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Targeting of DNA Gyrase in *Streptococcus pneumoniae* by Sparfloxacin: Selective Targeting of Gyrase or Topoisomerase IV by Quinolones

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***gyrA* and *parC* mutations have been identified in *Streptococcus pneumoniae* mutants stepwise selected for resistance to sparfloxacin, an antipneumococcal fluoroquinolone. GyrA mutations (at the position equivalent to resistance hot spot Ser-83 in *Escherichia coli* GyrA) were found in all 17 first-step mutants examined and preceded DNA topoisomerase IV ParC mutations (at Ser-79 or Glu-83), which appeared only in second-step mutants. The targeting of gyrase by sparfloxacin in *S. pneumoniae* but of topoisomerase IV by ciprofloxacin indicates that target preference can be altered by changes in quinolone structure.**

The 4-quinolones, and especially their fluoroquinolone congeners, are potent antibacterial agents that act by inhibiting DNA synthesis (30). Several studies have shown that the closely related enzymes DNA gyrase and DNA topoisomerase IV are inhibited by the drugs in vitro (8, 12, 15, 17, 18). Both topoisomerases are essential for bacterial growth, and both act by mediating the ATP-dependent crossing of one DNA duplex through a transient enzyme-bridged double-strand break in another DNA segment (9, 17, 21). Gyrase, an A_2B_2 complex encoded by the *gyrA* and *gyrB* genes, catalyzes negative supercoiling of DNA and is thought to bind ahead of the replication fork to remove positive supercoils resulting from DNA unwinding and fork movement (12, 33). By contrast, topoisomerase IV, a C_2E_2 complex encoded by the *parC* and *parE* genes, plays a specialized role in the segregation of daughter chromosomes at the end of a round of replication (1, 8, 33). In the case of gyrase, quinolones have been shown to interfere with DNA breakage and reunion by the GyrA subunits, leading to double-stranded DNA breakage (8, 9, 11); less is known about quinolone interactions with topoisomerase IV (15).

An important goal of recent work has been to establish whether gyrase or topoisomerase IV is the primary target of the quinolones. A variety of studies have shown that in *Escherichia coli*, DNA gyrase is the primary target of nalidixic acid and of fluoroquinolones, including ofloxacin, norfloxacin, and ciprofloxacin (8–11, 30). Resistance to these agents usually results from mutations in a defined region of the GyrA protein (residues 67 to 106) termed the quinolone resistance-determining region (QRDR), particularly at the highly conserved residues Ser-83 and Asp-87 (3, 10, 23, 32). These residues are close in the primary sequence to catalytic Tyr-122, which is involved in DNA breakage and reunion (14). Mutations at the equivalent positions of the ParC subunit of topoisomerase IV are secondary events and lead to very high-level resistance (13, 20). Similar results have been reported for *Neisseria gonorrhoeae*, another gram-negative pathogen (2). By contrast, recent work by our group and others on the stepwise acquisition of ciprofloxacin resistance in the gram-positive pathogens *Staphylococcus aureus* (5, 6, 16, 22, 28, 29, 31) and *Streptococcus pneumoniae* (24, 25) indicates that topoisomerase IV is the primary target. These observations, and evidence from the study of *Enterococcus faecalis* (19), could suggest a fundamental difference between the response of gram-positive organisms and that of their gram-negative cousins in that *parC* mutations precede *gyrA* mutations in the quinolone-selected mutants thus far examined.

However, analysis of the cross-resistance properties of our stepwise-selected ciprofloxacin-resistant *S. pneumoniae* mutants suggests that this hypothesis may not hold. Table 1 shows a typical set of results for mutants derived from parent 7785: first-step mutant 1C1 yielded second-step mutant 2C2, which generated third-step mutants 3C6 and 3C7. Clearly, in the stepwise challenge with ciprofloxacin, ParC mutations preceded those in GyrA (25). However, the ParC Ser-79-to-Tyr mutation in 2C2, though associated with increased resistance to ciprofloxacin, did not affect the susceptibility to sparfloxacin, a new fluoroquinolone which is being introduced for treatment of pneumococcal infections (4, 7, 26) (Fig. 1). Resistance to sparfloxacin increased markedly only on acquisition of GyrA mutations at Ser-83 or Glu-87 in third-step mutants (Table 1), perhaps suggesting that gyrase (and not topoisomerase IV) is the primary target of sparfloxacin (Fig. 1). The finding that different quinolones have different targets in the same bacterial species would be novel and would have important implications. Therefore, we decided to test this possibility by analyzing the *gyrA* and *parC* QRDRs in *S. pneumoniae* strains stepwise selected for resistance to sparfloxacin.

