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Contribution of Mutations in *gyrA* and *parC* Genes to Fluoroquinolone Resistance of Mutants of *Streptococcus pneumoniae* Obtained In Vivo and In Vitro

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We have analyzed by gene amplification and sequencing mutations in the quinolone resistance-determining regions of the *gyrA*, *gyrB*, and *parC* genes of fluoroquinolone-resistant *Streptococcus pneumoniae* mutants obtained during therapy or in vitro. Mutations leading to substitutions in ParC were detected in the two mutants obtained in vivo, BM4203-R (substitution of a histidine for an aspartate at position 84 [Asp-84→His]; *Staphylococcus aureus* coordinates) and BM4204-R (Ser-80→Phe), and in two mutants obtained in vitro (Ser-80→Tyr). An additional mutant obtained in vitro, BM4205-R3, displayed a higher level of fluoroquinolone resistance and had a mutation in *gyrA* leading to a Ser-84→Phe change. We could not detect any mutation in the three remaining mutants obtained in vitro. Total DNA from BM4203-R, BM4204-R, and BM4205-R3 was used to transform *S. pneumoniae* CP1000 by selection on fluoroquinolones. For the *parC* mutants, transformants with phenotypes indistinguishable from those of the donors were obtained at frequencies (5×10^{-3} to 8×10^{-3}) compatible with monogenic transformation. By contrast, transformants were obtained at a low frequency (4×10^{-5}), compatible with the transformation of two independent genes, for the *gyrA* mutant. Resistant transformants of CP1000 were also obtained with an amplified fragment of *parC* from BM4203-R and BM4204-R but not with a *gyrA* fragment from BM4205-R3. All transformants had mutations identical to those in the donors. These data strongly suggest that ParC is the primary target for fluoroquinolones in *S. pneumoniae* and that BM4205-R3 is resistant to higher levels of the drugs following the acquisition of two mutations, including one in *gyrA*.

Streptococcus pneumoniae is responsible for high levels of morbidity and mortality in humans throughout the world. It is the leading cause of bacterial pneumonia; for instance, more than a million cases of pneumococcal pneumonia may occur in the United States each year, with a fatality rate of 5 to 7% (2). It is also an important cause of otitis media and meningitis. In view of the pandemic spread of penicillin resistance in this species (3), there is an urgent need for the development of new antibiotics effective against penicillin-resistant clinical isolates. "Old" fluoroquinolones, such as ciprofloxacin and ofloxacin, show only marginal activity against *S. pneumoniae*, with the MICs of these agents being either above or at the breakpoint that delineates the susceptible and resistant clinical categories (6).

Sparfloxacin, a new fluoroquinolone that has recently become commercially available in Europe, exhibits improved activity against gram-positive bacteria, in particular against *S. pneumoniae* (MIC at which 90% of isolates are inhibited [MIC_{90}], 0.5 μg/ml) (6). A randomized comparative clinical trial has established that sparfloxacin is at least as effective as amoxicillin-clavulanic acid and erythromycin in the treatment of community-acquired pneumonia (19). This drug may therefore represent a useful alternative in the treatment of pneumococcal pneumonia, especially when penicillin-resistant isolates are involved.

However, in vivo selection of fluoroquinolone-resistant *S. pneumoniae* has been reported (5, 27), and a minority of clinical isolates, e.g., 0.25% in 1993 in France, belonging to this species have already developed resistance to sparfloxacin (MICs, ≥4 μg/ml) (11, 20). One can thus wonder if, as has been observed with staphylococci and ciprofloxacin, the use of sparfloxacin will lead to a rapid increase in resistance in pneumococci.

The mechanisms involved in the fluoroquinolone resistance of *S. pneumoniae* have not been investigated. In *Staphylococcus aureus* resistance appears to be mainly due to mutational alterations of the intracellular targets of fluoroquinolones, the type II DNA topoisomerases gyrase and topoisomerase IV (topo IV). The latter enzyme is thought to be the primary target of the drugs in this species, since mutations in the so-called quinolone resistance-determining region (QRDR) of the *parC* gene, which encodes the A subunit of topo IV, confer low levels of resistance to single-step mutants (8). The occurrence of a second mutation in the QRDR of *gyrA*, which encodes the A subunit of DNA gyrase, is responsible for higher levels of resistance (8). Mutations in *gyrB*, which encodes the B subunit of DNA gyrase, have also been implicated in the fluoroquinolone resistance of certain mutants obtained in vitro (13). A fourth mechanism of resistance, enhanced active efflux of hydrophilic quinolones such as norfloxacin, mediated by the membrane-associated protein NorA, has been found in a few strains (14).

