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Alterations in DNA Gyrase and Topoisomerase IV in Resistant Mutants of *Clostridium perfringens* Found after In Vitro Treatment with Fluoroquinolones

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To compare mutations in the DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*) genes of *Clostridium perfringens*, which are associated with in vitro exposure to fluoroquinolones, resistant mutants were selected from eight strains by serial passage in the presence of increasing concentrations of norfloxacin, ciprofloxacin, gatifloxacin, or trovafloxacin. The nucleotide sequences of the entire *gyrA*, *gyrB*, *parC*, and *parE* genes of 42 mutants were determined. DNA gyrase was the primary target for each fluoroquinolone, and topoisomerase IV was the secondary target. Most mutations appeared in the quinolone resistance-determining regions of *gyrA* (resulting in changes of Asp-87 to Tyr or Gly-81 to Cys) and *parC* (resulting in changes of Asp-93 or Asp-88 to Tyr or Ser-89 to Ile); only two mutations were found in *gyrB*, and only two mutations were found in *parE*. More mutants with multiple *gyrA* and *parC* mutations were produced with gatifloxacin than with the other fluoroquinolones tested. Allelic diversity was observed among the resistant mutants, for which the drug MICs increased 2- to 256-fold. Both the structures of the drugs and their concentrations influenced the selection of mutants.

The intracellular targets of fluoroquinolones are DNA gyrase and topoisomerase IV, two essential enzymes that regulate DNA topology in bacteria (9, 21). Whereas the older quinolones lack activity against anaerobic bacteria, newer derivatives are more effective (1, 17). Resistance to the newer quinolones has been found among anaerobes (16), including *Bacteroides* spp. (25) and *Clostridium difficile* (2, 3). Resistance in various bacteria has been attributed to a multidrug efflux pump, mutations that alter the target enzymes, and other mechanisms (12, 32). Among the anaerobes, mutations in gyrase or the presence of the efflux pump have been associated with resistance in *Bacteroides* spp. (23, 28) and *C. difficile* (2, 7), but the important pathogen *Clostridium perfringens* has not yet been examined. *C. perfringens* is a significant member of the anaerobic microflora of humans that has the potential to cause serious endogenous and exogenous infections (22).

One useful approach to the identification of the resistance mechanism is the analysis of mutations produced in target genes in response to different fluoroquinolones (15, 26). DNA gyrase or topoisomerase IV, or both, could be the primary targets of fluoroquinolones in a particular bacterium (11, 36). This could be established by evaluating the substitution of amino acids in resistant strains generated in vitro. The hot spots for mutations in *gyrA* (33, 34) and *parC* (10, 36), called quinolone resistance-determining regions (QRDRs), are assumed to include the quinolone-binding domains (33, 34).

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In the present study, fluoroquinolone-resistant mutants of eight strains of *C. perfringens* were generated by serial passage in the presence of increasing concentrations of four different fluoroquinolones with various activities against anaerobes. We found that both the structures and the concentrations of the fluoroquinolones affected the selection of mutations in the genes encoding the target enzymes.

MATERIALS AND METHODS

Growth of bacterial strains. The strains of *C. perfringens* used in this study and their sources are listed in Table 1. Cultures were grown anaerobically overnight in a glove box under an 85% N_2, 10% CO_2, and 5% H_2 atmosphere at 37°C in brain heart infusion (BHI) broth from Remel (Lenexa, Kans.) containing vitamin K (1 μg/ml), hemin (5 μg/ml), and L-cysteine (5 μg/ml) from Sigma Chemical Co. (St. Louis, Mo.) (29). Trovafloxacin was a kind gift from David Hecht, ciprofloxacin was from Serological Protein, Inc. (Kankakee, Ill.), gatifloxacin was from Bristol-Myers Squibb Co. (Princeton, N.J.), and norfloxacin was from Sigma. The MICs of these drugs were measured by a microdilution assay according to the guidelines of NCCLS (24). Various concentrations of each of the drugs were added to BHI broth in microtiter plates and inoculated with 10^5 to 10^6 bacteria per well, as determined by cell counting. After incubation under anaerobic conditions, cell growth was monitored by measurement of the turbidity at 600 nm with a PowerWave X spectrophotometer (Bio-Tek Instruments Inc.). The MICs of ciprofloxacin, gatifloxacin, levofloxacin, moxifloxacin, and sparfloxacin were also measured with Etest strips (AB BIODISK, Solna, Sweden) (18). For ciprofloxacin and gatifloxacin, the MICs were measured by both the microdilution and the Etest methods. The control was *Bacteroides thetaiotaomicron*.

