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High-Level Fluoroquinolone Resistance in *Streptococcus pneumoniae* Requires Mutations in *parC* and *gyrA*

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The mechanism of high-level fluoroquinolone resistance was studied in strains of *Streptococcus pneumoniae*, either selected in vitro or isolated from clinical samples. By using DNA from these high-level-resistant strains, low-level-resistant transformants (MIC of pefloxacin, ≥32 μg/ml; MIC of ciprofloxacin, 4 μg/ml; MIC of sparfloxacin, 0.50 μg/ml) were obtained at high frequencies (ca. 10^{-2}), while high-level-resistant transformants (MIC of pefloxacin, ≥64 μg/ml; MIC of ciprofloxacin, 16 to 64 μg/ml; MIC of sparfloxacin, ≥8 μg/ml) were obtained only at low frequencies (ca. 10^{-4}). This suggested that mutations in at least two unlinked genes were necessary to obtain high-level resistance. Low-level resistance was associated with ParC mutations (change from Ser to Tyr at position 79 [Ser79Tyr], Ser79Phe, or Asp83Gly). ParC mutations were associated, in high-level-resistant strains and transformants, with alterations in the quinolone resistance-determining region of GyrA (Ser84Tyr, Ser84Phe, and/or Glu88Lys). Low-level resistance was shown to be necessary for expression of the *gyrA* mutations. No mutation in the region corresponding to the quinolone resistance-determining region of GyrB and no alteration of drug accumulation were found.

The wide spectrum of fluoroquinolones, which includes gram-negative respiratory tract pathogens, associated with their improved activity against gram-positive pathogens explains why they are prescribed for respiratory tract infections (35). However, their limited activity against *Streptococcus pneumoniae* and the increasing β-lactam resistance observed in this species worldwide (1) have led to the continued search for more active fluoroquinolones. Among these, sparfloxacin appears to be one of the most efficient, with MICs at least two- to eightfold lower than those of ofloxacin and ciprofloxacin (6, 7, 24). Not unexpectedly, however, it was possible to select mutants in vitro with decreased susceptibility and cross-resistance to other fluoroquinolones, including sparfloxacin (21, 30, 34). Strains of *S. pneumoniae* less susceptible to ofloxacin and ciprofloxacin were also isolated in vivo (3, 29), and a trend toward increasing fluoroquinolone resistance in *S. pneumoniae* has been reported (10).

Three events, occurring either alone or in combination, have been found to be responsible for decreased fluoroquinolone activity in gram-negative and gram-positive organisms: (i) mutations in the DNA gyrase, mostly in a small region of the N-terminal portion of the GyrA subunit, the so-called quinolone resistance-determining region (QRDR) (8, 19, 31, 33, 38, 40), or more rarely in a similar region of the GyrB subunit (16, 37, 39); (ii) mutations in DNA topoisomerase IV (2, 11, 12, 14, 18); and (iii) a decrease in fluoroquinolone accumulation (5, 15, 17).

We report here on the mechanisms involved in high-level fluoroquinolone resistance in in vitro-selected mutants and clinical isolates of *S. pneumoniae*.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. The *S. pneumoniae* strains used in this study are listed in Table 1. Two wild-type fluoroquinolone-susceptible strains, R6 and 5455, were used for selection of spontaneous quinolone-resistant mutants. The rest were clinical isolates. Strains were grown either in Todd-Hewitt broth (Difco) supplemented with 0.5% yeast extract (Difco) or in C medium (20) supplemented with 0.2% yeast extract at 37°C.

Isolation of spontaneous quinolone-resistant mutants. The colonies of *S. pneumoniae* from two heavily inoculated agar plates were suspended in 20 ml of buffered glucose broth supplemented with 20% horse serum (Biomérieux, Craponne, France), incubated for 2 h at 37°C, and harvested by centrifugation. The pellet was resuspended, spread on Mueller-Hinton agar plates (Difco) supplemented with 4% horse blood (Biomérieux) and containing antibiotics, and incubated at 30°C for 3 days. Colonies appearing after 2 to 3 days were restreaked onto agar plates containing the same amount of antibiotic as the initial plates.