The aim of the study described here was to analyze the contributions of mutations in the *gyrA*, *gyrB*, and *parC* genes to the fluoroquinolone resistance of mutants of *S. pneumoniae* obtained in vivo and in vitro.

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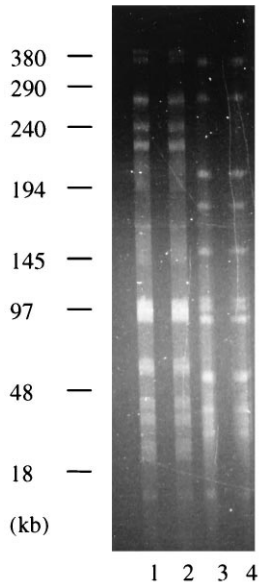

FIG. 1. Pulsed-field gel electrophoresis patterns of *Sma*I-digested genomic DNA from *S. pneumoniae* isolates. Lanes: 1, BM4203; 2, BM4203-R; 3, BM4204; and 4, BM4204-R. Size markers (bacteriophage lambda DNA concatemers) are indicated on the left.

MATERIALS AND METHODS

Isolation of spontaneous fluoroquinolone-resistant mutants of *S. pneumoniae*. Approximately 10^9 bacteria were plated onto Mueller-Hinton agar (Diagnostics Pasteur, Marnes-la-Coquette, France) supplemented with 10% horse blood and containing various concentrations of fluoroquinolones. After 48 h of incubation at 37°C, the colonies were counted and the frequencies of mutation were determined relative to the total viable count of organisms that were plated. Resistant clones were replated once on plates containing the same concentration of fluoroquinolone as that used for selection, and quinolone resistance phenotypes were determined.

Antibiotic susceptibility testing. The MICs of the fluoroquinolones were determined by the agar dilution method on Mueller-Hinton agar supplemented with 10% horse blood and by using an inoculum of 10^4 CFU per spot. Cultures were incubated at 37°C for 24 h in an atmosphere enriched with 10% CO_2. The following antimicrobial agents were provided by the indicated manufacturers: pefloxacin and sparfloxacin, Rhône-Poulenc Rorer (Vitry-sur-Seine, France); ciprofloxacin, Bayer AG (Leverkusen, Germany); temafloxacin, Abbott Laboratories (Chicago, Ill.); and PD131628, Parke-Davis Pharmaceutical Research (Ann Arbor, Mich.). The breakpoints used for ciprofloxacin (25) and sparfloxacin (1) were as published previously. Susceptibilities to other classes of antibiotics were tested on the same medium by the disk diffusion method with disks provided by Diagnostics Pasteur and an inoculum of 10^7 CFU/ml.

Analysis of chromosomal DNA by pulsed-field gel electrophoresis. Agarose plugs were prepared (18) and digested with *Sma*I restriction endonuclease (United States Biochemicals, Cleveland, Ohio) according to the manufacturer's recommendations. The software-assisted ZIFE (zero integrated field gel electrophoresis) apparatus AutoBase (Techgen, Les Ulis, France) was used with ROM card no. 2, which optimizes DNA fragment separation in the 8- to 200-kb size range (34).

