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DNA Gyrase and Topoisomerase IV Are Dual Targets of Clinafloxacin Action in *Streptococcus pneumoniae*

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We examined the response of *Streptococcus pneumoniae* 7785 to clinafloxacin, a novel C-8-substituted fluoroquinolone which is being developed as an antipneumococcal agent. Clinafloxacin was highly active against *S. pneumoniae* 7785 (MIC, 0.125 μg/ml), and neither *gyrA* nor *parC* quinolone resistance mutations alone had much effect on this activity. A combination of both mutations was needed to register resistance, suggesting that both gyrase and topoisomerase IV are clinafloxacin targets in vivo. The sparfloxacin and ciprofloxacin MICs for the *parC-gyrA* mutants were 16 to 32 and 32 to 64 μg/ml, respectively, but the clinafloxacin MIC was 1 μg/ml, i.e., within clinafloxacin levels achievable in human serum. *S. pneumoniae* 7785 mutants could be selected stepwise with clinafloxacin at a low frequency, yielding first-, second-, third-, and fourth-step mutants for which clinafloxacin MICs were 0.25, 1, 6, and 32 to 64 μg/ml, respectively. Thus, high-level resistance to clinafloxacin required four steps. Characterization of the quinolone resistance-determining regions of the *gyrA*, *parC*, *gyrB*, and *parE* genes by PCR, *Hin*fI restriction fragment length polymorphism, and DNA sequence analysis revealed an invariant resistance pathway involving sequential mutations in *gyrA* or *gyrB*, in *parC*, in *gyrA*, and finally in *parC* or *parE*. No evidence was found for other resistance mechanisms. The *gyrA* mutations in first- and third-step mutants altered GyrA hot spots Ser-83 to Phe or Tyr (*Escherichia coli* coordinates) and Glu-87 to Gln or Lys; second- and fourth-step *parC* mutations changed equivalent hot spots Ser-79 to Phe or Tyr and Asp-83 to Ala. *gyrB* and *parE* changes produced novel alterations of GyrB Glu-474 to Lys and of Pro-454 to Ser in the ParE PLRGK motif. Difficulty in selecting first-step gyrase mutants (isolated with 0.125 [but not 0.25] μg of clinafloxacin per ml at a frequency of 5.0×10^{-10} to 8.5×10^{-10}) accompanied by the small (twofold) MIC increase suggested only a modest drug preference for gyrase. Given the susceptibility of defined *gyrA* or *parC* mutants, the results suggested that clinafloxacin displays comparable if unequal targeting of gyrase and topoisomerase IV. Dual targeting and the intrinsic potency of clinafloxacin against *S. pneumoniae* and its first- and second-step mutants are desirable features in limiting the emergence of bacterial resistance.

Streptococcus pneumoniae is an important human pathogen. It is the main cause of community-acquired pneumonia and is frequently involved in exacerbations of chronic bronchitis and in meningitis, acute otitis media, and sinusitis (3). Treatment of *S. pneumoniae* infections relies heavily on antimicrobial therapy with penicillin or other beta-lactams. Over the past two decades, the emergence and, in some areas, the prevalence of pneumococci with decreased susceptibility to penicillins have emphasized the need for new therapeutic agents and have focused attention on the fluoroquinolones (14, 25, 34). However, ciprofloxacin, the main quinolone in current clinical use, has modest activity against gram-positive bacteria such as *S. pneumoniae*. Consequently, it has had relatively little impact on the treatment of respiratory tract infections.

Clinafloxacin (AM-1091, CI-960, and PD127391) is a novel fluoroquinolone with potent broad-spectrum in vitro activity against gram-positive, gram-negative, and anaerobic pathogens (reviewed in reference 18). The drug has a structure somewhat different from that of ciprofloxacin—notably, the presence of a chlorine C-8 substituent—and is much more active than ciprofloxacin against gram-positive species, including *S. pneumoniae*. Clinafloxacin has been identified as the most active fluoroquinolone against *S. pneumoniae* when compared with ofloxacin, levofloxacin, sparfloxacin, grepafloxacin, and trovafloxacin (22a) and is currently being evaluated as an antipneumococcal agent. However, its mechanism of action has yet to be examined in detail.