Selection of fluoroquinolone-resistant mutants. Mutants were selected by the addition of twice the MIC of each fluoroquinolone to BHI broth (with vitamin K, hemin, and L-cysteine), inoculation of the broth with 10^6 cells, and incubation under anaerobic conditions at 37°C until growth was observed. Only ciprofloxacin and gatifloxacin were used for mutant selection with strains F5603, B40, and 222. After incubation, the bacteria were transferred to media with increasing concentrations of the same fluoroquinolone until the development of mutants that were resistant to 5 and 50 μg of norfloxacin per ml (3.2- to 32-fold increase in the MIC), 5 and 50 μg of ciprofloxacin per ml (10- to 400-fold increase in the

TABLE 1. MICs of fluoroquinolones for eight parent strains of *C. perfringens,* as measured by microdilution assays for norfloxacin and trovafloxacin[a] and by Etests for the other fluoroquinolones

Strain	Source	MIC (μg/ml)						
		Ciprofloxacin	Norfloxacin	Trovafloxacin	Gatifloxacin	Moxifloxacin	Levofloxacin	Sparfloxacin
ATCC 3624	American Type Culture Collection	0.5	3.12	0.31	0.25	0.38	0.38	0.5
ATCC 3626	American Type Culture Collection	0.125	1.56	0.08	0.125	0.19	0.25	0.125
ATCC 13124	American Type Culture Collection	0.25	1.56	0.16	0.19	0.25	0.25	0.25
NCTR	Isolated at NCTR[b]	0.25	1.56	0.08	0.125	0.19	0.19	0.125
VPI	Virginia Polytechnic Institute	0.25	1.56	0.08	0.125	0.19	0.19	0.094
F5603[c]	Sarker et al. (30)	0.19	ND[d]	ND	0.19	ND	ND	ND
B40[c]	Sarker et al. (30)	0.125	ND	ND	0.19	ND	ND	ND
222[e]	Sarker et al. (30)	0.25	ND	ND	0.19	ND	ND	ND

[a] Twofold serial microdilutions of up to 10 μg of trovafloxacin per ml and 50 μg of norfloxacin per ml were used.
[b] NCTR, National Center for Toxicological Research, U.S. Food and Drug Administration.
[c] Human clinical isolates.
[d] ND, not done.
[e] Veterinary isolate.

MIC), 1 and 10 μg of gatifloxacin per ml (4- to 80-fold increase in the MIC), or 1 and 10 μg of trovafloxacin per ml (3.2- to 125-fold increase in the MIC), depending on the strain and the drug concentration. Bacteria that grew in the presence of these concentrations for three passages were examined for mutations in DNA gyrase and topoisomerase IV.

Comparison of DNA gyrase and topoisomerase IV gene sequences. Genomic DNA was extracted by the DNAzol protocol (Molecular Research Center, Cincinnati, Ohio).

The sequences of the genes for DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*) from *C. perfringens* strain 13 (GenBank accession number NC003366) and the PRIMERSELECT program from the DNA* Lasergene analysis software (DNASTAR, Inc., Madison, Wis.) were used to design the primers (Table 2). Two types of primers were designed for each of the genes: one that amplified the entire coding region of the gene and another that amplified only part of the gene.

An Applied Biosystems thermocycler and *Taq* polymerase were used to amplify the genes. The reaction mixture consisted of 1 to 2 μg of template, 1 μM each forward and reverse primers, 1 mM deoxynucleoside triphosphates, 3 mM $MgCl_2$, and 2.5 U of AmpliTaq DNA polymerase (Applied Biosystems) per 100 μl. After the samples were heated at 94°C for 5 min, the DNA was amplified for 30 cycles, as follows: 94°C for 30 s, 40°C for 1 min, and 72°C for 2.5 min. An additional incubation at 72°C for 7 min ended the amplification. The amplified PCR products were gel purified and then sequenced by use of an Applied Biosystems DNA sequencer with fluorescent dideoxy terminators. After the sequences were analyzed by using the DNA analysis programs from DNASTAR, the entire sequence of each gene of the resistant strains was compared with the corresponding sequence of the wild type. When a doubtful mutation was found in any of the regions sequenced, its presence was confirmed by using different primers and again sequencing both strands of the DNA containing that region.