Approximately 5×10^{10} CFU of the quinolone-susceptible *S. pneumoniae* clinical isolate 7785 were plated on brain heart infusion agar plates containing 10% horse blood and 1 μg of sparfloxacin (kind gift of Dainippon Pharmaceutical Co., Suita, Japan) per ml and were incubated aerobically at 37°C for 40 h. The frequency of mutant selection was determined from the number of colonies that grew on plates containing the drug compared to the number of colonies obtained in the absence of the drug. Colonies appearing on drug-containing plates were restreaked onto plates containing the same level of sparfloxacin, grown, and used in subsequent drug challenges (Fig. 2). Exposure of strain 7785 to three independent drug challenges yielded first-step mutants 1S1 to 1S6, 1S7 to 1S10, and 1S11 to

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TABLE 1. Cross-resistance profile of ciprofloxacin-selected *S. pneumoniae* mutants[a]

Strain	MIC (μg/ml) of:		Mutation	
	SPAR	CIP	GyrA	ParC
7785	0.25	1		
1C1	0.25	3	None	None
2C2	0.25	8	None	Ser-79 → Tyr
3C6	16	64	Glu-87* → Lys	Ser-79 → Tyr
3C7	16	64	Ser-83* → Tyr	Ser-79 → Tyr

[a] Strains 1C1 through 3C7 were obtained from the quinolone-susceptible parent 7785 by stepwise selection with ciprofloxacin (25). MICs were determined by the standard twofold dilution method. Inocula of 10^4 CFU were spotted onto brain heart infusion agar plates supplemented with 5% horse blood and either sparfloxacin or ciprofloxacin. MICs were read after aerobic incubation for 18 h at 37°C. *, GyrA residue identified by analogy with the equivalent residue in *E. coli* GyrA. SPAR, sparfloxacin; CIP, ciprofloxacin.

1S17 at frequencies of 5.0×10^{-10} to 8.0×10^{-10}. First-step mutant 1S1 was challenged with sparfloxacin at 2 and 4 μg/ml, yielding second-step mutants 2S1 to 2S4 and 2S5 to 2S10, respectively (Fig. 2). The mutant selection frequencies were 1.4×10^{-8} (2S1 to 2S4) and 7.8×10^{-9} (2S5 to 2S10).

Chromosomal DNA, isolated by a standard procedure (24) from strain 7785 and its mutants 1S1 to 1S17 and 2S1 to 2S10, was used as a template for PCR to amplify the QRDR of the *gyrA* and *parC* genes. Oligonucleotide primer pairs, VGA3 plus VGA4 and M0363 plus M4721, allowed amplification and isolation of 382-bp *gyrA* products (encoding residues 46 to 172) and 366-bp *parC* products (encoding residues 35 to 157), respectively. The primers and PCR conditions for analysis of ciprofloxacin-resistant *S. pneumoniae* mutants have been described previously (25). Initially, the status of the *gyrA* and *parC* QRDRs was examined by digesting PCR products with *Hin*fI (29) and examining the resulting DNA fragments by electrophoresis in 2% low-gelling agarose (data not shown). The 382-bp *gyrA* PCR product from 7785 underwent cleavage at a single *Hin*fI site (overlapping coding sequence for the conserved Ser equivalent to resistance hot spot Ser-83 in *E. coli*), generating 110- and 272-bp fragments. Interestingly, none of the *gyrA* PCR products derived from the 17 first-step mutants, 1S1 to 1S17, was cleaved by *Hin*fI, indicating that each had undergone mutation at either codon 82 or 83. The 366-bp *parC* product from the wild-type gene has *Hin*fI sites at nucleotide positions 232 and 288 and on digestion with *Hin*fI generated 183-, 127-, and 56-bp fragments. Acquisition of a quinolone resistance mutation altering mutational hot spot Ser-79 in ParC leads to a loss of the position 232 *Hin*fI site and generation of two 183-bp fragments (25). The *parC* genes of 1S1 to 1S17 in each case retained all their *Hin*fI sites, which is consistent with the absence of Ser-79 codon changes. Analysis by *Hin*fI restriction fragment length polymorphism (RFLP) of the second-step mutants revealed that five—2S1, 2S2, 2S5, 2S8,