Previous studies showed that quinolones inhibit DNA gyrase and DNA topoisomerase IV (reviewed in reference 6). Both enzymes act by a double-strand DNA break mechanism and are essential for bacterial growth (19, 35). They cooperate in DNA replication to facilitate DNA unlinking and chromosome segregation (41). Gyrase, an A_2B_2 tetramer encoded by the *gyrA* and *gyrB* genes, catalyzes negative DNA supercoiling (10, 35) and is thought to act ahead of the replication fork, neutralizing positive supercoils arising from DNA unwinding and thereby promoting fork movement (41). Topoisomerase IV, a C_2E_2 complex specified by the *parC* and *parE* genes, allows the segregation of daughter chromosomes at cell division (1, 17, 41). Point mutations in discrete regions of the gyrase and topoisomerase IV genes—the quinolone resistance-determining regions (QRDRs)—are responsible for the development of resistance (22, 39, 40). Ciprofloxacin resistance in *Staphylococcus aureus* and *S. pneumoniae* arises through mutation of the *parC* or *parE* genes before changes in the gyrase genes take place, suggesting that topoisomerase IV is the primary ciprofloxacin target and that gyrase is the secondary target in these gram-positive species (7, 15, 21, 24, 26, 27, 33). Interestingly, in *Escherichia coli* and other gram-negative bacteria, gyrase is the primary target (5, 6, 9, 13, 22), initially suggesting that there could be fundamental differences in drug responses within the bacterial kingdom. However, in recent work, we have shown

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that whereas ciprofloxacin targets topoisomerase IV in *S. pneumoniae*, sparfloxacin targets gyrase, indicating that the molecular structure of the quinolone determines the target preference (28). We and others have proposed that a quinolone acting equally through gyrase and topoisomerase IV would be desirable, as the onset of resistance would require selection for two mutations, which would be a rare event (24, 26). It is not known which if any of the new antipneumococcal fluoroquinolones exhibits this property.

Given the potency of clinafloxacin against *S. pneumoniae*, we have sought to determine its mechanism of action in this pathogen. We have studied the activity of clinafloxacin against representative *S. pneumoniae* mutants with characterized mutations in topoisomerase genes. We also report a detailed analysis of the *gyrA*, *parC*, *gyrB*, and *parE* QRDRs of *S. pneumoniae* mutants selected stepwise for resistance to clinafloxacin.

MATERIALS AND METHODS

Bacterial strains. *S. pneumoniae* 7785, a quinolone-susceptible clinical isolate, has been described previously (27). Characterization of mutants of strain 7785 selected in vitro for resistance to ciprofloxacin (1C1, 2C6, 2C7, and 3C4) or to sparfloxacin (1S1, 1S4, 2S1, and 2S4) also has been reported (26, 28).

Drug susceptibilities. Clinafloxacin hydrochloride was from Parke-Davis, Ann Arbor, Mich. A stock solution was made up in water and stored at −70°C. Ciprofloxacin was provided by Bayer U.K., Newbury, United Kingdom. Sparfloxacin was obtained from Dainippon Pharmaceutical Co., Suita, Japan. MICs were determined by the twofold dilution method with brain heart infusion medium supplemented with 10% horse blood. Approximately 10^5 CFU of bacteria was spotted on plates, which were examined after overnight aerobic incubation at 37°C.

Bacterial growth rates. Strains 7785 and 4CLN9 were taken from plates, diluted to 50 to 100 CFU/ml in T broth (Sanofi Diagnostics Pasteur), and grown aerobically on an orbital shaker at 37°C. At time zero and at 1-h intervals over a 7-h period, 100-μl samples of the culture were removed and, after appropriate dilution, spread on brain heart infusion plates containing 10% horse blood. Plates were incubated overnight at 37°C for the determination of viable counts. Experiments were done in duplicate, and doubling times were obtained from semilog plots of CFU versus time.

Stepwise selection of clinafloxacin-resistant *S. pneumoniae* strains. Mutants were selected by plating approximately 10^{11} CFU of strain 7785 or its drug-resistant derivatives on brain heart infusion plates containing 10% horse blood and clinafloxacin. Plates were incubated aerobically at 37°C for 24 to 48 h. Mutant frequencies were determined by comparing the number of colonies that grew on plates containing drug with the number of colonies obtained in the absence of drug. All procedures were as described previously (26, 28).

PCR and RFLP analysis. Conditions for bacterial growth and the protocol for the isolation of genomic DNA were as described previously (27). PCR was used to amplify DNA from the QRDRs of the gyrase and topoisomerase IV genes of clinafloxacin-selected *S. pneumoniae* mutants. PCR conditions have been reported previously, as have the primers: VGA3 and VGA4 for *gyrA*, M0363 and M4721 for *parC*, H4025 and H4026 for *gyrB*, and S6398 and S6399 for *parE* (28). Restriction fragment length polymorphism (RFLP) analysis by *Hin*fI digestion of the PCR products was carried out as reported previously (27).

