and *marA* was estimated using QuantiFast SYBR green PCR Mastermix (Qiagen) and the primer pairs shown in Table 5. The PCR cycling conditions were as follows: initial activation at 95°C for 5 min, followed by 40 cycles at 95°C for 10 s and 60°C for 30 s. Reactions were performed in a LightCycler 480 II (Roche, Mannheim, Germany). *E. coli* strain ATCC 25922 was used as a control. Endogenous reference genes *rrsE* and *gyrB* were used to normalize expression ratios. Data were calibrated against the expression levels of ATCC 25922 at baseline, and fold changes in expression were calculated using the comparative threshold cycle (C_T) method. Data were expressed as the mean ± standard deviation from three independent experiments.

Accumulation assays. Intracellular TGC concentrations were measured in a fluorometric uptake assay (31), with slight modifications. Briefly, 50 mg of wet cells suspended in 5 ml of 0.05 M phosphate buffer (pH 7.0) on ice were incubated for 10 min at 37°C. TGC was then added to each sample (final concentration, 50 mg/liter) and incubated for 5 min at 37°C. Next, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (Sigma-Aldrich, St. Louis, MO) (final concentration, 150 μM) or dimethyl sulfoxide was added and incubated for 5 min at 37°C. The cells were washed three times with cold phosphate buffer, and 1 ml of 0.1 M glycine-HCl (pH 3.0) was added to extract intracellular TGC. After an overnight incubation at room temperature, the solution was centrifuged at 10,000 × *g* for 10 min. The supernatant was collected, and the fluorescence of the TGC was measured at excitation and emission wavelengths of 355 and 830 nm, respectively, using an Infinite M1000 Pro fluorescence spectrophotometer (Tecan). Data were expressed as the mean ± standard deviation from at least three independent experiments.

Construction of *acrAB*-deficient mutants and *acrAB* expression vectors. HUE1 mutants deficient in *acrAB* and *acrF* were prepared as described previously (31). Other *acrAB*-deficient mutants were generated by homologous Red/ET recombination (52) using pKD46-Hyg that was ligated with a hygromycin resistance cassette (Gene Bridges GmbH, Heidelberg, Germany) into the PvuI-digested site of pKD46 (purchased from The *E. coli* Genetic Stock Center, Yale University, New Haven, CT). The hygromycin resistance cassette was amplified by PCR using the primers pKD46Hyg-F1-2 (5′-AGCTCCTTCGGT CCTCCGATATTCTACCGGGTAGGGGAGGCGC-3′) and pKD46-HygR2 (5′-ACTTCTGACAACGATCTACTACTAT

TCCTTTGCCCTCGGAC-3′). The *acrAB* genes were replaced with minigenes containing the kanamycin resistance cassette (Gene Bridges GmbH) and 50 nucleotides corresponding to the upstream and downstream regions of the target gene. These were amplified by PCR using the primers acrARed-F (5′-TTAACTTTTGACCATTGACCAATTTGAAATCGGACACTCGAGGTTTACATAATAATTAACCCTCACTAAAGG GCG-3′) and acrBRed-R (5′-GTTATGCATAAAAAAGGCCGCTTACGCGGCCTTAGTGATTACACGTTGTATAATAC GACTCACTATAGGGCTC-3′) to disrupt the *acrA-acrB* open reading frame.

For complementation of *acrAB* into the *acrAB* deletion mutants, *acrAB* expression vectors were constructed using the low-copy-number plasmids pHSG576 (obtained from National BioResource Project, Mishima, Japan) and pMW219 (Wako Pure Chemical). These contained the nucleotide sequences from bp −188 upstream to +545 downstream of *acrAB* (named pHSGacrAB or pMWacrAB). Amplification of *acrA* was performed by PCR using forward primers acrApHSG-cloF (5′-GCCAGTGAATTCGATGTGTTGGCGCGT TTCTTGCG-3′) or acrApMW-cloF (5′-GGATCCTCTAGAGATGTGTTGGCGCGTTTCTTGCG-3′), corresponding to each plasmid, and reverse primer acrA-cloR (5′-GTCTTAACGGCTCCTGTTTAAGTTAAGACTTGGAC-3′). To amplify *acrB*, forward primer acrB-cloF (5′-CTTAAACAGGAGCCGTTAAGACATGCCTAATTTC-3′) and reverse primer acrB-cloR (5′-TGATTACGCCAAGCTTAACGCGTCCCCTTCTTAGCGGTTGAACTAAC-3′) were used. These two fragments were ligated into the EcoRI and HindIII sites of pHSG576 or the XbaI- and HindIII-digested site of pMW219 using NEBuilder HiFi DNA Assembly master mix (New England BioLabs Japan, Tokyo, Japan).

Selection of TGC-resistant mutants by multistep CIP exposure *in vitro*. Selection of CIP-resistant mutants was performed using LB broth containing several concentrations of CIP according to a published protocol with slight modifications (53). Briefly, parent TGC-susceptible strains were grown in LB broth overnight at 37°C. Each 10 μl (approximately 1×10^7 CFU) of cell culture was inoculated into 1 ml of LB broth (1:100, vol/vol) containing CIP and incubated overnight at 37°C. The CIP concentrations were sub-MIC for the parent strains. After overnight culture, 10 μl of the cell suspension was subjected to the second step of CIP exposure (the CIP concentration was 1× the MIC for the parent strains). With each passage, three colonies were isolated per condition and subcultured on LB agar. These steps were repeated with increasing 2-fold-higher CIP concentrations until the cells did not grow in CIP-containing broth or the mutants acquired TGC resistance (TGC MIC, 4 μg/ml).

Statistical analysis. Statistical significance was determined using the Student *t* test. A *P* value of ≤0.05 was considered significant.

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