Combination of known and unknown mechanisms confers high-level resistance to fluoroquinolones in *Enterococcus faecium*

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In order to elucidate the mechanisms of fluoroquinolone resistance in *Enterococcus faecium*, spontaneous mutants isolated from *Ent. faecium* ATCC 19434 by stepwise selection with sparfloxacin (SPX) or norfloxacin (NOR) and 13 clinical isolates of *Ent. faecium* were characterized by analysing quinolone-resistance-determining regions (QRDRs) of the *gyrA*, *gyrB*, *parC* and *parE* genes and examining changes in MICs of SPX and NOR in the presence of efflux pump inhibitors. The SPX-selected first-step mutant had a point mutation only in *gyrA*, and the mutants QR7-18 and QR7-39, and clinical isolates that had point mutations in *parC*, showed NOR resistance. These results indicate that the primary targets of SPX and NOR are DNA gyrase and topoisomerase IV, respectively, and therefore that the primary target of fluoroquinolones in *Ent. faecium* differs depending on the structure of the compound used. The characterization of the spontaneous mutants and the clinical isolates demonstrates that in addition to the previously reported alterations in GyrA and ParC, an alteration in GyrB, a NorA-like pump, an unknown efflux pump, which excretes both SPX and NOR from bacterial cells, and probably other unknown mechanism(s) all contribute to fluoroquinolone resistance in *Ent. faecium*.

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INTRODUCTION

Enterococci are important nosocomial pathogens, capable of causing serious life-threatening infections, such as bacteraemia, endocarditis and meningitis (Moellering, 1992). In particular, infections caused by *Enterococcus faecium* have been on the rise over the past few years (Low *et al.*, 2001; Treitman *et al.*, 2005). *Ent. faecium* is equipped with a variety of antibiotic-resistance genes, some of which are inherent, while others are acquired. Since many clinical isolates of *Ent. faecium* are resistant to the majority of antibacterial agents, including vancomycin and fluoroquinolones, therapeutic options for *Ent. faecium* infections are very limited.

Fluoroquinolone resistance is mediated by alteration of the target enzymes, i.e. DNA gyrase (a heterotetramer in the form A₂B₂) and topoisomerase IV (a heterotetramer in the form C₂E₂), and/or by decreased intracellular accumulation

of fluoroquinolones (Piddock, 1999). It has already been demonstrated that fluoroquinolone resistance in *Enterococcus faecalis* and *Ent. faecium* is associated with mutations in the quinolone-resistance-determining regions (QRDRs) (Yoshida *et al.*, 1990a, 1991) of the *gyrA* and/or *parC* genes (Amin *et al.*, 1999; Brisse *et al.*, 1999; Kanematsu *et al.*, 1998; Korten *et al.*, 1994; Tankovic *et al.*, 1996). High-level fluoroquinolone-resistant enterococci have been shown to have amino acid substitutions in GyrA and/or ParC, while no amino acid change has been detected in either GyrA or ParC in the low-level resistant strains (Brisse *et al.*, 1999). Although mutations in other subunit genes in DNA gyrase and topoisomerase IV (i.e. *gyrB* and *parE*) have been reported to contribute to the fluoroquinolone resistance of some bacteria, such as *Escherichia coli*, *Staphylococcus aureus* and *Streptococcus pneumoniae* (Piddock, 1999), no report has investigated the association between these mutations and fluoroquinolone resistance in enterococci.

Interestingly, in Gram-negative bacteria such as *E. coli*, a strain must have a mutation that renders DNA gyrase resistant in order to acquire a subsequent mutation in topoisomerase IV genes, since the primary target of fluoroquinolones in *E. coli*

Abbreviations: CCCP, carbonyl cyanide 3-chlorophenylhydrazone; CIP, ciprofloxacin; EtBr, ethidium bromide; MDR, multidrug resistance efflux pump; NOR, norfloxacin; QRDR, quinolone-resistance-determining region; SPX, sparfloxacin.

is DNA gyrase (Heisig, 1996). On the other hand, it has been demonstrated that in some Gram-positive organisms, the hierarchy is reversed, and topoisomerase IV is the primary target (Ferrero *et al.*, 1995; Pan *et al.*, 1996). Other studies have shown that the primary target depends on the type of fluoroquinolone (Takenouchi *et al.*, 1995; Morrissey & George, 1999; Pan *et al.*, 2001).