AsPCR and DNA sequence analysis. Asymmetric PCR (AsPCR) was used to generate single-stranded DNA for direct DNA sequencing by the chain termination method (32). AsPCR conditions, purification of AsPCR products, and DNA sequencing were as described previously (28).

RESULTS

Activity of clinafloxacin against *S. pneumoniae gyrA* and *parC* mutants. As an initial step in defining the target specificity of clinafloxacin, we examined the susceptibilities of *S. pneumoniae* 7785 and its mutants bearing characterized quinolone resistance mutations in *parC*, in *gyrA*, and in both *parC* and *gyrA* (Table 1). These mutants were obtained previously by stepwise selection for resistance to ciprofloxacin (strains 1C1, 2C6, 2C7, and 3C4) or sparfloxacin (strains 1S1, 1S4, 2S1, and 2S4) (28). The mutations in these strains affect hot spots for quinolone resistance (Ser-79 and Asp-83 in ParC and the residue in *S. pneumoniae* GyrA equivalent to Ser-83 in *E. coli* GyrA). From Table 1, it can be seen that the *parC* mutants were about threefold more resistant to ciprofloxacin (compared to parent 1C1) and that the *gyrA* mutants were eightfold more resistant to sparfloxacin. Mutations in *gyrA* did not affect the response to ciprofloxacin, and *parC* changes had no effect on susceptibility to sparfloxacin (Table 1). Strains expressing both *parC* and *gyrA* mutations were extremely resistant to both ciprofloxacin and sparfloxacin; MICs were 16 to 64 μg/ml, i.e., ~64 to 128-fold higher than those for the wild type.

TABLE 1. Quinolone susceptibilities of *S. pneumoniae* mutants

Strain	MIC (μg/ml) of[a]:			Mutation identified in the QRDRs of the gene encoding	
	CIP	SPAR	CLN	GyrA[b]	ParC
7785	1	0.25	0.125		
1C1	3	0.25	0.125	None	None
2C6	8	0.25	0.25	None	Ser-79→Tyr
2C7	8	0.25	0.25	None	Ser-79→Phe
1S1	1	2	0.25	Ser-83→Phe	None
1S4	1	2	0.25	Ser-83→Tyr	None
3C4	64	32	1	Ser-83→Tyr	Ser-79→Tyr
2S1	64	32	1	Ser-83→Phe	Ser-79→Tyr
2S4	32	16	1	Ser-83→Phe	Asp-83→Asn

[a] CIP, ciprofloxacin; SPAR, sparfloxacin; CLN, clinafloxacin.
[b] GyrA residue identified by analogy with the equivalent residue in *E. coli* GyrA.

The pattern of clinafloxacin susceptibility was strikingly different. First, wild-type strain 7785 was highly susceptible to clinafloxacin (MIC, 0.125 μg/ml, twofold lower than that of sparfloxacin and eightfold lower than that of ciprofloxacin). Second, in contrast to the results obtained with ciprofloxacin and sparfloxacin, neither *parC* mutations (in strains 2C6 and 2C7) nor *gyrA* mutations (in strains 1S1 and 1S4) had much effect on clinafloxacin activity; in each case, the clinafloxacin MICs were twofold higher than those for the parent, within experimental error (Table 1). Third, for strains 3C4, 2S1, and 2S4, bearing both *gyrA* and *parC* mutations, the clinafloxacin MIC was 1 μg/ml, an eightfold increase over that for the wild type (compared with 16- to 64-fold increases in the ciprofloxacin and sparfloxacin MICs). These observations indicate that gyrase and topoisomerase IV are each targeted appreciably by clinafloxacin and that mutations in both enzymes are needed to register a significant increase in resistance. Moreover, clinafloxacin retained useful activity even against the double mutants (Table 1).

Stepwise selection of clinafloxacin-resistant *S. pneumoniae* mutants. Studies with characterized *gyrA* and *parC* mutants have provided evidence that clinafloxacin targets gyrase and topoisomerase IV with approximate parity in vivo but are insufficiently sensitive to distinguish whether one or the other target is favored. Were clinafloxacin to exert a target preference, then in a series of mutants selected in a stepwise manner, we should expect resistance mutations to appear first and invariably in that target. To examine this question, we generated *S. pneumoniae* 7785 mutants by stepwise challenge using increasing concentrations of clinafloxacin, an approach similar to that adopted in studies of other quinolones (26, 28) (Fig. 1).