TABLE 2. Primers and primer sequences used for amplification of gyrase and topoisomerase IV from *C. perfringens*

Gene	Primer name	Sequence	Size of PCR product (bp)
gyrA	*gyrA* F1	AATGAACGCAGGACAACTTT	2,580
	gyrA R1	TTATTTCTTCTTCCTCTTCATTAG	
	gyrA F2	GTGCCAGAATAGTTGG	340
	gyrA R2	GCCATACCAACAGCTATACC	
	gyrA F3	TGGAAGACATAAAATAATAGT	1,078
	gyrA R3	TAGTTCCCATAAATAAGT	
	gyrA F5	ATGGGCGATAAAGTAGAG	651
	gyrA R5	ATGTGGTGGTATATTAGTAGC	
	gyrA F6	ATGGCACAGGATTTCTCT	1,317
	gyrA R6	TATTCCCTTACCACCTCTC	
	gyrA F7	TTAATAATAGCCCTTGATA	984
	gyrA R7	TAATTTTTCGTCTTCTACAG	
parC	*parC* F1	TCTTCATGACCATACCTCCTACTT	2,967
	parC R1	CTTTATTAATGGGTGATGTTGTTC	
	parC F2	TTCCCTTTTTATTTATTCCTATGA	1,548
	parC R2	GGCCAGACCTTCCAACAG	
	parC F3	GTTTCTTTATTAATGGGTGATGTT	1,390
	parC R3	GAATTGCCTCTGCCTGTAAGT	
	parC F4	CCCACATGGAGATAGTTCAGTTTA	2,632
	parC R4	TCCTCTTCCCGCTCTATTTTGTA	
gyrB	*gyrB* F1	TAGGATTGCCACATTTATTTTG	2,052
	gyrB R1	GGTATTACTTTTCCCTCATTT	
	gyrB F2	TAAATAAGGGAATAAACATAA	1,335
parE	*parE* F1	TAGAGATTAGAGCAGAGCGAT	2,552
	parE R1	CTTCCCCAGTTTCCGTGTC	
	parE F2	AAGATGGCTAGAAGTTGAAGTA	1,270
	parE R2	ATAAAGAGGTGGTTGTGCTAAATA	

TABLE 3. Deduced amino acids at various positions in the DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*) proteins of fluoroquinolone-resistant strains of *C. perfringens* and fold increase in MICs of different fluoroquinolones for the resistant mutants