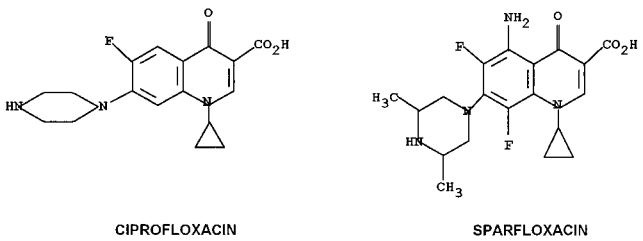

FIG. 1. Structures of ciprofloxacin and sparfloxacin.

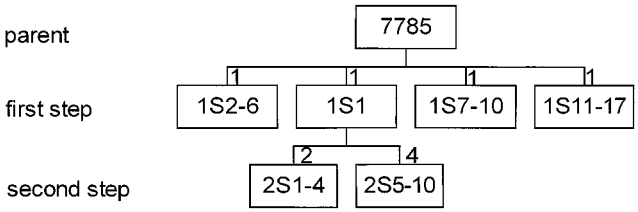

FIG. 2. Relationships among *S. pneumoniae* 7785 and quinolone-resistant mutants 1S1 through 2S10, selected by stepwise challenge with sparfloxacin. First- and second-step mutants are designated by the prefixes 1 and 2. Numbers are the concentrations of sparfloxacin (in micrograms per milliliter) used in each step of selection.

and 2S10—gave a single 183-bp band, suggesting acquisition of a Ser-79 change. *parC* PCR products from the remaining five second-step mutants yielded the wild-type *Hin*fI digestion pattern. However, this observation did not exclude the presence of *parC* mutations at other positions. Indeed, *parC* mutations were found in these strains at codon 83.

The RFLP analysis provided evidence for *gyrA* mutations in all 17 first-step mutants and for *parC* mutations in some second-step mutants. To analyze the respective QRDRs at the nucleotide level, PCR products from first-step mutants 1S1, 1S4, 1S7, and 1S11 and second-step mutants 2S1, 2S4, 2S6, and 2S10 (derived from different drug selections) were subjected to direct DNA sequencing. Asymmetric PCR (AsPCR) was used to generate single-stranded DNA. AsPCR conditions were as described previously for conventional PCR (25), except 2 μl of PCR products was used as the template and only one of the initial two primers was included in the reaction. AsPCR products were purified by selective ammonium acetate precipitation and used as templates for DNA sequence reactions. The DNA sequence was determined on both strands by the chain termination method (27) with Sequenase version 2.0 (Amersham) according to the manufacturer's instructions. The results are shown in Table 2.

Consistent with the RFLP data, all first-step mutants that were examined had a mutation in GyrA at the position equivalent to Ser-83 of *E. coli* GyrA (Table 2): 1S1 and 1S7 each had a Ser-83 (TCC)-to-Phe (TTC) change, whereas 1S4 and 1S11 each had a Ser-83 (TCC)-to-Tyr (TAC) mutation. These substitutions were each associated with an eightfold increase in resistance to sparfloxacin. No mutations were found in the

TABLE 2. Identification of GyrA and ParC mutations in sparfloxacin-selected mutants[a]

Strain	MIC (μg/ml) of:		Mutation	
	SPAR	CIP	GyrA	ParC
7785	0.25	1		
1S1	2	1	Ser-83* → Phe	None
1S4	2	1	Ser-83* → Tyr	None
1S7	2	1	Ser-83* → Phe	None
1S11	2	1	Ser-83* → Tyr	None
2S1	32	64	Ser-83* → Phe	Ser-79 → Tyr
2S4	16	32	Ser-83* → Phe	Asp-83 → Asn
2S6	16	32	Ser-83* → Phe	Asp-83 → Asn
2S10	32	64	Ser-83* → Phe	Ser-79 → Tyr

[a] Mutants 1S1 through 2S10 were derived from strain 7785 by stepwise selection with sparfloxacin. MICs were determined as for Table 1. SPAR, sparfloxacin; CIP, ciprofloxacin.