Efflux is the major cause of decreased intracellular accumulation of fluoroquinolones. For example, in the case of *Staph. aureus*, the efflux pump involved in fluoroquinolone resistance is NorA (Ng *et al.*, 1994; Yoshida *et al.*, 1990b), while for *Strep. pneumoniae*, the pump involved is PmrA (Gill *et al.*, 1999). Although a multidrug efflux pump (EmeA), which shows 32 % identity to NorA, has been shown to be associated with *Ent. faecalis* resistance to fluoroquinolones (Jonas *et al.*, 2001), no information has been provided on the mechanism of *Ent. faecium* resistance to fluoroquinolones.

In order to elucidate the mechanisms of fluoroquinolone resistance in *Ent. faecium*, we isolated, in this study, spontaneous fluoroquinolone-resistant mutants from *Ent. faecium* ATCC 19434 by stepwise selection with sparfloxacin (SPX), a hydrophobic fluoroquinolone, and norfloxacin (NOR), a hydrophilic fluoroquinolone, and characterized the mutants by analysing point mutations in QRDRs of the *gyrA*, *gyrB*, *parC* and *parE* genes, and by comparing MICs of SPX and NOR between the mutants with or without known multidrug efflux pump inhibitors. Furthermore, we characterized 13 clinical isolates of *Ent. faecium* to examine the extent to which the mechanism(s) of fluoroquinolone resistance found in the mutants isolated *in vitro* is similar to that in the clinical isolates.

METHODS

Bacterial strains and culture conditions. *Ent. faecium* ATCC 19434 was obtained from the American Type Culture Collection (ATCC). The clinical isolates used in this study were collected between 1994 and 1996, identified by an API 20 Strep system (API

system) and maintained at Kitasato University. All *Ent. faecium* strains were grown aerobically at 37 °C in brain heart infusion broth (Difco Laboratories).

Chemicals and antibacterial agents. SPX and NOR were synthesized at our Chemistry Research Laboratories. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP), ethidium bromide (EtBr), verapamil, lansoprazole and reserpine were purchased from Sigma-Aldrich Chemical Co. Other reagents were purchased from Nacalai Tesque, unless otherwise indicated.

Determination of MIC. MIC was determined by an agar dilution method with brain heart infusion agar, as recommended by the National Committee for Clinical Laboratory Standards (2003) (now renamed Clinical and Laboratory Standards Institute).

Selection of fluoroquinolone-resistant mutants. Approximately 10¹⁰ c.f.u. of *Ent. faecium* ATCC 19434 was spread onto brain heart infusion agar containing SPX or NOR at a concentration four times that of the MIC and incubated aerobically at 37 °C for 48 h. Second-step mutants were selected in a similar manner from the first-step mutants. All the mutants were derived from selection using a single bacterial culture.

Preparation of total DNA. *Ent. faecium* total DNA was prepared as described by Hudson & Curtiss (1990).

PCR amplification and DNA sequence analysis. DNA fragments containing QRDRs of the *gyrA*, *gyrB*, *parC* and *parE* genes were amplified by PCR with the primers shown in Table 1. PCR was performed with a GeneAmp PCR System 9600 using TAKARA Ex Taq polymerase (Takara Shuzo). The reactions were repeated for 30 cycles (15 s at 94 °C for denaturation, 30 s at 55 °C for annealing, and 1 min at 72 °C for polymerization). PCR-amplified fragments were sequenced by the cycle sequencing method using an ABI Prism Big Dye Terminator version 3.1 Cycle Sequencing kit and then applied to a Genetic analyser 3100 (Applied Biosystems).

Accumulation assays. Accumulation assays were carried out essentially as described by Celesk & Robillard (1989). The amount of accumulated fluoroquinolones was measured by an agar well diffusion bioassay with *E. coli* KP as assay organism (Gonzalez *et al.*, 1985).