DNA amplification and sequencing. We initially amplified by PCR a 310-bp fragment of *gyrA* from *S. pneumoniae* BM4203, from positions 70 to 379 (*S. aureus* coordinates). The degenerate oligodeoxynucleotide primers used, designated P1 and P2, were designed from conserved regions flanking the QRDR of *gyrA* from *S. aureus* (21) and consisted of the sequences 5′-GAITA(TC)GCIAT GAG(CT)GT and 5′-CGIGC(TC)TCIGT(AG)TA(AT)CG, respectively (where I is inosine). The amplified DNA was purified on Microspin S-400 HR columns (Pharmacia LKB Biotechnology, Uppsala, Sweden), and the sequences of both strands were determined directly by the dideoxy-chain termination method (29). The sequence data were used to design the P3 primer internal to the fragment amplified by P1 and P2 and consisting of the sequence 5′-AGCACTATCTCC ATCCATGGA. Oligonucleotides P1 and P3 were used to amplify a 285-bp *gyrA* fragment from positions 70 to 354 (*S. aureus* coordinates). A 334-bp fragment of *gyrB*, from positions 1188 to 1521, was amplified with oligonucleotide primers 5′-TGCGCGTGAAGTCACACGTA and 5′-GCATCGGTCATCAAAACGAG, designed from the published sequence of the *gyrB* gene of *S. pneumoniae* 533 (24). We also amplified a 254-bp fragment of *parC* of *S. pneumoniae* from positions 109 to 362 (*S. aureus* coordinates) with the degenerate oligonucleotide primers 5′-GG(ACGT)TT(AG)AA(AG)CC(ACGT)GT(ACGT)CAG and 5′-TC(AT)GT(AG)TA(AT)C(GT)CAT(AT)GC, designed from conserved regions of the *S. aureus parC* gene (9).

All amplifications were carried out in a 100-μl volume containing 50 pmol of each oligonucleotide primer, 50 nmol of each 2′-deoxynucleoside 5′-triphosphate, reaction buffer (Bioprobe Systems, Montreuil-sous-Bois, France), 5 μl of a template DNA sample (containing 100 ng of DNA), and 2 U of *Taq* DNA polymerase (Bioprobe Systems). The reactions were performed in a DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, Conn.) for 30 cycles. The conditions were 30 s at 95°C for denaturation, 30 s at 50°C (*gyrA* and *parC*) or 60°C (*gyrB*) for annealing, and 30 s at 72°C for polymerization. The PCR products were purified and sequenced directly on both strands (29).

Genetic transformation of *S. pneumoniae* CP1000. Frozen competent cells (22) were gently thawed, suspended in the transformation medium (26), and incubated at 37°C for 15 min. Chromosomal or purified PCR-amplified DNA (0.1 to 1 μg) was added, and the mixture was incubated at 30°C for 30 min, plated onto blood agar, and incubated at 37°C for 2 h. Transformants were selected with ciprofloxacin at 2 μg/ml or sparfloxacin at 1 μg/ml by the overlay procedure. Transformation of a mutation that confers resistance to rifampin was used as a control of monogenic transformation. Total DNA from *S. pneumoniae* 119 resistant to rifampin (*rif-r23*) (33) was used, and transformants were selected with rifampin at 2 μg/ml.

Sequence analysis. The programs included in the GCG package (Genetics Computer Group, Madison, Wis.) were used for the construction of alignments. Phylogenetic analysis was carried out with the PHYLIP program package, version 3.5c (University of Washington, Seattle, Wash.).

Nucleotide sequence accession numbers. The nucleotide sequences were submitted to GenBank and were assigned the accession numbers U49087 (*gyrA*) and U49088 (*parC*).

RESULTS AND DISCUSSION

Clinical strains. Two fluoroquinolone-susceptible and -resistant matched pairs of clinical isolates of *S. pneumoniae*, BM4203–BM4203-R and BM4204–BM4204-R, were examined. Strain BM4203 was isolated from the pleural fluid of an 84-year-old man admitted for community-acquired pneumonia and empyema (27). This patient was treated for 4 weeks with ciprofloxacin (200 mg given intravenously on the first day and then 500 mg given orally twice a day) and his symptoms resolved. Ten days later, the patient was readmitted for recurrence of his pleuropulmonary infection, and a fluoroquinolone-resistant isolate of *S. pneumoniae*, BM4203-R, was cultured from the pleural fluid. Strain BM4204 was isolated from the sputum of a 34-year-old AIDS patient with community-acquired pneumonia. Clinical cure was obtained by therapy with penicillin G. However, several months after this episode, the patient was admitted again for pneumonia and a fluoroquinolone-resistant isolate of *S. pneumoniae*, BM4204-R, was obtained from the sputum. Between the two pulmonary episodes, the patient had been treated with pefloxacin for a cutaneous staphylococcal infection.

