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NOTES

Role of Topoisomerase Mutations and Efflux in Fluoroquinolone Resistance of *Bacteroides fragilis* Clinical Isolates and Laboratory Mutants

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Twelve laboratory mutants and 32 ciprofloxacin-resistant isolates of *Bacteroides fragilis* were examined for the mechanism(s) of fluoroquinolone resistance. Five mutants had mutations in *gyrA*. One mutant and two clinical isolates contained a mutation in *gyrB*. Eight mutants and five clinical isolates accumulated significantly less ciprofloxacin than did wild-type isolates; the mutants and clinical isolates were restored to wild-type characteristics when carbonyl cyanide *m*-chlorophenylhydrazone was used.

DNA gyrase is the primary target of fluoroquinolones for most gram-negative bacteria (3, 4, 6). DNA topoisomerase IV is also a primary target for specific fluoroquinolones in certain gram-positive bacteria (15, 16). Studies to explore mechanisms of fluoroquinolone resistance in *Bacteroides fragilis* have identified mutations in *gyrA* as well as active efflux of norfloxacin (2, 7, 9, 10). However, analysis of the literature suggests that *gyrA* is a secondary (not primary) target of fluoroquinolones in *B. fragilis* (2, 10). The aim of this study was to identify the mechanism(s) of resistance in fluoroquinolone-resistant *B. fragilis* selected in vitro from agar and a pharmacodynamic (PD) model compared with those of 32 ciprofloxacin-resistant clinical isolates from the United States.

The MIC of each antibiotic was determined for each strain with the agar doubling-dilution method described by the British Society for Antimicrobial Chemotherapy (1). Mutants were selected as described previously (5) from *B. fragilis* NCTC9343/ATCC25285 (Z14) on Wilkins and Chalgren agar in a single step at 2× MIC of trovafloxacin, moxifloxacin, and ciprofloxacin at mutation frequencies of 2.6×10^{-10}, 6.6×10^{-9}, and 4.5×10^{-9}, respectively. In vitro mutants were also selected in a PD model (11, 12) from Z14, a clinical isolate (Z54), and ATCC 23745 (Z55). From Z14, three mutants (Z50, Z72, and Z73) were selected with trovafloxacin and two mutants (Z69 and Z70) were selected with moxifloxacin. From Z54, one mutant (Z51) was selected with levofloxacin and one mutant (Z52) was selected with trovafloxacin. From Z55, two mutants (Z48 and Z49) were selected with sparfloxacin.

Except for garenoxacin and isolate Z72, the MICs of all agents were 2- to 16-fold higher for all mutants than those seen with their parent strain (Table 1). Mutants selected with trovafloxacin on agar (isolate Z56) had a smaller decrease in susceptibility to garenoxacin than mutants selected in the PD model (Z50 and Z72). To study any role of efflux in fluoroquinolone resistance, the efflux inhibitor carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was used. For all parent strains and mutants (except Z69 and Z70), CCCP lowered the MIC of ciprofloxacin two- to fourfold. A total of 32 ciprofloxacin-resistant strains isolated in 1999 from a tertiary hospital in the United States were also investigated. Two isolates were inhibited by ≥8 μg of ciprofloxacin/ml, 15 were inhibited by ≥16 μg of ciprofloxacin/ml, 10 were inhibited by ≥32 μg of ciprofloxacin/ml, and 5 were inhibited by ≥64 μg of ciprofloxacin/ml (Table 1). Compared with the laboratory mutants, CCCP had a smaller effect on the ciprofloxacin MICs for these isolates. For comparison, six ciprofloxacin-sensitive isolates collected in the United Kingdom in 2002 were examined; all required 4 μg of ciprofloxacin/ml for inhibition.