MIC determination. MICs were determined by the standard method of twofold antibiotic dilution in agar. Inocula of 5×10^3 to 1×10^4 CFU were spotted onto Mueller-Hinton agar plates supplemented with 4% horse blood and containing the following antibiotics: pefloxacin, sparfloxacin (Rhône-Poulenc Rorer, Vitry-sur-Seine, France), ciprofloxacin, Bay y3118 (Bayer Pharma, Puteaux, France), and ofloxacin (Roussel-Uclaf, Romainville, France). MICs were read after 18 h of incubation at 37°C.

Preparation of chromosomal DNA and transformation experiments. DNA was prepared as previously described (22), except that the proteinase K concentration was changed (100 μg/ml). DNA was resuspended either in Tris-EDTA buffer for transformation experiments or in distilled water for PCR. The susceptible strain R6 was used as a recipient for transformation experiments. DNA of strain R119 (36), carrying a streptomycin resistance marker (*str-41*), was used for monitoring the competence of R6. Transformation was carried out as previously described (27, 36). Chromosomal DNA (1 μg/ml) or PCR-generated fragments (10 μg/ml) were added, and the mixtures were incubated for 30 min at 30°C. Phenotypic expression was allowed to occur for 2 h at 37°C before plating of the transformants onto blood agar plates containing fluoroquinolones at different concentrations or streptomycin at 200 μg/ml. After 48 h of incubation at 37°C, the fluoroquinolone susceptibilities of at least five transformants were tested by disk diffusion and MIC determination. In negative control experiments, DNA from all donors was replaced with sterile water and competent cells were plated onto the same selector as the transformants to evaluate the background of selection of spontaneous mutants.

PCR experiments and DNA sequencing. Two degenerate consensus oligonucleotide primers were chosen, on the basis of alignment of the *gyrA* genes of *Escherichia coli, Staphylococcus aureus*, and *Bacillus subtilis*, to encompass the QRDR encoded by the *S. pneumoniae gyrA* gene. According to the numbering for *S. aureus gyrA* (4, 25), primer PNC1 (5′-GACGGTTTAAAACCGGTA/TCAT-3′) corresponds to nucleotides 118 to 138 and PNC2 (5′-CGTTGCCAT ACCTACCGCA/GAT-3′) is complementary to nucleotides 543 to 523. After

TABLE 1. *S. pneumoniae* strains used in this study

Strain	Relevant characteristic(s)[a]	Reference or source
R6	FQ^s	27
R6p16	FQ^r; derived from R6, selected in vitro on pefloxacin (16 μg/ml)	This study
R6p16b1	FQ^r; derived from R6p16, selected in vitro on Bay y3118 (1 μg/ml)	This study
5455	FQ^s; clinical isolate from sputum	This study
5456	FQ^r; derived from 5455, selected in vitro on sparfloxacin (1 μg/ml)	This study
6304	FQ^r; clinical isolate from sputum, selection unknown	3
6305	FQ^r; derived from 6304, isolated from sputum and blood culture from a patient treated with ofloxacin and ciprofloxacin	3
30201	FQ^r; clinical isolate from pus, selection unknown	This study
5486	FQ^r; clinical isolate from sputum, selection unknown	This study

[a] FQ^s, fluoroquinolone susceptible; FQ^r, fluoroquinolone resistant.