Mutant strain[a]	Mutation(s)[b]			MIC change (μg/ml [fold increase])[c]				
	gyrA	*parC*	*gyrB* or *parE*	Ciprofloxacin	Gatifloxacin	Moxifloxacin	Levofloxacin	Sparfloxacin
Nor-3624-5	—[d]	—	ND[e]	ND	ND	ND	ND	ND
Nor-3624-50	—	—	172V in *gyrB*	ND	ND	ND	ND	ND
Nor-3626-5	A119E	—	ND	4 (32)	0.5 (4)	0.75 (4)	1.5 (6)	1.5 (12)
Nor-3626-50	G81C	D93Y	—	32 (256)	0.75 (6)	0.75 (4)	1.5 (6)	1.5 (12)
Nor-13124-5	—	—	ND	1.5 (6)	0.25 (1.3)	0.38 (1.5)	1 (4)	0.25 (1)
Nor-13124-50	A119E	—	ND	>32 (>128)	0.75 (4)	0.5 (2)	1.5 (6)	0.75 (3)
Nor-NCTR-5	D87Y	—	ND	0.38 (1.5)	0.19 (1.5)	0.38 (2)	0.38 (2)	0.25 (2)
Nor-NCTR-50	D87Y	—	—	8 (32)	1 (8)	1.5 (8)	4 (21)	2 (16)
Nor-VPI-5	D87Y	V196F	ND	3 (12)	0.5 (4)	0.5 (2.6)	0.75 (4)	1 (10.6)
Nor-VPI-50	D87Y	V196F	ND	8 (32)	0.5 (4)	0.5 (2.6)	1.5 (8)	0.38 (4)
Cip-3624-5	D87Y	—	ND	ND	ND	ND	ND	ND
Cip-3624-50	D87Y	—	V637 in *parE*	ND	ND	ND	ND	ND
Cip-3626-5	D82N	—	ND	6 (48)	1 (8)	0.5 (2.6)	1 (4)	1 (8)
Cip-3626-50	D87Y	D93Y	—	>32 (>256)	32 (256)	1 (5)	>32 (>128)	>32 (>256)
Cip-13124-5	D87Y	S89I	ND	6 (24)	0.75 (4)	1 (4)	2 (8)	0.5 (2)
Cip-13124-50	D87Y	S89I	ND	>32 (>128)	2 (10.5)	1.5 (6)	6 (24)	3 (12)
Cip-NCTR-5	D87Y	—	—	8 (32)	2 (16)	2 (10.5)	12 (63)	6 (48)
Cip-NCTR-50	D87Y	—	—	>32 (>128)	12 (96)	8 (42)	32 (168)	24 (192)
Cip-VPI-5	D87Y	D93Y	ND	24 (96)	0.75 (6)	0.75 (4)	2 (10.5)	3 (32)
Cip-VPI-50	D87Y	D93Y	—	>32 (>128)	1 (8)	1.5 (8)	3 (16)	8 (85)
Cip-222-10	G81C, D87Y	S89I	—	>32 (>128)	8 (42)	ND	ND	ND
Cip-F5603-10	D87Y	D93Y	—	>32 (>128)	>32 (>128)	ND	ND	ND
Cip-B40-10	D87Y	D93Y	—	ND	ND	ND	ND	ND
Gat-3624-1	—	—	ND	6 (12)	2 (8)	2 (5)	2 (5)	2 (4)
Gat-3624-10	G81C	—	—	>32 (>64)	6 (24)	4 (10.5)	32 (84)	6 (12)
Gat-3626-1	G81C, D87Y	—	ND	32 (256)	16 (128)	>32 (>168)	>32 (>128)	>32 (>256)
Gat-3626-10	G81C, D87Y	D93Y, A131S	—	>32 (>256)	>32 (>256)	>32 (>168)	>32 (>128)	>32 (>256)
Gat-13124-1	D87Y	—	ND	2 (8)	0.75 (4)	1 (4)	1.5 (6)	1 (4)
Gat-13124-10	G81C, D87Y	S89I	A431S in *gyrB*	>32 (>128)	>32 (>168)	>32 (>128)	>32 (>128)	>32 (>128)
Gat-NCTR-1	G81C	—	ND	8 (32)	1.5 (12)	0.75 (4)	4 (21)	1.5 (12)
Gat-NCTR-10	G81C, D87Y	—	E486K in *parE*[f]	>32 (>128)	>32 (>256)	>32 (>168)	>32 (>168)	>32 (>256)
Gat-VPI-1	G81C	D93Y, D502Y	ND	>32 (>128)	2 (16)	2 (10.5)	8 (42)	2 (21)
Gat-VPI-10	G81C	D93Y, D502Y	ND	>32 (>128)	8 (64)	32 (168)	>32 (>168)	>32 (>340)
Gat-B40-10	G81C, D87Y	S89I	ND	ND	ND	ND	ND	ND
Tro-3624-1	—	—	ND	ND	ND	ND	ND	ND
Tro-3624-10	G81C	—	—	ND	ND	ND	ND	ND
Tro-3626-1	S83L	D88Y	ND	8 (64)	2 (16)	1.5 (8)	12 (48)	0.75 (6)
Tro-3626-10	S83L	D88Y	—	>32 (>256)	8 (64)	8 (42)	32 (128)	3 (24)
Tro-13124-1	D87Y	S89I	ND	32 (128)	4 (21)	8 (32)	32 (128)	24 (192)
Tro-13124-10	G81C, D87Y	S89I	—	>32 (>128)	>32 (>168)	>32 (>128)	>32 (>128)	>32 (>256)
Tro-NCTR-1	D87Y	D11Y, V22F	ND	6 (24)	0.5 (4)	0.75 (4)	2 (10.5)	2 (16)
Tro-NCTR-10	D87Y	D11Y, V22F	—	ND	ND	ND	ND	ND
Tro-VPI-1	D87Y	D88Y	ND	4 (16)	0.75 (6)	1.5 (8)	2 (10.5)	2 (21)
Tro-VPI-10	D87Y	D11Y, V22F	—	>32 (>128)	32 (256)	8 (42)	>32 (>168)	>32 (>340)