Table 1. Primers used for PCR and sequencing

Name	Sequence (5'–3')	Location
EFFGA-1*	CGGCGGCACCGTCACCGTCAACAG	bp 139–162 of <i>gyrA</i>
EFFGA-2*	GAATTGGGTGTGACACCGGATAAAG	bp 558–579 of <i>gyrA</i>
EFFPC-1*	TTCCCGTGCAATTCGATCAGTACTTC	bp 185–204 of <i>parC</i>
EFFPC-2*	CGTATGACAAAGGATTCCGTAAATC	bp 554–573 of <i>parC</i>
EFFGB-1†	AGGAAAATTAGCGGACTGCTCT	bp 1242–1263 of <i>gyrB</i>
EFFGB-2†	GACATCGGCATCAGTCATGA	bp 1511–1530 of <i>gyrB</i>
EFFPE-1‡	CCGTAAAGCAATCAAAGCCAG	bp 1086–1106 of <i>parE</i>
EFFPE-2‡	CGCATCGGTCATGATGATTAC	bp 1450–1470 of <i>parE</i>

*Brisse *et al.* (1999).
†RefSeq accession number ZP_00286242.
‡RefSeq accession number ZP_00285124.

RESULTS AND DISCUSSION

Isolation of fluoroquinolone-resistant mutants of *Ent. faecium*

In order to examine the mechanisms of fluoroquinolone resistance in *Ent. faecium*, a variety of spontaneous fluoroquinolone-resistant mutants were isolated from *Ent. faecium* ATCC 19434 with SPX, a hydrophobic fluoroquinolone, and NOR, a hydrophilic fluoroquinolone (Fig. 1). When $2 \times$ MIC of SPX and NOR was used, many colonies were obtained, but some were not fluoroquinolone resistant, suggesting that $2 \times$ MIC was too low a concentration to isolate fluoroquinolone-resistant mutants. Therefore, $4 \times$ MIC was used for isolation of the mutants. As shown in Table 2, first-step mutants were obtained at a frequency of 4.2×10^{-8} , regardless of the selection agent. When second-step mutants were selected from the first-step mutants with the same fluoroquinolone that was used for the selection of the first-step mutants, mutants were obtained at a frequency of 1.6×10^{-7} with SPX and 1.2×10^{-6} with NOR. However, the second-step mutants derived from SPX-selected first-step mutants with NOR selection were obtained at a frequency as low as 9.0×10^{-9} , and SPX selection from NOR-selected first-step mutants was not successful. The reason for this difference in second-step mutant frequency is unclear.

Characterization of first- and second-step mutants

To characterize the first- and second-step mutants, the association between mutations in the QRDRs of *gyrA*, *gyrB*, *parC* and *parE* genes and susceptibility to fluoroquinolones was examined. At each selection step, MICs of NOR and SPX for 60 randomly selected isolates were determined, and three mutants selected from the isolates that showed similar MIC patterns were subjected to QRDR analysis. Since the three selected mutants had the same amino acid substitution(s) in QRDR(s) in all cases, one of the mutants was selected as a representative strain (Fig. 1). The characteristics of the

Table 2. Mutant frequency by stepwise selection at four-times fluoroquinolone MIC

ND, Not detected.

Selection agent		Mutant frequency	
First step	Second step	First-step mutant	Second-step mutant
SPX	SPX	4.2×10^{-8}	1.6×10^{-7}
	NOR		9.0×10^{-9}
NOR	SPX	4.2×10^{-8}	ND
	NOR		1.2×10^{-6}

mutants are summarized in Table 3. SPX-selected first-step mutant QR7 was four times more resistant to SPX than the parent strain, *Ent. faecium* ATCC 19434, but was as susceptible to NOR as the parent strain. Sequencing analysis of QRDRs revealed that strain QR7 had a Glu-87 (GAG) to Lys (AAG) substitution in *GyrA*, but no substitution in the QRDRs of *GyrB*, *ParC* or *ParE*. The amino acid change at position 87 of *GyrA* has already been reported in fluoroquinolone-resistant *Ent. faecium* and *Ent. faecalis* (Amin *et al.*, 1999; Kanematsu *et al.*, 1998; Korten *et al.*, 1994). QR19, another first-step mutant, had eightfold decreased susceptibility to NOR, but its susceptibility to SPX was unchanged compared to that of the parent strain. From the results of nucleotide sequencing, QR19 had no mutation in QRDRs of the *gyrA*, *gyrB*, *parC* or *parE* genes. Since hydrophilic quinolones, such as NOR and ciprofloxacin (CIP), are considered to be easily pumped out from bacterial cells by NorA and PmrA, our results suggest that an efflux system like NorA is activated in QR19.