sequencing of part of the 426-bp fragment amplified with these oligonucleotides from R6 DNA, primers PNC7 (5′-TTCTCTACGGAATGAATG-3′) (nucleotides 146 to 163) and PNC6 (5′-GATATCACGAAGCATTTCCAG-3′), complementary to nucleotides 417 to 397, were chosen. Two further pairs of primers, PNC8 (5′-CATGGAAAATCCACAGATTG-3′) (1110 to 1129) and PNC9 (5′-ATCGGCATCGGTCATCAAAA-3′) (1524 to 1505) for *gyrB* (26) of *S. pneumoniae*, and PNC10 (5′-TGGGTTGAAGCCGGTTCA-3′) (104 to 122) and PNC11 (5′-CAAGACCGTTGGTTCTTTC-3′) (469 to 450) for *parC* (28) of *S. pneumoniae* were chosen. For each pair of oligonucleotides, PCR was carried out in a PHC-2 thermal cycler (Techne, Cambridge, United Kingdom) in a 100-μl volume containing primers (0.5 μM), deoxynucleoside triphosphates (200 μM), reaction buffer containing magnesium chloride (1.5 mM) (Appligene, Illkirch, France), and 0.6 U of *Taq* polymerase (Appligene). The initial cycle of denaturation (4 min at 94°C) was followed by 40 cycles including 1 min of denaturation at 92°C, 1 min of annealing at 52°C, 1 min of extension at 70°C, and a final cycle of 10 min at 70°C. Amplified fragments of the correct size were purified from 1.2% agarose gels with the QIAquick gel extraction kit (Qiagen) and sequenced with a PCR sequencing kit (Promega) according to the manufacturers' recommendations. Mutations were verified by nucleotide sequencing in at least two independent experiments.

Uptake of fluoroquinolones by whole cells. Cells were grown at 37°C in a complex medium (Biocase "low salt" [Biomérieux], 10 g/liter; tryptone [Difco], 5 g/liter; yeast extract, 4 g/liter; NaCl, 5 g/liter; K_2HPO_4, 17 mM; glucose, 0.2% [wt/vol] [adjusted to pH 7.5]) to an optical density at 550 nm of 0.4 to 0.5, washed once with uptake medium (NaCl, 110 mM; NH_4Cl, 50 mM; KCl, 7 mM; Na_2HPO_4, 0.4 mM; Tris base, 52 mM; HCl, 50 mM [pH 7.5]), and concentrated 20-fold in this medium to approximately 2×10^9 cells per ml. The cell suspension was kept at 4°C until use. For uptake measurements, the suspension was supplemented with 0.2% glucose and warmed at 37°C for 5 min. Fluoroquinolones were added to a final concentration of 20 μg/ml. In some experiments, 100 μg of the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) per ml was added after 5 min. Accumulation was then determined as previously described (9), and the concentrations of ofloxacin and pefloxacin in the supernatant were determined by measuring the fluorescence. The accumulation of sparfloxacin was measured with [^{14}C]sparfloxacin (12 mCi/mM; Rhône-Poulenc Rorer) at 5 μCi/ml.

Nucleotide sequence accession number. The sequence data obtained in this study have been submitted to the EMBL database under accession number 407845.

RESULTS AND DISCUSSION

Selection and characterization of spontaneous fluoroquinolone-resistant mutants. R6p16 was a derivative of R6, isolated on pefloxacin (16 μg/ml) at a very low frequency ($<10^{-9}$). It was cross-resistant to all fluoroquinolones tested, with MICs between 32 and 128 μg/ml, with the exception of Bay y3118; the MIC of this drug, although increased 10-fold, remained below 1 μg/ml (Table 2). R6p16b1 was obtained from R6p16 after selection on Bay y3118 (1 μg/ml). The susceptible clinical isolate *S. pneumoniae* 5455 yielded mutant 5456 after one round of selection on sparfloxacin (1 μg/ml), again at a very low frequency and with a phenotype similar to that of R6p16 (Table 2). The clinical strain *S. pneumoniae* 6304 was spontaneously resistant to pefloxacin (MIC, 32 μg/ml), while the MICs of the other fluoroquinolones were 4- to 16-fold lower than those for the in vitro-selected mutants. Strain 6305, derived in vivo from 6304 and similar in its pulsed-field gel electrophoretic profile (data not shown), had a resistance phenotype similar to that of the in vitro-selected mutants R6p16 and 5456 and the resistant clinical strains 30201 and 5486 (Table 2). All of these mutants were stable after subculture on antibiotic-free medium, in contrast with a previous report (34).

For convenience, all strains for which the MICs of sparfloxacin and ciprofloxacin were ≥8 μg/ml are called high-level resistant (HLR), while the association of sparfloxacin and ciprofloxacin MICs of ≤8 μg/ml with pefloxacin MICs of ≥32 μg/ml is taken to define low-level resistance (LLR).