Amplification of a 267-bp product of *gyrA* was achieved using primers BFA1 (5′-ACTACTCCATGTCGGTCATC-3′) and BFA2 (5′-CAGAACCGAAGTTACCTTGC-3′). Amplification of a 260-bp product of *gyrB* was achieved using primers BFB1 (5′-CTATGTCAGGTGGCGGTCTT-3′) and BFB2 (5′-GTCTTCTTCCGTTCCGATAG-3′). The PCR parameters were initial denaturation at 95°C for 5 min followed by 30 cycles of 95°C for 30 s, 52°C for 30 s (*gyrA*) or 53°C for 30 s (*gyrB*), and extension at 72°C for 30 s, with a final extension at 72°C for 10 min. DNA sequences of amplimers were determined commercially on an LI-COR 4200 automatic DNA sequencer (MWG Biotech, Ebersberg, Germany).

The only strains of *B. fragilis* in the present study to have mutations in *gyrA* were the 12 mutants selected in the PD model. A total of 5 of the 12 mutants had a substitution in GyrA (Ser82 to Phe; equivalent to Ser83 of *Escherichia coli*) and these strains (Z49, Z50, Z51, Z52, and Z72) were the

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TABLE 1. Phenotype and genotype of *B. fragilis* laboratory mutants and clinical isolates

Group and strain[b]	MIC (μg/ml)[a]									Mutation in QRDR of:		SSC (ng of CIP/mg [dry wt] of cells)	
	CIP	CIP + C	NOR	TVA	MXF	CLX	SPX	LVX	GAR	*gyrA*	*gyrB*	CIP	CIP + CCCP
Wild type													
Z14	4	2	64	0.25	0.5	0.12	2	2	0.12	WT[c]	WT	92.6 ± 1.4	135 ± 6.5
Mutants selected on agar (n = 3)													
Z56 (TVA)	16	16	64	1	1	0.12	8	4	0.12	WT	WT	56.4 ± 4.6	91.4 ± 5.2
Z57 (MXF)	16	8	64	1	1	0.12	4	2	0.12	WT	WT	65.3 ± 7.1	88.4 ± 4.1
Z58 (CIP)	16	8	128	0.5	1	0.25	4	2	0.5	WT	WT	61.7 ± 6.8	93.5 ± 6.6
Mutants selected in a PD model (n = 5)													
Z50 (TVA)	32	8	256	8	8	1	16	16	2	Ser82→Phe	WT	40.3 ± 4.6	76.4 ± 5.9
Z72 (TVA)	32	8	128	4	4	2	16	32	0.12	Ser82→Phe	WT	44.8 ± 3.6	85.8 ± 5.8
Z69 (MXF)	16	16	128	2	2	0.5	4	8	0.25	Asp81→Asn	WT	51.4 ± 7.1	82.6 ± 7.2
Z70 (MXF)	8	8	128	2	1	0.25	4	8	0.5	Asp81→Gly	WT	45 ± 3.8	78.5 ± 9.5
Z73 (TVA)	16	8	128	2	2	0.5	4	8	0.5	WT	Glu478→Lys	49.6 ± 5.1	86.4 ± 8.9
Z54 (n = 2)	4	1	64	0.5	0.5	0.12	2	2	0.12	WT	WT	31.1 ± 5.8	58.1 ± 6.6
Z51 (LVX)	32	16	256	8	8	1	16	16	2	Ser82→Phe	WT	54.7 ± 6.8	69.9 ± 5.7
Z52 (TVA)	32	8	256	8	8	1	16	16	2	Ser82→Phe	WT	43.7 ± 5.6	89.7 ± 8.2
Z55 (n = 2)	8	2	64	0.5	0.5	0.25	2	2	0.25	WT	WT	40.2 ± 6.9	81.4 ± 3.5
Z48 (SPX)	16	8	128	4	4	1	16	32	0.5	WT	WT	60.6 ± 5.7	86.5 ± 6.6
Z49 (SPX)	32	4	256	8	8	1	16	16	0.5	Ser82→Phe	WT	46.9 ± 6.5	73.6 ± 6.6
Ciprofloxacin-sensitive isolates (n = 6)													
Z74[d]	4	2	32	0.25	0.5	0.06	2	4	0.12	WT	WT	42.3 ± 5.4	76.8 ± 5.3
Ciprofloxacin-resistant isolates (n = 32)													
Z19 (n = 14)[d]	8	8	64	0.25	0.5	0.12	2	2	0.5	WT	SM[e]	49.8 ± 6.4	81.4 ± 5.9
Z20	16	8	64	0.25	0.25	0.25	1	2	0.5	WT	SM[f]	29.8 ± 3.4	85.3 ± 4.7
Z17	16	8	64	1	2	0.25	2	4	0.5	WT	WT	45.6 ± 4.8	78.6 ± 5.9
Z39	16	8	64	4	4	1	8	32	0.5	WT	WT	35.6 ± 6.3	80.2 ± 6.5
Z21 (n = 4)[d]	32	16	64	0.25	0.25	0.12	1	1	0.5	WT	Leu415→Val	32.5 ± 5.7	82.6 ± 4.1
Z35	32	16	128	0.25	0.5	0.12	2	2	0.5	WT	WT	34.4 ± 3.5	76.3 ± 8.2
Z46	32	16	64	2	1	0.5	1	8	0.5	WT	Leu415→Val	30.4 ± 5.8	79.3 ± 7.3
Z29 (n = 4)[d]	32	16	64	0.5	0.5	0.25	2	4	1	WT	WT	31.5 ± 6.3	78.6 ± 4.1
Z23	64	16	64	1	1	0.5	4	4	1	WT	WT	29.9 ± 3.6	74.4 ± 6.3
Z28	64	16	64	1	1	1	4	8	2	WT	SM[e]	27.7 ± 5.4	77.3 ± 7.6
Z30 (n = 2)[d]	64	32	64	0.25	0.25	0.12	2	2	2	WT	SM[f]	30.8 ± 5.9	76.2 ± 4.6
Z31	64	16	64	1	1	0.5	2	4	2	WT	SM[f]	14.4 ± 6.4	92.4 ± 5.1