Typing of clinical isolates. The fluoroquinolone-susceptible and -resistant members of each of the two pairs of clinical strains belonged to the same serotype: serotype 3 for BM4203–

TABLE 1. Selection of fluoroquinolone-resistant mutants from *S. pneumoniae* BM4203 and BM4205

Selecting agent	Concn (multiple of MIC)	Mutation frequency	
		BM4203	BM4205
Ciprofloxacin	2	1.1×10^{-7}	7.9×10^{-7}
	4	1.8×10^{-8}	2.3×10^{-8}
	8	$<1 \times 10^{-9}$	1 clone
Temafloxacin	2	7.1×10^{-8}	$<1 \times 10^{-9}$
	4	$<1 \times 10^{-9}$	$<1 \times 10^{-9}$
PD131628	2	2.9×10^{-8}	$<1 \times 10^{-9}$
	4	$<1 \times 10^{-9}$	$<1 \times 10^{-9}$

TABLE 2. Susceptibilities of *S. pneumoniae* strains to selected fluoroquinolones and mutations in the *gyrA* and *parC* genes

Strain	Selecting agent (concn [μg/ml])	MIC (μg/ml)[a]					Amino acid substitution (mutation)[b]	
		PEF	CIP	SPA	TEM	PD	GyrA	ParC
BM4203	None	8	2	0.5	0.25	0.25	—[c]	—
BM4203-R	CIP (in vivo)	32	16	1	2	1	—	Asp-84→His (GAT-CAT)
BM4203-R1	PD (0.5)	32	16	1	2	1	—	Ser-80→Tyr (TCT-TAT)
BM4203-R2	TEM (0.5)	32	16	1	2	1	—	Ser-80→Tyr (TCT-TAT)
BM4203-R3	CIP (4)	16	8	0.5	2	1	—	—
BM4204	None	4	1	0.25	0.5	0.25	—	—
BM4204-R	PEF (in vivo)	16	8	0.5	1	0.5	—	Ser-80→Phe (TCT-TTT)
BM4205	None	8	2	0.5	0.5	0.5	—	—
BM4205-R1	CIP (8)	32	8	0.5	1	1	—	—
BM4205-R2	CIP (8)	32	16	1	2	2	—	—
BM4205-R3	CIP (16)	128	64	4	8	8	Ser-84→Phe (TCC-TTC)	—

[a] CIP, ciprofloxacin; PD, PD131628; PEF, pefloxacin; SPA, sparfloxacin; TEM, temafloxacin.
[b] Positions of substitutions are according to the *S. aureus* coordinates.
[c] —, no change.

BM4203-R and serotype 6 for BM4204–BM4204-R. Capsular type is not a reliable criterion for determining the genetic relatedness of *S. pneumoniae* strains (30). In contrast, pulsed-field gel electrophoresis of total DNA digested with *Sma*I allows for the differentiation of strains within serotypes and is thus suitable for identifying clones of pneumococci (18). The two members of each pair had indistinguishable *Sma*I-generated DNA profiles (Fig. 1), indicating that BM4203-R and BM4204-R were in vivo mutants selected under therapy with fluoroquinolones.

Selection of fluoroquinolone-resistant mutants. Spontaneous fluoroquinolone-resistant mutants were selected by plating clinical *S. pneumoniae* isolates BM4203 and BM4205 onto medium containing ciprofloxacin, temafloxacin, and PD131628. Resistant mutants were selected from both strains on ciprofloxacin at two and four times the MICs at frequencies of 10^{-7} to 10^{-8} (Table 1). In addition, a single resistant mutant of BM4205, BM4205-R3, was obtained on ciprofloxacin at eight times the MIC. By using PD131628 and temafloxacin as selecting agents, only resistant mutants of BM4203 were obtained at twice the MIC. Six in vitro mutants, including BM4205-R3, were selected for further studies.

Susceptibility to fluoroquinolones. The MICs of certain fluoroquinolones for the strains are summarized in Table 2. In comparison with the other mutants, in vitro mutant BM4205-R3 displayed a higher level of resistance to fluoroquinolones, including ciprofloxacin and sparfloxacin. By contrast, the other mutants had a low degree of resistance to ciprofloxacin and remained susceptible to sparfloxacin. There were no differences between the susceptibilities of the parental strains and of the derived mutants to other classes of antibiotics, as tested by disk diffusion.