Transformation experiments. R6 was transformed with DNA from in vitro-selected HLR mutants (R6p16 and 5456) and clinical HLR isolates (6305, 30201, and 5486). Whatever the origin of the DNA, two types of populations were obtained. The first, LLR, was selected on pefloxacin (20 μg/ml) at high frequencies ($\geq 10^{-2}$) compared with those of spontaneous mutants ($<10^{-7}$) and comprised transformants $R6trLL_{R6p16}$, $R6trLL_{5456}$, and $R6trLL_{30201}$ (Table 2). These were characterized by eightfold-increased MICs of pefloxacin and ciprofloxacin and only a twofold-increased MIC of sparfloxacin. Their phenotypes were similar to that of LLR clinical isolate 6304. With DNA from the latter, only LLR transformants were obtained, as expected. The second population, HLR, selected on 4 μg of sparfloxacin per ml, was obtained at lower frequencies (ca. 10^{-4}). The resistance pattern of transformants $R6trHL_{R6p16}$, $R6trHL_{5456}$, and $R6trHL_{30201}$ was similar to that of the parental HLR strains (Table 2). This difference in selection frequency suggests that one resistance marker was integrated by the LLR transformants and at least two were integrated by the HLR transformants. To test this hypothesis, LLR transformant $R6trLL_{R6p16}$ was retransformed with DNA from HLR strains R6p16, 5456, and 5486. In all cases, HLR transformants were obtained at high frequencies ($\geq 10^{-2}$) compared with those of spontaneous mutations (ca. 10^{-5}). This suggested that one additional recombinational event was sufficient for expression of an HLR phenotype in the LLR strain.

Sequencing of the QRDR of GyrA and similar regions in ParC and GyrB. A 306-bp sequence spanning nucleotides 138 to 444 (*S. aureus* numbering [4, 25]) of the R6 DNA fragment amplified with primers PNC1 and PNC2 was determined. The deduced amino acid identity between *S. pneumoniae* and *S. aureus* was 82% for the whole fragment and 90% when only the QRDR (amino acids 67 to 106, *E. coli* coordinates) was considered. The respective identities with GyrA from *Enterococcus faecalis* were 88 and 90%.

Sequencing of the region encoding the QRDR of GyrA was carried out with primers PNC6 and PNC7. Mutations were found in all HLR strains (Table 2). The three HLR clinical strains harbored a silent mutation in the codon encoding the amino acid at position 78 (TAC → TAT) and a C-to-T transition leading to a substitution of Phe for Ser at position 84 (Ser84Phe) (*S. aureus* numbering), a substitution which has been described for quinolone-resistant *Neisseria gonorrhoeae* (8), *Staphylococcus epidermidis* (31), and *S. pneumoniae* (33). In HLR in vitro-selected mutant 5456, a C-to-A transversion

TABLE 2. Nucleotide and amino acid changes in the QRDR of GyrA and in the analogous region of ParC in in vitro-selected mutants, clinical isolates, and their respective LLR and HLR transformants

Strain	Nucleotide[a]/amino acid at position				MIC (μg/ml)[b]			
	Gyrase (GyrA)[c]		Topoisomerase IV (ParC)[d]		PEF	CIP	SPX	BAY
	84	88	79	83				
R6	TCC/Ser	GAA/Glu	TCT/Ser	GAT/Asp	8	0.5	0.25	0.06
R6p16	—[e]	**A**AA/Lys	T**A**T/Tyr	—	128	64	32	0.5
R6trLL$_{R6p16}$	—	—	T**A**T/Tyr	—	64	4	0.5	0.12
R6trHL$_{R6p16}$	—	**A**AA/Lys	T**A**T/Tyr	—	128	64	32	0.5
R6p16b1	T**A**C/Tyr	**A**AA/Lys	T**A**T/Tyr	—	128	64	64	2
5455	—	—	—	—	4	1	0.5	0.06
5456	T**A**C/Tyr	—	—	G**G**T/Gly	64	16	8	0.5
R6trLL$_{5456}$	—	—	ND[f]	ND	64	4	0.5	0.12
R6trHL$_{5456}$	T**A**C/Tyr	—	ND	ND	64	32	8	0.5
30201	T**T**C/Phe	—	T**T**T/Phe	—	64	64	16	1
R6trLL$_{30201}$	—	—	ND	ND	64	4	0.5	0.12
R6trHL$_{30201}$	T**T**C/Phe	—	ND	ND	64	64	16	0.5
6304	—	—	T**A**T/Tyr	—	32	8	0.5	0.12
6305	T**T**C/Phe	—	T**A**T/Tyr	—	64	32	16	1
5486	T**T**C/Phe	—	T**T**T/Phe	—	64	32	16	0.5