From the results shown in Table 1, we realized that it might be difficult to select mutants from strain 7785 using clinafloxacin at >0.25 μg/ml, as two mutations (in *gyrA* and *parC*) would be needed for growth. Therefore, approximately 10^{11} CFU of strain 7785 was plated on brain heart infusion agar plates containing 10% horse blood and clinafloxacin at 0.125 and 0.25 μg/ml, i.e., one and two times the MIC. Resistant mutants appeared on the plates containing drug at 0.125 μg/ml after 48 h of aerobic incubation at 37°C. (No mutants were obtained

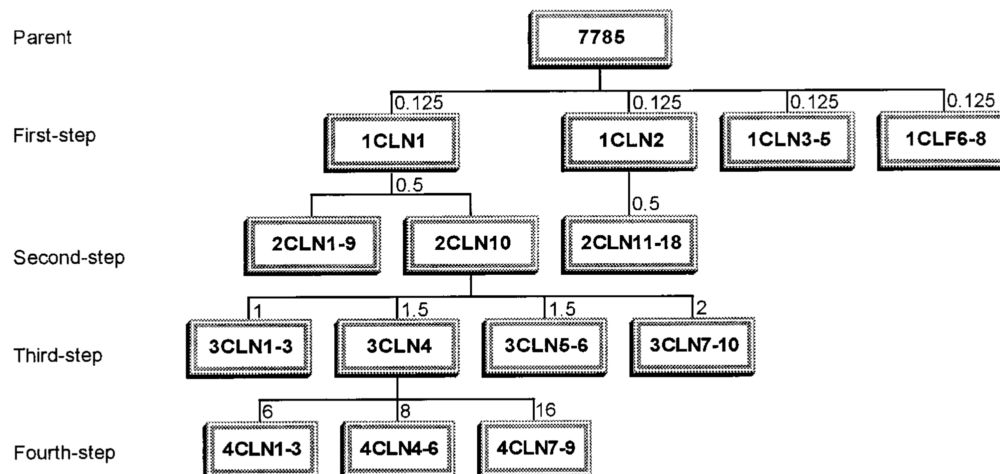

FIG. 1. Relationships among *S. pneumoniae* 7785 and its resistant mutants selected by stepwise exposure to clinafloxacin. First-, second-, third-, and fourth-step mutants are designated by the prefixes 1, 2, 3, and 4, respectively. Numbers above the boxes indicate the concentrations of clinafloxacin (in micrograms per milliliter) used for each selection step.

with clinafloxacin at 0.25 μg/ml, even when 5×10^{11} CFU was screened). Colonies were restreaked on plates containing the same level of clinafloxacin. From two independent experiments, eight of the first-step clinafloxacin-resistant mutants—1CLN1 to 1CLN5 and 1CLN6 to 1CLN8—were chosen for analysis (Fig. 1). Strains 1CLN1 and 1CLN2 were challenged independently with clinafloxacin at 0.5 μg/ml, yielding second-step mutants 2CLN1 to 2CLN10 and 2CLN11 to 2CLN18, respectively (Fig. 1). Third-step mutants 3CLN1 to 3CLN3, 3CLN4 to 3CLN6, and 3CLN7 to 3CLN10 were generated from strain 2CLN10 by exposure to clinafloxacin at 1, 1.5, and 2 μg/ml, respectively. Finally, fourth-step mutants were selected from strain 3CLN4 with clinafloxacin at 6, 8, and 16 μg/ml (Fig. 1). The mutant frequencies were similar for all steps of selection and were in the range of 5×10^{-10} to 1.2×10^{-9} (Table 2). These frequencies were very low, despite selection with clinafloxacin concentrations that were only one to three times the MICs for the parent strains. By comparison, the frequency of mutants selected with ciprofloxacin at similar multiples of the ciprofloxacin MIC was higher by one to several orders of magnitude (12, 26).

TABLE 2. Frequencies of clinafloxacin (CLN)-selected mutants generated from *S. pneumoniae* 7785 and its derivatives

Strain	CLN MIC (μg/ml)	CLN concn (μg/ml) used for selection	Mutant frequency
7785	0.125	0.125	5×10^{-10}–8.5×10^{-10}
7785	0.125	0.25	$<10^{-11}$
1CLN1	0.25	0.5	1×10^{-9}
1CLN2	0.25	0.5	5×10^{-10}
2CLN10	1.0	1.0	1.2×10^{-9}
2CLN10	1.0	1.5	5.5×10^{-10}
2CLN10	1.0	2.0	5.5×10^{-10}
3CLN4	6	6	7×10^{-10}–8×10^{-10}
3CLN4	6	8	7×10^{-10}–8×10^{-10}
3CLN4	6	16	7×10^{-10}–8×10^{-10}

Clinafloxacin has a target preference for DNA gyrase in *S. pneumoniae*. Resistant strains were characterized for their drug susceptibilities, and the status of their *gyrA*, *parC*, *gyrB*, and *parE* QRDRs was determined by DNA sequence analysis of PCR products generated with genomic DNA as a template (see reference 26 for details). A rapid RFLP method was particularly useful in the initial screening of *S. pneumoniae gyrA* and *parC* PCR products for resistance mutations affecting codons for Ser-83 and Ser-79 (26).