Nucleotide sequence of the QRDRs of *gyrA* and *parC* from *S. pneumoniae* BM4203. Two pairs of oligodeoxynucleotide primers were used to amplify by PCR and sequence the QRDRs of the *gyrA* and *parC* genes from *S. pneumoniae* BM4203. The deduced amino acid sequence of the 285-bp PCR fragment (Fig. 2) had a higher percentage of identity with portions of GyrA from *S. aureus* (84%), *Enterococcus faecalis* (88%), and *Bacillus subtilis* (76%) than with ParC from *S. aureus* (45%) (Table 3). Conversely, the derived amino acid sequence of the 254-bp fragment (Fig. 2) showed a higher percentage of identity with ParC from *S. aureus* (59%) than with GyrA from *S. aureus* (45%), *E. faecalis* (29%), and *B. subtilis* (36%) (Table 3). Furthermore, the histidine residue at position 46 (His-46; *S. aureus* coordinates) specifically conserved in GyrA and the glutamine residue at position 42 (Gln-42; *S. aureus* coordinates) specifically conserved in ParC were also present in the corresponding sequences from *S. pneumoniae* (Fig. 2). The phylogeny of these sequences was determined by the neighbor-joining (28) and the maximum parsimony (10) methods, and the topologies of the trees obtained were identical. Analysis of the statistical significance of the tree topology was performed by bootstrapping (7). In the tree obtained (Fig. 3), the putative GyrA sequence of *S. pneumoniae* was included in a cluster of GyrA sequences from other gram-positive bacteria, and the putative ParC sequence of *S. pneumoniae* was closely related to that of *S. aureus*. Taken together, these data indicate that the 285-bp and 254-bp PCR fragments amplified from *S. pneumoniae* BM4203 correspond to the QRDRs of *gyrA* and *parC*, respectively.

Detection of mutations in the *gyrA*, *gyrB*, and *parC* genes by amplification and then sequencing. We first tested the possibility that the two fluoroquinolone-resistant mutants obtained in vivo and the six mutants obtained in vitro carried mutations in the QRDR of the *gyrA* or the *parC* gene. A mutation was found in *gyrA* of the high-level ciprofloxacin-resistant mutant BM4205-R3 (Table 2). The position of the resulting Ser→Phe substitution corresponded to Ser-84 in *S. aureus* (Ser-83 in *Escherichia coli*). Changes at this site are most commonly as-

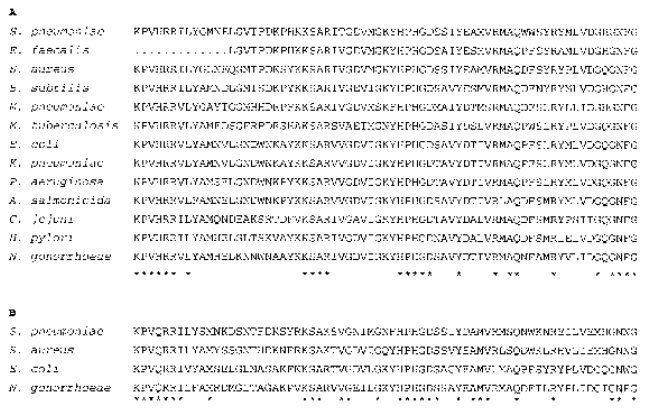

FIG. 2. Sequence similarity among the deduced amino acid sequences of the QRDRs of the *gyrA* and *parC* genes. (A) GyrA sequences; the sequence of *S. aureus* corresponds to amino acids 43 to 111. (B) ParC sequences; the sequence of *S. aureus* corresponds to amino acids 39 to 107. Asterisks indicate the positions where amino acids are identical in all the sequences.