The primary target for fluoroquinolones differs among micro-organisms, depending on the structure of the compound. Which type II topoisomerase is the primary target for the fluoroquinolones in *Ent. faecium* has not been determined (Amin *et al.*, 1999; Brisse *et al.*, 1999). However, our findings that the SPX-selected first-step mutant

Fig. 1. Schematic representation showing how the first- and second-step mutants were derived from their parent strains.

Table 3. Topoisomerase subunit changes in first- and second-step fluoroquinolone-resistant *Ent. faecium*

Strain	MIC ($\mu\text{g ml}^{-1}$)		Amino acid substitution in QRDR			
	SPX	NOR	GyrA	GyrB	ParC	ParE
<i>Ent. faecium</i> ATCC 19434	1	8				
First-step mutants						
QR7	4	8	Glu-87 (GAG)→Lys (AAG)	None	None	None
QR19	1	64	None	None	None	None
QR7-derived second-step mutants						
QR7-11	32	32	Glu-87 (GAG)→Lys (AAG)	None	None	None
QR7-16	8	32	Glu-87 (GAG)→Lys (AAG)	None	None	None
QR7-18	128	> 128	Glu-87 (GAG)→Lys (AAG)	None	Ser-80 (AGC)→Ile (ATC)	None
QR7-39	128	> 128	Glu-87 (GAG)→Lys (AAG)	None	Glu-84 (GAA)→Lys (AAA)	None
QR19-derived second-step mutant						
QR19-55	2	> 128	None	None	None	None

QR7 had a point mutation only in the *gyrA* gene, and that the NOR-selected first-step mutant QR19 had no mutation in QRDRs of type II topoisomerase genes, demonstrate that the primary target at least for SPX is DNA gyrase.

The second-step mutants QR7-18 and QR7-39 selected with NOR had 32-fold and > 16-fold decreased susceptibility to SPX and NOR, respectively, and had amino acid changes in ParC that have previously been found in fluoroquinolone-resistant *Ent. faecium* and *Ent. faecalis*, i.e. Ser-80 (AGC) to Ile (ATC) in QR7-18 and Glu-84 (GAA) to Lys (AAA) in QR7-39 (Amin *et al.*, 1999; Brisse *et al.*, 1999; Kanematsu *et al.*, 1998). No QRDR mutation was associated with resistance to NOR, although the *gyrA* mutant (QR7) possessed the same NOR MIC as the parent strain, suggesting that the primary target for NOR might be topoisomerase IV. As no single *parC* mutant was obtained, this was not proven conclusively. Taken together with the finding that the primary target for SPX is DNA gyrase, it is suggested that the primary fluoroquinolone target in *Ent. faecium* as well as in *Strep. pneumoniae* differs depending on the structure of the compound used (Morrissey & George, 1999; Pan *et al.*, 2001). The second-step mutants QR7-11 and QR7-16 isolated from the SPX-selected first-step mutant QR7 by SPX and NOR selection, respectively, were two to eight times more resistant to SPX and four times more resistant to NOR than the parent strain, which had a mutation in *GyrA*. Surprisingly, QR7-11 and QR7-16 had no additional amino acid substitution in QRDRs of *GyrA*, *GyrB*, *ParC* and *ParE*. The second-step mutant QR19-55 isolated from the NOR-selected first-step mutant QR19 by NOR selection was two times or more resistant to SPX and NOR than the parent strain. No mutation in QRDRs of *gyrA*, *gyrB*, *parC* and *parE* genes from QR19-55 was detected. Thus far, the mechanisms of acquisition of fluoroquinolone resistance cannot be explained by QRDR mutations only. Apart from the possibility of mutations in regions other than QRDRs, a multidrug resistance efflux pump (MDR) might be involved in the development of resistance to fluoroquinolone (Gill

et al., 1999; Jonas *et al.*, 2001; Ng *et al.*, 1994; Yoshida *et al.*, 1990b).