[a] Changed nucleotides are in boldface.
[b] PEF, pefloxacin; CIP, ciprofloxacin; SPX, sparfloxacin; BAY, Bay y3118.
[c] Amino acid position corresponding to that of *S. aureus* GyrA (4, 25).
[d] Amino acid position corresponding to that of *S. pneumoniae* ParC (28).
[e] —, no difference from the wild-type strain, R6.
[f] ND, not done.

leading to a Ser84Tyr change was found. This replacement of an aliphatic amino acid by an aromatic amino acid has not been described previously. The G-to-A transition, leading to a Glu88Lys change in HLR mutant R6p16, has previously been associated with high-level resistance to ciprofloxacin in clinical isolates of *E. faecalis* (19) and *S. aureus* (13, 32). In HLR *S. pneumoniae*, it caused higher-level resistance to sparfloxacin than the Ser84Phe or Ser84Tyr change. The association of the Ser84Tyr with the Glu88Lys change in R6p16b1 caused a further increase in resistance, especially to sparfloxacin and Bay y3118. Mutations identical to those of the donor DNA were found in all HLR transformants (Table 2). In contrast, neither LLR transformants nor clinical LLR isolate 6304 had such changes (Table 2), suggesting that the first level of resistance was not due to a mutation in the QRDR of GyrA. Using PCR-generated gyrA fragments from the different HLR strains, we could not select any resistant transformant of R6. However, with LLR transformant R6trLL$_{R6p16}$ as the recipient, HLR transformants were obtained repeatedly at frequencies that were low (5×10^{-5} to 10^{-4}) but at least fivefold higher than those of spontaneous HLR mutants. Such low frequencies are not surprising, considering the small fragment size (23). All these results suggested that the *gyrA* mutations in the HLR strains were acquired or expressed only when the mutation responsible for the LLR phenotype was present.

Sequencing of the region of ParC corresponding to the QRDR, where mutations leading to fluoroquinolone resistance have been described for several species (2, 11, 12, 14, 18), was performed with oligonucleotides chosen on the basis of the recently published sequence of the *parC* gene (28). Mutations at amino acid positions 79 and 83 were found in all clinical and in vitro-selected LLR and HLR strains (Table 2), as well as in LLR and HLR transformants R6trLL$_{R6p16}$ and R6trHL$_{R6p16}$. The Ser79Tyr change observed in the R6 mutants and in clinical strains 6304 and 6305 has previously been associated with low-level resistance in a first-step *S. aureus* mutant (11) and in an apparent second-step mutant of *S. pneumoniae* with low-level ciprofloxacin resistance (28). The Ser79Phe substitution in clinical HLR mutants 30201 and 5486 has been found only in a resistant clinical isolate of *S. aureus* (12). The novel Asp83Gly change is analogous to the Glu84Gly change in ParC of *E. coli* (14).

Nucleotide sequencing (data not shown) of *gyrB*, encoding a region similar to the QRDR, of the HLR strains revealed no difference with respect to the sequence published by Muñoz et al. (26).

Fluoroquinolone accumulation. Accumulation of pefloxacin, ofloxacin, ciprofloxacin, and sparfloxacin was tested in all strains. No significant differences in accumulation were observed among the wild-type strains, their respective mutants, and the LLR and HLR transformants. Representative accumulation kinetics (means of at least three independent assays) are shown in Fig. 1. Addition of the protonophore CCCP led to only a twofold increase in accumulation, regardless of whether the strains were susceptible or resistant (data not shown).