TABLE 3. Sequence identity between the deduced amino acid sequences of the QRDRs of the *gyrA* or *parC* genes from various species[a]

Sequence compared	% Sequence identity								
	SAGYR	EFGYR	BSGYR	ECGYR	NGGYR	SPPAR	SAPAR	ECPAR	NGPAR
SPGYR	84	88	76	61	51	45	45	59	48
SAGYR		80	76	63	56	45	42	63	56
EFGYR			82	67	52	29	33	64	45
BSGYR				72	63	36	48	59	59
ECGYR					82	26	36	59	45
NGGYR						18	33	56	42
SPPAR							59	14	26
SAPAR								36	42
ECPAR									61

[a] Portions of GyrA from *S. pneumoniae* (SPGYR), *S. aureus* (SAGYR), *E. faecalis* (EFGYR), *B. subtilis* (BSGYR), *E. coli* (ECGYR), and *N. gonorrhoeae* (NGGYR) and of ParC from *S. pneumoniae* (SPPAR), *S. aureus* (SAPAR), *E. coli* (ECPAR), and *N. gonorrhoeae* (NGPAR) were compared. All the sequences but that of EFGYR are 69 amino acids in length and correspond to residues 43 to 111 and 39 to 107 of *S. aureus* GyrA and ParC, respectively. The sequence of EFGYR has a length of 56 amino acids and corresponds to residues 56 to 111 of *S. aureus* GyrA.

sociated with fluoroquinolone resistance in the two latter species (8, 9, 36), as well as in other species that have been examined (4, 16, 17, 23). The Ser→Phe change is not the most prevalent substitution at this position, but it has already been described in *B. subtilis* (23). Mutations in *parC* were detected in the two resistant mutants obtained in vivo (BM4203-R and BM4204-R) as well as in two (BM4203-R1 and BM4203-R2) of the six mutants obtained in vitro (Table 2). These mutations led to Ser-80→Tyr or Phe or to Asp-84→His substitutions (*S. aureus* coordinates) that are either identical or similar to those found in low-level-resistant *parC* mutants of *S. aureus*: Ser-80→Tyr or Phe and Glu-84→Lys or Leu (8, 9).

We also amplified and sequenced the region of *gyrB* corresponding to codons 400 to 460 (*S. aureus* coordinates) in which lie all the mutations associated with quinolone resistance described in *E. coli* (35), *S. aureus* (13), and *Neisseria gonorrhoeae* (31). None of the eight resistant mutants examined had mutations in that portion of *gyrB*.

Transfer of fluoroquinolone resistance by transformation. Chromosomal DNA from fluoroquinolone-resistant *parC* mutants BM4203-R and BM4204-R was used to transform *S. pneumoniae* CP1000. When selection for resistance was with sparfloxacin at 1 μg/ml, no transformants were obtained (transformation frequency, $<10^{-6}$ per competent cell). By contrast, on plates containing 2 μg of ciprofloxacin per ml, transformants with the same resistance phenotype as that of the donor (MICs of ciprofloxacin, 8 to 16 μg/ml; MICs of sparfloxacin, 0.5 to 1 μg/ml) were obtained at frequencies of 8×10^{-3} for BM4203-R and 5×10^{-3} for BM4204-R (Table 4). These frequencies are similar to that obtained in the control experiment (7×10^{-3}) performed with total DNA of mutant 119 resistant to rifampin following a single mutational event (33). Thus, total DNAs from BM4203-R and BM4204-R yielded transformants at frequencies compatible with monogenic transformation. In addition, when CP1000 was transformed with the 254-bp *parC* PCR fragment from BM4203-R and BM4204-R, frequencies of transformation (1×10^{-3} and 4×10^{-3}, respectively) similar to those obtained with total DNA were obtained (Table 4). The *parC* QRDRs of two transformants from each experiment that was carried out in duplicate were amplified and sequenced. The eight transformants were found to harbor the same mutation as that present in the donor DNA. Control experiments with H_2O or total DNA from parental strain BM4203 did not yield any resistant colony (transformation frequency, $<10^{-6}$). Taken together, these data strongly suggest that the mutations in *parC* present in mutants BM4203-R and BM4204-R obtained in vivo are solely responsible for fluoroquinolone resistance.