Involvement of an efflux pump in the development of resistance to fluoroquinolones

Reserpine (a competitive pump blocker), verapamil (a calcium-channel blocker) and lansoprazole (a H^+ and K^+ ATPase pump inhibitor) are known to inhibit various bacterial MDRs, including NorA (Aeschlimann *et al.*, 1999) and PmrA (Gill *et al.*, 1999). Therefore, we examined the susceptibility of the first- and second-step mutants to SPX, NOR and EtBr, some of the major substrates of NorA, with or without these inhibitors. As shown in Table 4, the susceptibility of the NOR-selected first-step mutant QR19 and the QR19-derived second-step mutant QR19-55 to NOR and EtBr increased two- to fourfold in the presence of reserpine, verapamil or lansoprazole. However, the susceptibility of these two mutants to SPX did not change. The susceptibility changes observed for QR19 and QR19-55 in the presence of MDR inhibitors were very similar to those observed for *Staph. aureus* RN4220 and its NorA-over-expressed derivative in the presence of the same inhibitors. It has been reported that NorA actively transports NOR and other hydrophilic fluoroquinolones out of bacterial cells, resulting in a decrease in the intracellular concentration of these drugs (Yoshida *et al.*, 1990b). Indeed, decreased NOR accumulation was detected in the mutants QR19 and QR19-55. In addition, when the protonophore CCCP was added, the intracellular concentration of NOR in QR19 and QR19-55 became almost the same as that in the wild-type strain ATCC 19434 (data not shown). These results are similar to those of previous reports that show that NorA mediates fluoroquinolone efflux in *Staph. aureus* (Ng *et al.*, 1994; Yoshida *et al.*, 1990b). Furthermore, our findings strongly suggest that a NorA-like efflux pump exists in *Ent. faecium* and that this pump is involved in NOR resistance in the strains QR19 and QR19-55. It would be interesting to examine whether NorA-like efflux pump

Table 4. MICs of SPX, NOR and EtBr with or without reserpine, verapamil and lansoprazole

Abbreviations: R, reserpine; V, verapamil; L, lansoprazole.

Strain	MIC (µg ml ⁻¹)											
	SPX	SPX + R	SPX + V	SPX + L	NOR	NOR + R	NOR + V	NOR + L	EtBr	EtBr + R	EtBr + V	EtBr + L
<i>Ent. faecium</i> ATCC 19434												
First-step mutants												
QR7	4	2	4	4	8	4	8	8	16	8	8	16
QR19	1	1	1	1	64	16	16	32	64	16	16	16
QR7-derived second-step mutants												
QR7-11	32	32	32	32	32	16	32	32	16	8	8	16
QR7-16	8	2	4	2	32	8	16	16	64	16	32	64
QR7-18	128	128	128	128	>128	>128	>128	>128	16	8	8	16
QR7-39	128	128	128	128	>128	>128	>128	>128	16	8	8	16
QR19-derived second-step mutant												
QR19-55	2	2	2	2	128	32	32	64	128	32	32	32
<i>Staph. aureus</i>												
RN4220	0.125	0.125	0.125	0.125	2	0.25	0.5	0.5	8	1	2	1
RN4220/pRK1*	0.5	0.25	0.25	0.25	32	4	8	8	64	16	16	16

*Strain which overexpresses NorA (Yamagishi *et al.*, 1996).