The combined data suggest that high-level fluoroquinolone resistance in *S. pneumoniae* requires at least two distinct mechanisms and that their acquisition is stepwise, just as in *S. aureus* (11) and presumably *E. faecalis* (19). The mutations in GyrA necessary to obtain the expression of high-level resistance could not arise or be expressed unless the topoisomerase IV was mutated first, as demonstrated by the transformation experiments with PCR-generated *gyrA* fragments. Therefore, topoisomerase IV appears to be the primary essential target of the fluoroquinolones in *S. pneumoniae*, similar to what was found for *S. aureus* (12) but in contrast to what seems to be the

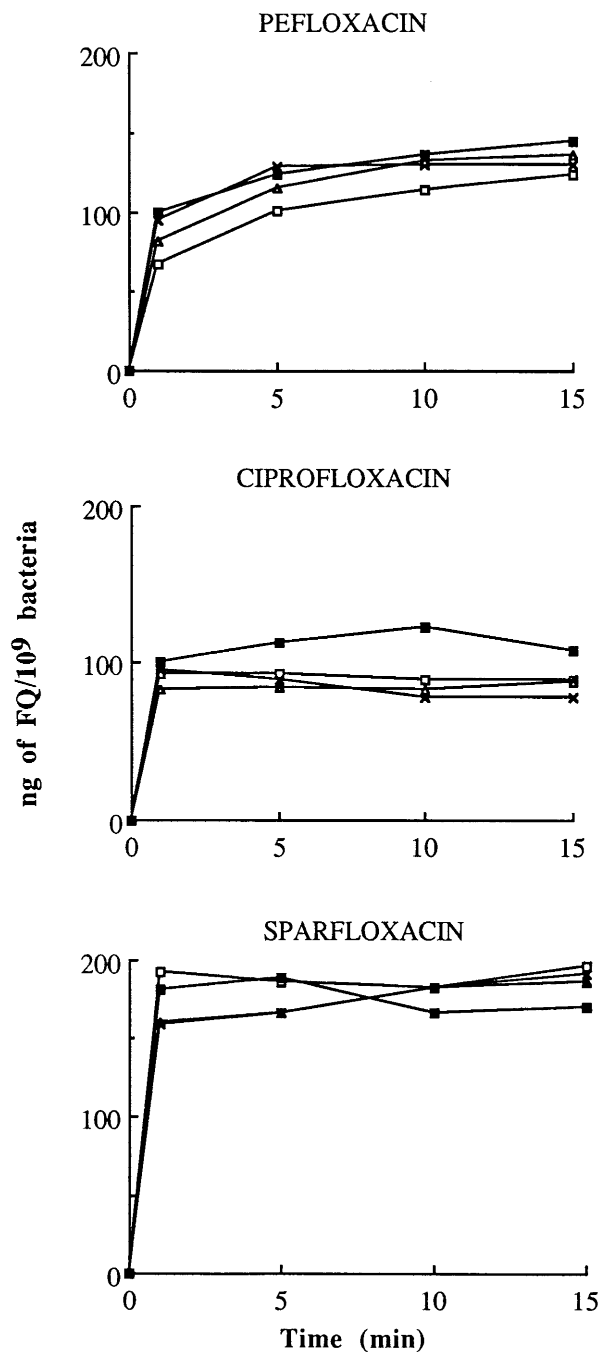

FIG. 1. Kinetics of accumulation of pefloxacin, ciprofloxacin, and sparfloxacin by R6 (□), R6p16 (■), and their respective LLR and HLR transformants $R6trLL_{R6p16}$ (**X**) and $R6trHL_{R6p16}$ (△). FQ, fluoroquinolone.

case for the gram-negative species *E. coli* and *N. gonorrhoeae* (2, 14, 18). It may be anticipated for *S. pneumoniae* that the use of fluoroquinolones with only marginal activity against this species, such as ofloxacin or ciprofloxacin, could compromise the efficacy of the more active compounds.

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