[a] TVA, trovafloxacin; MXF, moxifloxacin; CIP, ciprofloxacin; CIP + C, ciprofloxacin plus 100 μM CCCP; LVX, levofloxacin; SPX, sparfloxacin; NOR, norfloxacin; CLX, clinafloxacin; GAR, garenoxacin.

[b] Z14 is NCTC 9343/ATCC 252852; Z54 is M97-117 (quinolone-sensitive clinical isolate); Z14 is ATCC 23745. Bold type indicates parent strains or ciprofloxacin-sensitive clinical isolates. Agents used to select mutants are shown in parentheses.

[c] WT, wild type.

[d] Only one mutant or clinical isolate of each phenotype is shown.

[e] Silent mutation at Asp426.

[f] Silent mutations at Asp426 and Leu432.

strains most resistant to fluoroquinolones. Two mutants (Z69 and Z70) had different substitutions in GyrA at position 81 (Asp to Asn and Asp to Gly, respectively). The MICs of fluoroquinolones for mutants with substitutions at Asp81 were lower than those for mutants with substitutions at Ser82. Only one laboratory mutant selected in the PD model, Z73, contained a mutation in *gyrB*, replacing Gly478 with Lys. Five of the clinical isolates contained a mutation in *gyrB*, replacing Leu415 with Val (Table 1). The increases in fluoroquinolone MICs for mutants with mutations in *gyrB* were only increased two- to eightfold compared to the results seen with the controls. It is hypothesized that as so few ciprofloxacin-resistant mutants and clinical isolates contained a mutation in the quinolone resistance-determining regions (QRDR) of *gyrA* or *gyrB*, all mutants from the PD model and clinical isolates with mutations in these genes are at least second-step mutants. Previously reported studies focused upon identifying mutations in the QRDR of *gyrA* in either laboratory mutants or clinical isolates of *B. fragilis* (2, 9, 10), with only one of these studies exploring the QRDR of both *gyrA* and *gyrB* (10). Analyses of data from all studies indicate that mutations in *gyrA* or *gyrB* are usually not the first step to resistance.