[a] The mutant strain name designations are as follows: the abbreviation for the fluoroquinolone used to generate mutant is used as the prefix for each parent strain (Nor, norfloxacin; Cip, ciprofloxacin; Gat, gatifloxacin; and Tro, trovafloxacin), and the concentration of fluoroquinolone (in micrograms per milliliter) that was used to select mutants is shown as a suffix; for example, Nor-3626-5 designates a resistant mutant of ATCC 3626 which was selected with 5 μg of norfloxacin per ml.
[b] All multiple mutations listed were found in the same fluoroquinolone-resistant mutant.
[c] MICs were measured with Etest strips (concentration, up to 32 μg/ml).
[d] —, no mutation was found.
[e] ND, not done.
[f] Sequencing was not repeated.

RESULTS

Fluoroquinolone susceptibilities of *C. perfringens* strains. The MICs of the various fluoroquinolones except norfloxacin for eight wild-type strains of *C. perfringens* ranged from 0.08 to 0.50 μg/ml (Table 1); the MICs of norfloxacin were higher (1.56 to 3.12 μg/ml). Mutants that had low-level to moderate resistance (MICs ≤32-fold the MIC for the parent strain) and those that had high-level resistant (MICs ≥32-fold the MIC for the parent strain) were examined for target mutations. Only one mutant that had survived in the presence of increasing concentrations of each fluoroquinolone was selected from each parent strain.

Mutants selected by different fluoroquinolones. The entire DNA sequences of the *gyrA*, *gyrB*, *parC*, and *parE* genes of the mutants were compared with those of the parent strains; the deduced amino acid changes are listed in Table 3. Most of the mutations were in *gyrA* and *parC*; only two mutations were observed in *gyrB*, and only two were observed in *parE*. DNA gyrase mutants were selected even in the presence of low concentrations of fluoroquinolones, but for some strains topo-

isomerase IV mutants were found only when high concentrations of fluoroquinolones were used.

The most common mutation in the QRDR of *gyrA* in response to ciprofloxacin and norfloxacin resulted in a change of Asp-87 to Tyr, which was found in 12 of 13 mutants selected with ciprofloxacin and 4 of 10 mutants selected with norfloxacin. Six of the mutants (five selected with ciprofloxacin and one selected with norfloxacin) also had a mutation in *parC* (Asp-93 to Tyr).

Mutations in the QRDRs of both *parC* and *gyrA* selected with gatifloxacin. In *gyrA* there were more mutations that resulted in changes of Gly-81 to Cys (9 of 11 strains) than in changes of Asp-87 to Tyr (6 of 11 strains). Gatifloxacin also selected for double mutations that resulted in changes of Asp-87 to Tyr and Gly-81 to Cys more often than the other fluoroquinolones (five mutants with double mutations were selected with gatifloxacin, and one mutant with double mutations was selected with each of ciprofloxacin and trovafloxacin). Some *gyrA* mutants selected with gatifloxacin also had *parC* mutations of Asp-93 to Tyr.

The MIC of trovafloxacin was lower than those of the other fluoroquinolones for at least four strains. Most of the mutations in *gyrA* selected with trovafloxacin resulted in changes of Asp-87 to Tyr, but one unique mutation resulted in a change of Ser-83 to Leu. Some of the mutants with mutations in *gyrA* selected with trovafloxacin also had mutations in *parC* that were different from those induced by the other fluoroquinolones.

Sensitivities of mutants. All of the mutants selected by the four fluoroquinolones tested also were resistant to moxifloxacin, levofloxacin, and sparfloxacin (Table 3). Strains with a single mutation in the QRDR of *gyrA* had 2- to 32-fold lower levels of susceptibility to fluoroquinolones. The highest increases in MICs (up to 256-fold), however, were observed for strains that had more than one mutation in the gene for DNA gyrase or topoisomerase IV, or both. Most of these double or triple mutations were generated by gatifloxacin.

DISCUSSION

This study shows that mutants of *C. perfringens* with different allelic variants of DNA gyrase and topoisomerase IV were selected in response to different fluoroquinolones and that DNA gyrase was the primary target of the four fluoroquinolones tested. More mutations in *gyrA* and *parC* were selected in the presence of higher concentrations of the most effective fluoroquinolones, gatifloxacin and trovafloxacin. Most of the *gyrA* mutations selected with gatifloxacin and the *parC* mutations selected with trovafloxacin were different from those selected with the other fluoroquinolones tested.