gene transcription increases, since some fluoroquinolone-resistant strains of *Staph. aureus* have an increased quantity of NorA that appears to result from either increased transcription of *norA* or increased stability of its mRNA (Kaatz & Seo, 1995). Interestingly, the second-step mutant QR7-16 became fourfold less susceptible to SPX, NOR and EtBr in the presence of reserpine, and showed about threefold decreased NOR accumulation compared to QR7. The intracellular concentration of NOR in QR7-16 increased when CCCP was added. On the other hand, the SPX concentration in QR7-16 was similar to that in *Ent. faecium* ATCC 19434 (data not shown). The reason for the difference in intracellular concentration between NOR and SPX in QR7-16 is unclear; however, as SPX is very hydrophobic and can easily bind bacterial membranes, it is possible that the amount of membrane-bound SPX was measured instead of the amount of intracellular SPX. These findings suggest the existence of a novel multidrug transporter that pumps out not only NOR but also SPX from bacterial cells, while NorA and PmrA transport only hydrophilic fluoroquinolones such as NOR and CIP. MDR inhibitors did not influence or only marginally influenced the susceptibility of the first-step mutant QR7 and the QR7-derived second-step mutants QR7-11, QR7-18 and QR7-39 to NOR and SPX. As mentioned above, the mutant QR7-11, which had the *gyrA* point mutation of its parent QR7, did not have other mutations in the QRDRs of the *gyrA*, *gyrB*, *parC* and *parE* genes. In addition, the results of the accumulation assays demonstrated that the intracellular concentration of NOR in QR7-11 was almost the same as that in its parent strain QR7 (data not shown). However, QR7-11 was eight times and four times more resistant to SPX and NOR, respectively, than its parent strain QR7. These findings indicate that a mechanism(s) other than an efflux pump is involved in QR7-11 resistance to fluoroquinolones.

Properties of clinical isolates of *Ent. faecium*

Finally, we characterized 13 fluoroquinolone-resistant clinical isolates of *Ent. faecium* to examine to what extent the mechanism(s) of fluoroquinolone resistance found in the mutants isolated *in vitro* was similar to that in the clinical isolates. Properties of the 13 clinical isolates of *Ent. faecium* are summarized in Table 5. The six isolates KU2391, KU2415, KU1861, KU1869, KU2386 and KU2411 that showed low-level resistance to SPX and NOR (MICs of SPX and NOR, $\leq 4 \mu\text{g ml}^{-1}$ and $\leq 16 \mu\text{g ml}^{-1}$, respectively) did not have any amino acid substitution in the QRDRs of GyrA, GyrB, ParC and ParE. Also, the susceptibility of these isolates to SPX and NOR did not change in the presence of reserpine, verapamil or lansoprazole (data not shown). Brisse *et al.* (1999) have suggested that an amino acid substitution(s) in GyrB and/or ParE confers low- or intermediate-level fluoroquinolone resistance. Of course, this might be true in some low- or intermediate-level fluoroquinolone-resistant isolates; however, our

Table 5. Topoisomerase subunit changes in fluoroquinolone-resistant clinical isolates of *Ent. faecium*

Strain	MIC ($\mu\text{g ml}^{-1}$)		Amino acid substitution in QRDR			
	SPX	NOR	GyrA	GyrB	ParC	ParE
KU2391	0.5	2	None	None	None	None
KU2415	1	8	None	None	None	None
KU1861	4	16	None	None	None	None
KU1869	4	16	None	None	None	None
KU2386	4	16	None	None	None	None
KU2411	4	16	None	None	None	None
KU1783	8	>128	Glu-87 (GAG)→Gly (GGG)	None	Ser-80 (AGC)→Ile (ATC)	None
KU2400	16	128	Glu-87 (GAG)→Lys (AAG)	None	Ser-80 (AGC)→Ile (ATC)	None
KU2420	16	128	Glu-87 (GAG)→Lys (AAG)	None	Ser-80 (AGC)→Ile (ATC)	None
KU2409	16	128	Ser-83 (AGT)→Ile (CGT)	None	Ser-80 (AGC)→Ile (ATC)	None
KU2377	64	>128	Ser-83 (AGT)→Arg (AGA)	Glu-475 (GAA) → Lys (AAA)	Ser-80 (AGC)→Ile (ATC)	None
KU2379	64	>128	Ser-83 (AGT)→Arg (AGA)	None	Ser-80 (AGC)→Ile (ATC)	None
KU1778	64	>128	Ser-83 (AGT)→Arg (AGA)	None	Ser-80 (AGC)→Ile (ATC)	None

results suggest that an unknown mechanism(s) is also associated with low-level resistance to fluoroquinolones.