For each of the first-step mutants 1CLN1 to 1CLN8, there was a twofold increase in the clinafloxacin MIC (Table 3). All eight mutants produced a 366-bp *parC* PCR product which retained the wild-type *Hin*fI digestion pattern, producing 183-, 127-, and 56-bp fragments; this result indicated the likely absence of a mutation affecting ParC Ser-79. All of the mutants (except for 1CLN2 and 1CLN8) yielded a 382-bp *gyrA* PCR product which did not undergo cleavage at an internal *Hin*fI site overlapping codon 83, suggesting that this codon carried a mutation. PCR products from 1CLN2 and 1CLN8 yielded the 110- and 272-bp fragments seen for the wild-type *gyrA* gene. By DNA sequence analysis of AsPCR products from the *gyrA*, *parC*, *gyrB*, and *parE* QRDRs, it could be shown that selected strains 1CLN1, 1CLN3, 1CLN6, and 1CLN7 carried an acquired mutation in *gyrA* resulting in a change of Ser-83 to Phe or Tyr at the protein level. Strain 1CLN2 carried a GyrA change of Asp-87 to Lys, and 1CLN8 carried a GyrB change of Glu-474 to Lys (Table 3). None of the strains carried *parC* mutations. These sequencing results are consistent with the RFLP analysis. As 1CLN1 to 1CLN8 were produced in two independent drug challenges, it would seem that the *gyrA* and *gyrB* mutations are consistently selected in the first step.

All 18 second-step mutants generated from two different parent strains, 1CLN1 (GyrA Phe-83) and 1CLN2 (GyrA Lys-87), produced *parC* PCR products that had lost the *Hin*fI site overlapping codon 79, yielding a 183-bp doublet on *Hin*fI digestion; this result indicated a substitution at ParC Ser-79. The *parC* and *gyrA* PCR products of five selected mutants (clinafloxacin MIC, 1 μg/ml) were sequenced. Strains 2CLN1, 2CLN6, 2CLN10, 2CLN11, and 2CLN14, in addition to the expected GyrA mutation, had all acquired a mutation altering Ser-79 to Tyr or Phe in ParC, consistent with the RFLP anal-

TABLE 3. Mutations identified in the QRDRs of the genes encoding the gyrase and topoisomerase IV proteins in clinafloxacin-selected *S. pneumoniae* mutants

Strain	MIC (μg/ml) of[a]:			Mutation(s)[b] in the QRDR of the gene encoding			
	CLN	SPAR	CIP	GyrA[c]	ParC	GyrB	ParE
7785	0.125	0.25	1				
1CLN1[d]	0.25	2	1–2	Ser-83→Phe	None	None	None
1CLN2[d]	0.25	2	1–2	Glu-87→Lys	None		
1CLN3	0.25	2	1–2	Ser-83→Tyr	None		
1CLN6	0.25	2	1–2	Ser-83→Tyr	None		
1CLN7	0.25	2	1–2	Ser-83→Phe	None		
1CLN8	0.25	1	1–2	None	None	Glu-474→Lys	None
2CLN1	1	32	64	Ser-83→Phe	Ser-79→Tyr		
2CLN6	1	32	64	Ser-83→Phe	Ser-79→Tyr		
2CLN10[d]	1	32	64	Ser-83→Phe	Ser-79→Tyr	None	None
2CLN11	1	16	32	Glu-87→Lys	Ser-79→Tyr		
2CLN14	1	16	32	Glu-87→Lys	Ser-79→Phe		
3CLN1	6	64	64	Ser-83→Phe Glu-87→Gln	Ser-79→Tyr	None	None
3CLN4[d]	6	64	64	Ser-83→Phe Glu-87→Lys	Ser-79→Tyr	None	None
3CLN10	6	64	64	Ser-83→Phe Glu-87→Gln	Ser-79→Tyr	None	None
4CLN3	32–64	64	128	Ser-83→Phe Glu-87→Lys	Ser-79→Tyr	None	Pro-454→Ser
4CLN9	64	64	128	Ser-83→Phe Glu-87→Lys	Ser-79→Tyr Asp-83→Ala	None	None