Gyrase and topoisomerase IV were both targeted by all the fluoroquinolones tested; however, while 42 mutants with mutations in *gyrA* were found, only 27 mutants with mutations in *parC* were selected. Higher concentrations of gatifloxacin and trovafloxacin generated *parC* mutations in some strains with *gyrA* mutations, indicating that gyrase was targeted first.

The *gyrA* mutations in the *C. perfringens* strains found in this study were different from those found in clinical strains and in vitro mutants of another member of the genus, *C. difficile*, which resulted in a change of Asp-71 to Val in one strain, Ala-118 to Thr in one strain, and Thr-82 to Ile in six strains (7). Some of the *C. perfringens* mutations, however, were similar to those in *Escherichia coli* (4, 19).

The most frequent mutation in *gyrA* resulted in a change of Asp-87 to Tyr, and the second most frequent mutation was for a change of Gly-81 to Cys. Gatifloxacin, which generally has 4- to 16-fold greater activity against most anaerobic bacteria than the other fluoroquinolones (14, 27), independently selected for both of these mutations in *C. perfringens* more often than the other fluoroquinolones did. Asp-87 and Gly-81 are both in the core (positions 81 to 87) of α helix 4 of the QRDR, which may be part of the drug-binding site (13, 31). The C-8 methoxy group of gatifloxacin improves its activity against GyrA and ParC (6, 35) and may alter the positioning of the drug on DNA gyrase (12). It increases the level of quinolone attack against mutants that have alterations at positions 83 and 87, potentially overcoming the protective effect of helix mutations (20). Therefore, a wild-type strain needs to acquire additional mutations for resistance (20). Gly-81, which is near the N-terminal end of the recognition helix (in the *E. coli* numbering system), is assumed to bind to the C-7 fluoroquinolone ring substituent (12). Mutations of Gly-81 to Asp confer resistance to ciprofloxacin but not to nalidixic acid, which lacks a C-7 piperazine ring (5). The QRDRs and α-helix sequences of *E. coli* and *C. perfringens* contain conserved amino acid sequences, including amino acids 81 and 87. The detection of double mutations in this region of *gyrA* produced by gatifloxacin supports the hypothesis that methoxy groups may alter quinolone positioning on DNA gyrase; the amino acids in α helix 4 in the QRDR of GyrA determine the binding of quinolones to the enzyme-DNA complex (20, 31).

As found by Lu et al. (20), gatifloxacin-resistant strains with multiple mutations were also resistant to the other fluoroquinolones. However, even strains with a single mutation produced by 5 μg of norfloxacin per ml had 2- to 32-fold increased levels of resistance to the other fluoroquinolones. While we have not ruled out other factors that may contribute to resistance, our data agree with those of other investigators (8, 10) that additional mutations in DNA gyrase and topoisomerase IV are associated with increased levels of resistance to fluoroquinolones. We have observed differences in the levels of resistance to different fluoroquinolones in strains that had the same mutations in target enzymes. Other factors, including drug efflux, could have contributed to resistance development.

Variations in the most common mutations selected in the DNA gyrase and topoisomerase IV sequences of *C. perfringens* mutants were associated not only with different fluoroquinolones but also with different parent strains; for instance, a mutation in *parC* that resulted in a change of Ser-89 to Ile was found in only two strains but occurred with three different fluoroquinolones. There were minor allelic variations in the wild-type strains, which may have had an effect. Also, we do not know if other mutants could have been selected if we had repeatedly attempted mutant selection using the same strain with different concentrations of various fluoroquinolones. The growth of *C. perfringens* ATCC 3624 in the presence of increasing concentrations of gatifloxacin twice resulted in the selection of mutants with the identical gyrase mutation.

Zhou et al. (37) reported on the allelic diversity among fluoroquinolone-resistant mutants of *Mycobacterium* spp., not-

ing that the selection of mutants depends on both the drug and its concentration. Our data agree; norfloxacin generated the fewest mutations, with no multiple mutations in *gyrA*, and trovafloxacin selected most of the mutants whose *parC* sequences differed. Whereas double mutations for fluoroquinolone resistance occur infrequently in *Staphylococcus aureus* (36), we found several double mutations in *C. perfringens*, especially by selection with gatifloxacin. It is likely that *C. perfringens* isolates residing in the gut could become resistant to fluoroquinolones used for the treatment of other bacterial infections.

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