Transformation experiments were also performed with total DNA from high-level fluoroquinolone-resistant *gyrA* mutant BM4205-R3, and two phenotypic classes of transformants were obtained. The first class of transformants, which were resistant to low levels of fluoroquinolones (MIC of ciprofloxacin, 16 μg/ml; MIC of sparfloxacin, 1 μg/ml), was obtained on ciprofloxacin at 2 μg/ml and at a frequency (10^{-3}) compatible with monogenic transformation. The second class of transformants, which had the same resistance phenotype as the donor (MIC of ciprofloxacin, 64 μg/ml; MIC of sparfloxacin, 4 μg/ml), was obtained on sparfloxacin at 1 μg/ml and at a low frequency (4×10^{-5}), compatible with the transformation of two independent genes. Two transformants of each class were studied for the presence of a *gyrA* mutation identical to that in the donor DNA which was found only in the second class of transformants. Furthermore, transformation with the 285-bp PCR fragment of *gyrA* from BM4205-R3 did not yield any resistant colony (transformation frequency, $<10^{-6}$). A transformant of the first class was retransformed with either total DNA or the *gyrA* PCR fragment from BM4205-R3. On plates containing sparfloxacin at 1 μg/ml, transformants with the phenotype of the second class of transformants and harboring the same mutation as the donor DNA were obtained in both cases at

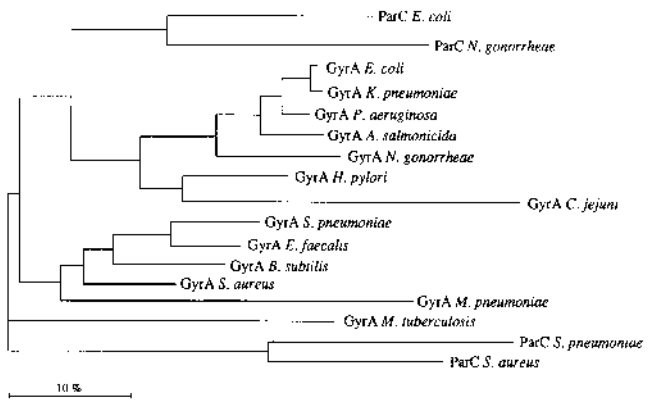

FIG. 3. Phylogenetic tree derived from the alignments presented in Fig. 2 and constructed by the neighbor-joining (28) and maximum parsimony (10) methods. The line below the alignment indicates the distance corresponding to approximately 10% sequence divergence.

TABLE 4. Transformation of *S. pneumoniae* CP1000 with total or PCR-amplified DNA

Donor	Amino acid substitution in donor (subunit)	Transforming DNA	Transformation frequency on ciprofloxacin at 2 μg/ml[a]	Amino acid substitution in transformant (subunit)[b]	Transformation frequency on sparfloxacin at 1 μg/ml[a]	Amino acid substitution in transformant (subunit)[b]
BM4203	None	Total	$<1 \times 10^{-6}$	NA[c]	$<1 \times 10^{-6}$	NA
BM4203-R	Asp-84→His (ParC)[d]	Total	8×10^{-3}	Asp-84→His (ParC)	$<1 \times 10^{-6}$	NA
		PCR (*gyrA*)[e]	$<1 \times 10^{-6}$	NA	$<1 \times 10^{-6}$	NA
		PCR (*parC*)[f]	1×10^{-3}	Asp-84→His (ParC)	$<1 \times 10^{-6}$	NA
BM4204-R	Ser-80→Phe (ParC)	Total	5×10^{-3}	Ser-80→Phe (ParC)	$<1 \times 10^{-6}$	NA
		PCR (*parC*)	4×10^{-3}	Ser-80→Phe (ParC)	$<1 \times 10^{-6}$	NA
BM4205-R3	Ser-84→Phe (GyrA)[d]	Total	1×10^{-3}	None	4×10^{-5}	Ser-84→Phe (GyrA)
		PCR (*gyrA*)	$<1 \times 10^{-6}$	NA	$<1 \times 10^{-6}$	NA