[a] CLN, clinafloxacin; SPAR, sparfloxacin; CIP, ciprofloxacin.
[b] The indicated GyrA mutations resulted from the following nucleotide changes: Ser-83→Phe, TCC→TTC; Ser-83→Tyr, TCC→TAC; Glu-87→Lys, GAA→AAA; Glu-87→Gln, GAA→CAA. The indicated ParC mutations resulted from the following changes: Ser-79→Tyr, TCT→TAT; Ser-79→Phe, TCT→TTT; Asp-83→Ala, GAT→GCT. The GyrB mutation Glu-474→Lys resulted from a GAA→AAA change. The ParE mutatioin Pro-454→Ser resulted from a CCT→TCT change.
[c] *S. pneumoniae* GyrA residues are identified by analogy with *E. coli* GyrA.
[d] Mutant strain used as a parent for the subsequent step of selection.

ysis (Table 3). There were no additional mutations in GyrA. For strain 2CLN10, there were no alterations in GyrB or ParE. Thus, it appears that the increased clinafloxacin resistance of second-step mutants is associated with mutation of the ParC protein.

For 3 of the 10 third-step mutants obtained from parent 2CLN10 (Fig. 1), the QRDRs of the four topoisomerase genes were sequenced. No mutations were detected in the *gyrB* or *parE* QRDRs of strains 3CLN1, 3CLN4, and 3CLN10, and no additional mutations were detected in the *parC* QRDR (Table 3). However, all three strains had acquired a second mutation in GyrA (Glu-87 to Gln or Lys) associated with a clinafloxacin MIC of 6 μg/ml. Finally, for the nine fourth-step mutants, the four topoisomerase gene QRDRs were examined for strains 4CLN3 and 4CLN9 derived from strain 3CLN4 by independent challenge with clinafloxacin at 6 and 16 μg/ml (Table 3). These strains were highly resistant to clinafloxacin (MICs, 32 to 64 μg/ml). In addition to the ParC and two GyrA mutations present in the parent, both mutants had acquired a second mutation affecting topoisomerase IV: a ParE Pro-454 alteration to Ser in 4CLN3 and a ParC Asp-83 change to Ala in 4CLN9. In summary, the development of high-level resistance occurred in four steps, each associated with a mutation in a topoisomerase gene and involving alternate changes in gyrase and topoisomerase IV.

Growth properties of quinolone-resistant mutants. Given that gyrase and topoisomerase IV are essential bacterial enzymes, it was of interest to examine whether the growth of resistant mutants was impaired. We found that in the absence of drug, the growth on plates and morphology of fourth-step mutants 4CLN3 and 4CLN9 were indistinguishable from those of the wild type (data not shown). In a more detailed analysis, the generation times for 7785 and 4CLN9 in liquid cultures, determined from duplicate experiments, were 26.6 ± 2.8 and 28.6 ± 3.2 min, respectively (data not shown). Thus, although we cannot rule out the possibility that fourth-step mutants exhibit other phenotypic changes, e.g., altered pathogenicity, it is clear that the altered topoisomerases in these mutants do not have a marked effect on bacterial growth in the laboratory.

DISCUSSION

We have determined the target specificity of clinafloxacin in *S. pneumoniae* by using defined *gyrA* and *parC* strains and through analysis of mutants selected in a stepwise manner. A key finding was that representative *gyrA* or *parC* mutations did not affect the activity of clinafloxacin (Table 1). The combined presence of the same mutations led to a small but significant increase in the clinafloxacin MIC. These results are consistent with the idea that clinafloxacin targets both gyrase and topoisomerase IV in *S. pneumoniae*. In further support of the dual-target hypothesis, it proved very difficult to isolate clinafloxacin-resistant mutants by stepwise challenge (Tables 2 and 3). First-step mutants could be obtained at a frequency of 5×10^{-10} only by challenge with clinafloxacin at 0.125 μg/ml, the MIC for the parent strain. These mutants exhibited an increase in resistance of only less than or equal to twofold (clinafloxacin MIC, 0.25 μg/ml); this finding was associated with the acqui-

sition of a *gyrA* or *gyrB* mutation, suggesting a modest drug preference for gyrase. The subsequent selection of second-, third-, and fourth-step mutants, again at low frequencies and involving small incremental changes in clinafloxacin MICs, was associated with alternate mutations in the topoisomerase IV and gyrase genes (Table 3). The drug retained potency against first- and second-step mutants; indeed, four topoisomerase IV mutations were necessary to generate high-level resistance. These results indicate that clinafloxacin is intrinsically highly active against *S. pneumoniae* and exhibits approximate parity in targeting gyrase and topoisomerase IV in vivo.