The isolates KU1783, KU2400, KU2420, KU2409, KU2379 and KU1778 had amino acid substitutions in both GyrA and ParC. The MICs of SPX and NOR for these isolates were 8–64 $\mu\text{g ml}^{-1}$ and $\geq 128 \mu\text{g ml}^{-1}$, respectively and were not influenced by efflux pump inhibitors (data not shown). Previous reports have shown that, in general, alterations in GyrA and/or ParC confer high-level fluoroquinolone resistance (Amin *et al.*, 1999; Brisse *et al.*, 1999). However, our analysis of the clinical isolates revealed that some combinations of alteration in GyrA and ParC (e.g. Ser-83 to Arg in GyrA and Ser-80 to Ile in ParC) confer high-level resistance to both NOR and SPX, while some other combinations (e.g. Glu-87 to Gly in GyrA and Ser-80 to Ile in ParC) are responsible for high- and intermediate-level resistance to NOR and SPX, respectively. Therefore, the SPX-resistance level seems to depend on the type and position of amino acid change in GyrA in *Ent. faecium*, as in *Staph. aureus* (Ito *et al.*, 1994; Takenouchi *et al.*, 1995), i.e. the amino acid change Ser-83 to Arg in GyrA confers high-level SPX resistance, while the amino acid change Glu-87 to Gly in GyrA confers low- or intermediate-level SPX resistance. On the other hand, alteration in ParC confers high-level resistance to NOR, regardless of the kind of amino acid change in GyrA. These findings suggest that alterations in DNA gyrase and topoisomerase IV are involved in SPX and NOR resistance, respectively, and strongly support the idea that the primary target of fluoroquinolones in *Ent. faecium* depends on the structure and/or physicochemical properties of the fluoroquinolone used.

It is worth noting that the mutants QR7-18 and QR7-39 with the amino acid changes Glu-87 to Lys in GyrA and Ser-80 to Ile in ParC, and Glu-87 to Lys in GyrA and Glu-84 to Lys in ParC, respectively, showed high-level resistance to both SPX and NOR (Table 3). These findings are

consistent with those that show that amino acid substitutions in GyrA and/or ParC confer high-level fluoroquinolone resistance (Brisse *et al.*, 1999). However, in these mutants, other unknown mechanisms might be involved in SPX resistance, since, as mentioned above, the amino acid change Glu-87 to Lys in GyrA seems to confer low-level SPX resistance.

Interestingly, the strain KU2377 had an extra amino acid substitution, i.e. Glu-475 (GAA) to Lys (AAA) in GyrB, in addition to the amino acid substitutions in GyrA and ParC. This extra amino acid substitution has so far not been reported, and is equivalent to the substitution Glu-474 (GAA) to Lys (AAA) in GyrB in *Strep. pneumoniae*, which confers low-level resistance to SPX (Pan & Fisher, 1998). Thus, there is a possibility that the substitution Glu-475 to Lys in GyrB is associated with low-level SPX resistance in *Ent. faecium*, although the MIC of SPX for KU2377 was identical to that for KU2379 or KU1778. In the case of KU2377, since the amino acid changes in GyrA and ParC confer high-level resistance to fluoroquinolones, the contribution of the amino acid change in GyrB to KU2377 resistance might be only slight.

The mechanism of acquisition of high-level resistance to fluoroquinolones in *Ent. faecium*, as in other Gram-positive organisms such as *Staph. aureus* and *Strep. pneumoniae*, i.e. alteration of target enzymes, is basically the same between *in vitro* mutants and clinical isolates. However, the efflux of fluoroquinolones, which leads to decreased intracellular accumulation, did not seem to occur in the clinical isolates examined in this study. Another unknown mechanism(s) is probably involved in fluoroquinolone resistance in the clinical isolates.

In summary, our analysis of fluoroquinolone-resistant *Ent. faecium* strains isolated both *in vitro* and clinically demonstrates that (i) the primary target for fluoroquinolones can differ depending on the structure of the compound used, (ii)

a previously unreported amino acid substitution, Glu-475 (GAA) to Lys (AAA) in GyrB, exists, (iii) there is not only a NorA-like efflux pump but also a non-NorA-like efflux pump which transports NOR and SPX, and (iv) unknown mechanisms are involved in the development of fluoroquinolone resistance. These unknown mechanisms might be explained by the existence of (a) a novel efflux pump that excretes hydrophobic fluoroquinolones, (b) a point mutation(s) in DNA gyrase and/or topoisomerase IV outside QRDRs, and (c) a novel fluoroquinolone-resistance gene(s), such as *qnr* (Tran & Jacoby, 2002).

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