[a] Values are the means of two independent experiments.
[b] Two transformants from each experiment were studied.
[c] NA, not applicable.
[d] *S. aureus* coordinates.
[e] PCR-amplified 285-bp fragment containing the QRDR of the *gyrA* gene of *S. pneumoniae.*
[f] PCR-amplified 254-bp fragment containing the QRDR of the *parC* gene of *S. pneumoniae.*

frequencies (8×10^{-3} and 3×10^{-3}, respectively) compatible with monogenic transformation. Transformants of the second class were also obtained at a high frequency (10^{-3}) when the *gyrA* PCR product of BM4205-R3 was used to transform a *parC* mutant derivative of CP1000. Taken together, these data strongly suggest that the fluoroquinolone resistance of BM4205-R3 is due to the sequential acquisition of two mutations located in different genes: the first one in a gene that is not yet identified and that causes low-level resistance and the second one in *gyrA* conferring high-level resistance. Surprisingly, these two mutations apparently occurred during a one-step selection. Strain BM4205-R3 was obtained on plates containing 16 μg of ciprofloxacin per ml, a concentration corresponding to the MIC for some mutants with single mutations obtained on plates with lower concentrations of ciprofloxacin (Table 2). Thus, the occurrence of a first mutation may have enabled the cells to survive the bactericidal effect of ciprofloxacin and even to grow slowly, whereas a second mutation in *gyrA* provided a selective advantage over the mutants with single mutations. Alternatively, since the resistant clones obtained after the primary plating were restreaked onto agar containing the same concentration of antibiotic to ensure resistance, we cannot eliminate the possibility that the second mutation occurred during replating.

In *E. coli*, inhibition of topo IV becomes apparent phenotypically only when the DNA gyrase is mutated to quinolone resistance (15). Thus, topo IV is a secondary target in *E. coli*, which also seems to be the case in *N. gonorrhoeae* (4). By contrast, in *S. aureus* topo IV appears to be the primary target for the drugs, since one-step mutants with low-level resistance always carry a mutation in *parC* but not in *gyrA* (8, 9). Our data indicate that in *S. pneumoniae*, the occurrence of a mutation in the QRDR of the *parC* gene confers low-level fluoroquinolone resistance. By contrast, mutations in the QRDR of *gyrA* seem to occur only in strains having previously undergone a mutation in another quinolone resistance gene and are responsible for higher levels of resistance. Our observations are consistent with those in the report by Gootz et al. (12), who studied the presence of *parC* or *gyrA* mutations in first- and second-step mutants of *S. pneumoniae* obtained in vitro. The 4 first-step mutants thus examined had a *parC* but not a *gyrA* mutation, whereas the 12 second-step mutants had mutations in both genes. All these data strongly suggest that topo IV is also the primary target for fluoroquinolones in *S. pneumoniae*. The observation that the *gyrB* gene is not implicated in resistance represents further support for the notion that DNA gyrase is not a primary target for fluoroquinolones in *S. pneumoniae*. It thus appears that, more generally, topo IV is the primary quinolone target in gram-positive organisms, whereas it is secondary to DNA gyrase in gram-negative bacteria. This could result from the fact that gyrases of gram-positive bacteria are naturally relatively resistant to inhibition by quinolones (32) or, alternatively, that the topo IV enzymes of these microorganisms are more susceptible to quinolones than are the type II topoisomerases from other bacterial genera.

In *S. aureus*, fluoroquinolone resistance is constantly associated with the presence of mutations in the *parC* gene (8, 9). Such mutations were present in the two mutants of *S. pneumoniae* obtained in vivo but in only two of the six mutants obtained in vitro. These results suggest that acquisition of fluoroquinolone resistance in *S. pneumoniae* may result, at least in vitro, from mutations in other genes. Mutations in the *parE* gene, which encodes the B subunit of topo IV, or active efflux of the drugs (14) could be involved. However, we cannot rule out the presence of *parC* mutations outside of the sequenced region. The sparfloxacin MICs for the seven low-level ciprofloxacin-resistant mutants remained unchanged or increased only by a factor of 2, and these strains remained susceptible to sparfloxacin (MICs, ≤1 μg/ml). By contrast, high-level-resistant mutant BM4205-R3, which exhibits two mutations including one in *gyrA*, was resistant to sparfloxacin (MIC, 4 μg/ml). This suggests that pneumococci become sparfloxacin resistant only after two mutations. The occurrence, in clinical settings, of sparfloxacin-resistant *S. pneumoniae* in two steps is consistent with our previous proposal (5). In conclusion, using gene amplification, sequencing, and transformation, we have shown that mutations in the *parC* and *gyrA* genes play a role in the fluoroquinolone resistance of *S. pneumoniae*. As in *S. aureus*, topo IV appears to be the primary target for fluoroquinolones in *S. pneumoniae.*

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