Access to a panel of *S. pneumoniae* mutants expressing ParC and/or GyrA proteins with characterized quinolone resistance mutations proved extremely useful in assessing the activities of clinafloxacin and its target preferences. The responses of mutant strains to clinafloxacin could be rapidly examined and compared with parallel data obtained for sparfloxacin and ciprofloxacin, agents that target gyrase and topoisomerase IV, respectively (Table 1). Clinafloxacin was the most potent of the three agents tested against wild-type *S. pneumoniae*, *parC*, or *gyrA* mutants, and particularly the *parC-gyrA* double mutants (Table 1). For the parent strain and single mutants, the clinafloxacin MICs were 0.125 and 0.25 μg/ml, respectively. For the double mutants, the clinafloxacin MIC was increased fourfold to 1 μg/ml. However, the sparfloxacin and ciprofloxacin MICs for the double mutants each climbed steeply to 16 to 64 μg/ml, i.e., beyond levels attainable in serum or tissue (Table 1). Although we did not examine trovafloxacin, recent studies with a similar approach have measured trovafloxacin MICs of 0.5 and 6 μg/ml for representative *parC* and *parC-gyrA S. pneumoniae* mutants isolated as first-step and second-step mutants by challenge with ciprofloxacin, respectively (12). The fourfold increase in the trovafloxacin MIC for *parC* mutants indicates that trovafloxacin targets topoisomerase IV, and from human pharmacokinetic data, it appears that the drug retains clinically relevant activity against the first-step mutants (12). For clinafloxacin, pharmacokinetic studies have shown that for twice-daily oral doses of 200 and 400 mg, respective peak concentrations in human serum reached 2.75 and 5.22 μg/ml, with half-lives for elimination of 5.7 and 7.6 h (18, 31). Although further pharmacokinetic studies may be needed, the data presented in Table 1 indicate that clinafloxacin should retain potent clinical activity against *gyrA*, *parC*, and *gyrA-parC* mutants.

One limitation in using mutant strains to test drug activities is that small preferences in drug targeting are not apparent. Thus, for both *gyrA* and *parC* mutants of *S. pneumoniae* there was a twofold increase in the clinafloxacin MIC (Table 1). These small changes are usually considered to be within experimental error for the twofold dilution method used for MIC determinations. The stepwise selection of mutants is a more sensitive approach for determining target preferences. The development of high-level resistance to clinafloxacin required multiple steps with a defined and invariant sequence of mutations of single residues in gyrase or topoisomerase IV (Table 3). That first-step mutants displaying an increase in the clinafloxacin MIC of less than or equal to twofold in association with a mutation in either *gyrA* or *gyrB* could be selected is consistent with dual targeting with a modest drug preference for gyrase. Recent work with trovafloxacin has shown that this agent selects resistant *S. pneumoniae* mutants at a frequency of $\sim 10^{-8}$ when used at the MIC. The MICs and QRDRs of the trovafloxacin-resistant mutants were not examined (12).

We found no evidence for clinafloxacin resistance mechanisms other than topoisomerase gene mutations. In contrast, selection with ciprofloxacin led to first-step mutants, such as 1C1, whose resistance was not attributable to topoisomerase changes (Table 1) (26). Interestingly, 1C1 was completely susceptible to clinafloxacin. We have speculated that 1C1 may be a permeation mutant whose resistance accrues from the up-regulation of an efflux pump, akin to the NorA protein of *S. aureus*, for which ciprofloxacin is a substrate. Basal-level expression of NorA in *S. aureus* is involved in setting wild-type susceptibility to ciprofloxacin (38), and several ciprofloxacin-resistant *S. aureus* strains in which NorA is upregulated have been identified (16, 23). *S. pneumoniae* is known to express one or more efflux pumps (2), and our failure to isolate such mutants could suggest that clinafloxacin is not a substrate, a property that would contribute to its intrinsic activity. These issues require further study.

The mutations selected by clinafloxacin challenge were predominantly those described previously for other quinolones (Tables 1 and 3) (22). Thus, the *gyrA* mutations altered GyrA Ser-83 (to Phe or Tyr) or Glu-87 (to Gln or Lys) (Table 3). The *parC* mutations resulted in ParC Ser-79 (to Phe or Tyr) and Asp-83 (to Ala) changes. These changes occurred at the expected GyrA and ParC hot spots. In the crystal structure of an N-terminal 59-kDa fragment of the *E. coli* GyrA protein, the equivalent residues lie in an α helix that is adjacent to the catalytic Tyr-122 residues involved in DNA breakage and reunion (20). This helix likely functions in DNA recognition and binding. It is not known how the resistance mutations interfere with quinolone action, although this interference may arise from steric inhibition of drug binding (5, 37).