Topoisomerase IV has been shown to be a secondary target for fluoroquinolone action in *E. coli*, in which high-level resistance (ciprofloxacin MICs ≥ 32 μg/ml) is commonly associated with at least one mutation in *gyrA* and another mutation in the gene encoding the A subunit of topoisomerase IV (*parC*) (3, 4, 6). In *Staphylococcus aureus*, however, mutations in the A subunit gene (*grlA*) can be selected prior to those in *gyrA* (16). Varon et al. (15) also suggested that *parC* and *gyrA* of *S. pneumoniae* might be interchangeable initial targets of certain fluoroquinolones, such as ciprofloxacin, sparfloxacin, and moxifloxacin. Due to the overwhelming amount of data supporting the idea of a role of topoisomerase IV (ParC/ParE) in

fluoroquinolone resistance in other species, the role of *parC* and *parE* in *B. fragilis* was investigated. A *parC*/*parE* homologue was identified, but as yet no role in fluoroquinolone resistance has been established (M. L. Peterson and L. J. V. Piddock, unpublished data).

Both Miyamae et al. and Ricci and Piddock have demonstrated that *B. fragilis* actively causes the efflux of norfloxacin (7, 13). More recently Miyamae et al. described *bexA* from *B. thetaiotaomicron* (8). The BexA transporter has been shown to be responsible for the efflux of norfloxacin, ciprofloxacin, and ethidium bromide and is a member of the multidrug and toxic compound extrusion family. The concentrations of ciprofloxacin accumulated by all strains in the present study were measured by a fluorescence assay as previously described (14). All experiments were performed in duplicate on three separate days to obtain the mean value, from which the standard deviation was calculated. Differences in accumulation values between different experiments were analyzed by Student's *t* test. A P value of <0.05 was considered significant.

All mutants selected from isolate Z14 accumulated significantly less ciprofloxacin than Z14 (Table 1). Addition of 100 μM CCCP significantly increased the steady-state concentrations (SSC) of ciprofloxacin accumulated by all strains, including Z14; Z14 accumulated approximately 120 ng of ciprofloxacin/mg (dry weight) of cells. The concentrations of ciprofloxacin accumulated by mutants of Z54 and Z55 were similar to the results seen with the respective parent strains (data not shown). Six ciprofloxacin-sensitive clinical isolates of ATCC 23745 (strain Z55) and M97-117 (Z54; ciprofloxacin-sensitive clinical isolates from the United States) accumulated significantly less ciprofloxacin (40 to 50 ng of ciprofloxacin/μg [dry weight] of cells) than did the type strain for the species NCTC 9343/ATCC 25285 (Z14). Compared with the results seen with respect to the mean concentration of ciprofloxacin accumulated by the sensitive isolates, only five ciprofloxacin-resistant isolates (Z20, Z35, Z23, Z28, and Z31) accumulated significantly lower concentrations. CCCP increased the SSC for all isolates (irrespective of susceptibility) to 80 to 100 ng of ciprofloxacin/mg (dry weight) of cells.

In summary, mutations were observed in *gyrA* and *gyrB* but for 22 clinical isolates no mutations were revealed. Further studies are in progress to identify the fluoroquinolone resistance mechanisms in these isolates. Eight laboratory mutants and five isolates accumulated significantly less ciprofloxacin than the parent strain. Addition of CCCP significantly increased these concentrations, suggesting that enhanced efflux might play a role in fluoroquinolone resistance in some strains of *B. fragilis*.

In conclusion, mutations were observed in *gyrA* and *gyrB*; for some mutants and isolates, the accumulation data suggest either enhanced efflux or decreased uptake. Current work is characterizing these strains further.