ParC QRDRs of these mutants (Table 2). Similarly, no changes were found in their ParE QRDRs (data not shown). However, the second-step mutants derived from 1S1 (which had a Ser-83-to-Phe alteration in GyrA) had all acquired a mutation in the ParC QRDR: 2S1 and 2S10 exhibited Ser-79 (TCT)-to-Tyr (TAT) changes, and 2S4 and 2S6 each had Asp-83 (GAT)-to-Asn (AAT) changes. These genetic changes are consistent with the *parC* RFLP results and are associated with 16- and 8-fold increases in sparfloxacin MIC over that for 1S1 (Table 2). No additional changes were detected in GyrA. The single mutations selected by stepwise drug challenge (Table 2) are identical to those at the equivalent positions of GyrA and ParC, mutations which are known to cause quinolone resistance in other species. Moreover, these mutations were found consistently in each of several independent drug selections and are therefore unlikely to be a statistical anomaly. Thus, it appears that gyrase is the primary target and topoisomerase IV is a secondary target of sparfloxacin in *S. pneumoniae*.

Interestingly, the cross-resistance profile of sparfloxacin-selected mutants (Table 2) mirrored that of the ciprofloxacin-selected mutants (Table 1). Thus, the Ser-83 GyrA mutations in 1S1, 1S4, 1S7, and 1S11 did not alter the ciprofloxacin MIC: all mutants remained susceptible to ciprofloxacin (Table 2). Resistance to ciprofloxacin was seen only in second-step mutants which had acquired ParC mutations. The respective patterns of cross-resistance for strains bearing GyrA but not ParC mutations and vice versa (Tables 1 and 2) reinforce the conclusion that sparfloxacin targets DNA gyrase whereas ciprofloxacin targets topoisomerase IV in *S. pneumoniae*. It may be noted from recent studies with *S. aureus* that transformation of a wild-type strain with a multicopy plasmid carrying a quinolone resistance *grlA* (*parC*) allele resulted in twofold and fourfold increases in MICs for sparfloxacin and ciprofloxacin, respectively (31). When a resistant strain bearing a chromosomal quinolone resistance-encoding *gyrA* allele and a plasmid-borne quinolone resistance-encoding *parC* allele was cured of the plasmid, the resulting strain, with quinolone-resistant gyrase and quinolone-susceptible topoisomerase IV, was entirely susceptible to sparfloxacin and ciprofloxacin. Although these experiments are more complicated than our *S. pneumoniae* studies, the results suggest that sparfloxacin targets *S. aureus* DNA topoisomerase IV more than *S. aureus* DNA gyrase.

How can the different target preferences of the quinolones be explained? Studies with *E. coli* have suggested that the primacy of gyrase as a target in this bacterium arises from two factors (18). First, quinolones, including ciprofloxacin, were uniformly some twofold more effective as inhibitors of purified *E. coli* gyrase enzyme than as inhibitors of topoisomerase IV. Second, inhibition of gyrase is more lethal to the cell than that of topoisomerase IV. This effect has been attributed to gyrase acting ahead of the replication fork and topoisomerase IV acting behind it, allowing more efficient repair of the drug-induced DNA damage caused by inhibition of topoisomerase IV. However, it is not known whether this replication model applies to other bacteria, e.g., gram-positive organisms such as *S. aureus* and *S. pneumoniae*. Our results with *S. pneumoniae* show that quinolone structure can determine whether topoisomerase IV or gyrase is responsible for cell killing. We suspect that the differences between the principal targets for sparfloxacin and ciprofloxacin in *S. pneumoniae* are determined by their relative intrinsic activities against the two enzymes and that affinity for either target determines potency. Confirmation of this idea must await the purification of topoisomerase IV and gyrase proteins from this organism.

The results presented here indicate for the first time that different quinolones can have different primary targets in the same bacterial species, i.e., quinolone structure determines the mode of antibacterial action. Although the structural features responsible for the interaction of quinolones with the drug binding pockets on gyrase or topoisomerase IV are unknown, it should be possible to design quinolones that selectively target one or the other enzyme or both (the latter could also be achieved by combining two quinolones with complementary target specificities). We note that *S. pneumoniae* mutants bearing *parC* (but not *gyrA*) mutations, though resistant to ciprofloxacin, were still susceptible to sparfloxacin (Table 1). The choice of an appropriate quinolone together with a knowledge of the *parC* and *gyrA* QRDR status may be useful in overcoming quinolone resistance in *S. pneumoniae* and other pathogens.

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