In contrast to the GyrA and ParC changes, the GyrB and ParE mutations acquired in first-step strain 1CLN8 and fourth-step strain 4CLN3 are novel. First, the Glu-474→Lys GyrB mutation does not occur in the EGDSA and P(I/L)RGK motifs of GyrB, commonly implicated in resistance and identified as the GyrB QRDR (40). Instead, the mutation lies C terminal to the PLRGK motif. Previous studies of quinolone-resistant *Salmonella typhimurium* identified a Ser-463→Lys alteration in GyrB located at a position similar to that which is altered in GyrB of strain 1CLN8 (11). Moreover, an Asn-470→Asp mutation lying C terminal to the PLRGK motif has been described for *S. aureus* ParE (8). Together, the data suggest that the GyrB QRDR may occupy a more extensive region of the protein than was previously defined by studies with *E. coli* (40). Second, the Pro-454→Ser ParE mutation lies within the PLRGK motif but is a novel change, as the Arg residue of this sequence usually is mutated, at least in GyrB. Other studies with *S. pneumoniae* have shown that first-step mutants resistant to ciprofloxacin carry ParE mutations of Asp-435 to Asn in the EGDSA motif (30). The functional role of the GyrB or ParE QRDR region is unknown. The equivalent region in the crystal structure of a fragment of *Saccharomyces cerevisiae* topoisomerase II lies distant from the DNA binding domain, and it is not clear how mutations in this region may affect drug susceptibility (4). Mutations in the conserved PLRGK motif and DNA recognition helix A′α4 of yeast topoisomerase II confer resistance to the anticancer agents amsacrine and doxorubicin, respectively (29, 36). Thus, there appear to be close similarities in the mechanisms of action of these topoisomerase-targeting antitumor agents and the antibacterial fluoroquinolones.

Interest in drugs that act on two or more targets stems in part from the possibility of limiting the emergence of resistance. Were a drug to act with equal potency through two targets, then simultaneous acquisition of two mutations would be required for the development of resistance. Given a typical frequency of 10^{-8} for single mutations, the frequency of double mutations would be 10^{-16}, an extremely rare occurrence. It may be difficult to obtain agents that act exactly equally through two topoisomerase targets. However, comparable if

unequal targeting is also advantageous. In this case, the level of resistance arising from mutation of the preferred target will be severely limited by the appreciable contribution to susceptibility of the second target. The results of the stepwise selection (Table 3) indicate that clinafloxacin acts in this manner. To our knowledge, clinafloxacin is the first reported example of a dual-targeting quinolone for *S. pneumoniae* and extends our previous work indicating that quinolone structure determines target preference in *S. pneumoniae* (28). Interestingly, recent studies with defined quinolone-resistant *S. aureus* mutants suggest that sparfloxacin and DU6895a also act against both gyrase and topoisomerase IV in *S. aureus* (8). In the absence of data from mutants selected in a stepwise manner, it is an open question whether these fluoroquinolones act equally or with approximate parity on their two *S. aureus* targets. However, the results suggest that dual-targeting quinolones may not be as rare as might be imagined. It is notable that sparfloxacin targets gyrase in *S. pneumoniae* but acts against both gyrase and topoisomerase IV in *S. aureus*. Evidently, targeting depends not only on drug structure but also on relative structural differences in topoisomerases. It remains to be seen whether dual targeting for one bacterial species can be retained for other pathogens.

Finally, the target preferences for clinafloxacin described in this paper indicate the relative importance of cell-killing pathways initiated through drug inhibition of gyrase or topoisomerase IV in *S. pneumoniae*. The molecular determinants favoring one or the other of these pathways are poorly understood. A C-8 substituent on the fluoroquinolone appears to favor cell killing through gyrase in *S. pneumoniae*. The gyrase-targeting agents sparfloxacin and clinafloxacin have fluorine and chlorine substituents at C-8, respectively. In contrast, neither ciprofloxacin nor trovafloxacin carries a C-8 substituent, and it is significant that both target topoisomerase IV in *S. pneumoniae*. We do not know whether these preferences arise from enhanced affinity for the target or enhanced lethality, as recently described for the 8-methoxy derivative of ciprofloxacin against *E. coli gyrA* mutants (42). Our working hypothesis is that the high potency of clinafloxacin against *S. pneumoniae* and its *gyrA*, *parC*, and *gyrA-parC* mutants arises from intrinsic tight binding of the drug to the target enzyme-DNA complexes, perhaps aided by poor efflux. Biochemical studies on clinafloxacin in progress in our laboratory should provide further information on the factors underlying its potent antipneumococcal activity.

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