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REFERENCES

1. **Andrews, J. M.** 2001. Determination of minimum inhibitory concentrations. J. Antimicrob. Chemother. **48**(Suppl. S1)**:**5–16.
2. **Bachoual, R., L. Dubreuil, C. J. Soussy, and J. Tankovic.** 2000. Roles of *gyrA* mutations in resistance of clinical isolates and in vitro mutants of *Bacteroides fragilis* to the new fluoroquinolone trovafloxacin. Antimicrob. Agents Chemother. **44:**1842–1845.
3. **Bagel, S., V. Hullen, B. Weidemann, and P. Heisig.** 1999. Impact of *gyrA* and *parC* mutations on quinolone resistance, doubling time, and supercoiling degree of *Escherichia coli.* Antimicrob. Agents Chemother. **43:**868–875.
4. **Everett, M. J., Y. F., Jin, V. Ricci, and L. J. V. Piddock.** 1996. Contributions of individual mechanisms to fluoroquinolone resistance in 36 *Escherichia coli* strains isolated from humans and animals. Antimicrob. Agents Chemother. **40:**2380–2386.
5. **Griggs, D. J., H. Marona, and L. J. V. Piddock.** 2003. Selection of moxifloxacin-resistant *Staphylococcus aureus* compared with five other fluoroquinolones. J. Antimicrob. Chemother. **51:**1403–1407.
6. **Heisig, P.** 1996. Genetic evidence for a role of *parC* mutations in development of high-level fluoroquinolone resistance in *Escherichia coli.* Antimicrob. Agents Chemother. **40:**879–885.
7. **Miyamae, S., H. Nikaido, Y. Tanaka, and F. Yoshimura.** 1998. Active efflux of norfloxacin by *Bacteroides fragilis.* Antimicrob. Agents Chemother. **42:** 2119–2121.
8. **Miyamae, S., O. Ueda, F. Yoshimura, J. Hwang, Y. Tanaka, and H. Nikaido.** 2001. A MATE family multidrug efflux transporter pumps out fluoroquinolones in *Bacteroides thetaiotaomicron.* Antimicrob. Agents Chemother. **45:** 3341–3346.
9. **Oh, H. C. E. Nord, L. Barkholt, M. Hedberg, and C. Edlund.** 2000. Ecological disturbances in intestinal microflora caused by clinafloxacin, an extended-spectrum quinolone. Infection **28:**272–277.
10. **Onodera, Y., and K. Sato.** 1999. Molecular cloning of the *gyrA* and *gyrB* genes of *Bacteroides fragilis* encoding DNA gyrase. Antimicrob. Agents Chemother. **43:**2423–2429.
11. **Peterson, M. L., L. B. Hovde, D. H. Wright, A. D. Hoang, J. K. Raddatz, P. J. Boysen, and J. C. Rotschafer.** 1999. Fluoroquinolone resistance in *Bacteroides fragilis* following sparfloxacin exposure. Antimicrob. Agents Chemother. **43:**2251–2255.
12. **Peterson, M. L., L. B. Hovde, D. H. Wright, G. H. Brown, A. D. Hoang, and J. C. Rotschafer.** 2002. Pharmacodynamics of trovafloxacin and levofloxacin against *Bacteroides fragilis* in an in vitro pharmacodynamic model. Antimicrob. Agents Chemother. **46:**203–210.
13. **Ricci, V., and L. J. V. Piddock.** 2000. Accumulation of norfloxacin by *Bacteroides fragilis.* Antimicrob. Agents Chemother. **44:**2361–2366.
14. **Ricci, V., and L. Piddock.** 2003. Accumulation of garenoxacin by *Bacteroides fragilis* compared with that of five fluoroquinolones. J. Antimicrob. Chemother. **52:**605–609.
15. **Varon, E., C. Janoir, M. D. Kitzis, and L. Gutmann.** 1999. ParC and GyrA may be interchangeable initial targets of some fluoroquinolones in *Streptococcus pneumoniae.* Antimicrob. Agents Chemother. **43:**302–306.
16. **Yamagishi, J., T. Kojima, Y. Oyamada, K. Fujimoto, H. Hattori, S. Nakamura, and M. Inoue.** 1996. Alterations in the DNA topoisomerase IV *grlA* gene responsible for quinolone resistance in *Staphylococcus aureus.* Antimicrob. Agents Chemother. **40:**1157–1163.