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ParC and GyrA May Be Interchangeable Initial Targets of Some Fluoroquinolones in *Streptococcus pneumoniae*

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To evaluate the role of known topoisomerase IV and gyrase mutations in the fluoroquinolone (FQ) resistance of *Streptococcus pneumoniae*, we transformed susceptible strain R6 with PCR-generated fragments encompassing the quinolone resistance-determining regions (QRDRs) of *parC* or *gyrA* from different recently characterized FQ-resistant mutants. Considering the MICs of FQs and the GyrA and/or ParC mutations of the individual transformants, we found three levels of resistance. The first level was obtained when a single target, ParC or GyrA, depending on the FQ, was modified. An additional mutation(s) in a second target, GyrA or ParC, led to the second level. The highest increases in resistance levels were seen for Bay y3118 and moxifloxacin with the transformant harboring a double mutation in both ParC and GyrA. When a single modified target was considered, only the ParC mutation(s) led to an increase in the MICs of pefloxacin and trovafloxacin. In contrast, the GyrA or ParC mutation(s) could lead to increases in the MICs of ciprofloxacin, sparfloxacin, grepafloxacin, Bay y3118, and moxifloxacin. These results suggest that the preferential target of trovafloxacin and pefloxacin is ParC, whereas either ParC or GyrA may both be initial targets for the remaining FQs tested. The contribution of the ParC and GyrA mutations to efflux-mediated FQ resistance was also examined. Active efflux was responsible for two- to fourfold increases in the MICs of ciprofloxacin for the transformants, regardless of the initial FQ resistance levels of the recipients.

Fluoroquinolone (FQ) resistance is essentially mediated by the inhibition of two closely related type II topoisomerases, DNA gyrase and DNA topoisomerase IV. Topoisomerases are involved in the regulation of chromosome supercoiling and decatenation. Gyrase, composed of the GyrA and GyrB subunits, is the only topoisomerase known to catalyze negative supercoiling of DNA, whereas topoisomerase IV, composed of the ParC and ParE subunits, is the principal decatenase that acts during replication (2, 11).

DNA gyrase has been established as the primary target of the quinolones in most gram-negative bacteria (6) and in mycobacteria (19, 20). Conversely, DNA topoisomerase IV appears to be the primary target of the FQs in gram-positive bacteria, such as *Staphylococcus aureus* (3), *Streptococcus pneumoniae* (7, 13, 15, 23), and enterococci (8, 9, 22). In pneumococci, it was recently reported that low-level FQ resistance was associated with mutations in the quinolone resistance-determining region (QRDR) of ParC at position 79 or 83 and that high-level resistance required additional mutations in the QRDR of GyrA (7, 13, 15, 23). Mutations in ParE have also been found in low-level FQ-resistant mutants (18). At an equivalent position (Asp435), a mutation in GyrB was found in a high-level-resistant mutant which already harbored mutations in the QRDRs of ParC and GyrA (15). In this background, the role of the GyrB mutation in FQ resistance must still be examined. A further mechanism of resistance involving enhanced active FQ efflux has also been reported in *S. pneumoniae* (1, 24).

In a previous study (7), different in vitro- and in vivo-selected FQ-resistant mutants of *S. pneumoniae* were characterized. To evaluate the impact on FQ resistance of the different mutations found in the QRDRs of ParC and GyrA, either alone or combined, we introduced the respective mutated regions by transformation into susceptible pneumococcal strain R6. The results allowed us to indicate some hierarchy of ParC and GyrA as initial targets, depending upon the individual FQ tested. Using the same strategy, we also determined the effect of increased active efflux on the FQ resistance in association with different ParC and GyrA mutations.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. The main characteristics of the pneumococcal strains used as recipients or donors in the transformation experiments are listed in Table 1. Strains were grown either in Todd-Hewitt broth (Difco) supplemented with 0.5% yeast extract (Difco) or in C-medium (10) supplemented with 0.2% yeast extract at 37°C.

MIC determinations. MICs were determined for all strains under the same conditions, and all the antibiotics were tested in the same assay. We used the agar dilution method, with 5×10^3 to 1×10^4 CFU per spot, on Mueller-Hinton agar plates supplemented with 4% horse blood and the following antibiotics: pefloxacin and sparfloxacin (Rhône-Poulenc Rorer, Vitry-sur-Seine, France), ciprofloxacin, Bay y3118, and moxifloxacin (Bayer Pharma, Puteaux, France), trovafloxacin (Pfizer, Orsay, France), and grepafloxacin (under license to GlaxoWellcome, Issy-les-Moulineaux, France, from Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan). In order to determine the MICs of ciprofloxacin, the following intermediate concentrations were used: 0.75, 1, 1.25, 1.5, and 1.75 μg/ml. All MICs were determined in triplicate.

PCR and transformation experiments. DNA was extracted as described previously (7). Fragments encompassing the QRDRs of *parC* (346 bp) or *gyrA* (251 bp) were amplified with the oligonucleotide primers PNC10-PNC11 and PNC6-PNC7, respectively (7). Each PCR was carried out in a PROGENE thermal cycler (Techne, Cambridge, United Kingdom) in a volume of 100 μl containing primers (0.5 μM), desoxynucleoside triphosphates (200 μM each), a reaction buffer containing magnesium chloride (1.5 mM), and 0.8 U of *Taq* polymerase (Appligène, Illkirch, France). The conditions used were those described previously (7). After purification (High pure PCR purification kit; Boehringer Mannheim), the PCR-generated fragments were added to competent pneumococci (14) at ca. 10 μg/ml.

The reaction mixtures were incubated for 30 min at 30°C and then for 2 h at 37°C to allow expression of the resistance before plating on selective media. By using these small PCR-generated fragments, transformants were generally ob-

TABLE 1. Characteristics of the pneumococcal strains

Strain	Origin	Amino acid at the indicated positions in the following proteins:				Active efflux[a]	Reference or source
		ParC[b]		GyrA[c]			
		79	83	84	88		
R6[d]	Susceptible pneumococcal strain	Ser	Asp	Ser	Glu	−	13
R6p16[e]	Derived from R6; selected on pefloxacin (16 μg/ml)	Tyr	—[f]	—	Lys	−	7
R6p16b1b4[e]	Derived in two steps from R6p16; R6p16b1 was selected on Bay y3118 (1 μg/ml), and R6p16b1b4 was selected on Bay y3118 (4 μg/ml)	Try	—	Tyr	Lys	−	7
		Tyr	Tyr	Tyr	Lys	−	23
5456[e]	Derived from a susceptible clinical isolate; selected on sparfloxacin (4 μg/ml)	—	Gly	Tyr	—	−	7
30201[e]	Clinical isolate; selection unknown	Phe	—	Phe	—	−	7
R6tr5929[d]	R6 transformed with total DNA of Spn5929; selected on ciprofloxacin (2 μg/ml)	—	—	—	—	+	23

[a] − and +, absence and presence of increased active efflux, respectively.
[b] Amino acid position according to the *S. pneumoniae* ParC coordinates (16).
[c] Amino acid position according to the *S. aureus* GyrA coordinates (12).
[d] Strain used as recipient in transformation experiments in this study.
[e] Strains used as donors in transformation experiments in this study.
[f] —, no difference from wild-type strain R6.

tained at a frequency of 10^{-4} to 10^{-3}, which was 10- to 50-fold less than that observed when total DNA was used. The concentrations of the selecting agents used were at least twice the MIC for the recipient except for the selection of resistant transformants of R6 with *gyrA* fragments from resistant donors, which required concentrations of sparfloxacin very close to the MIC for R6. After 48 to 96 h of incubation at 37°C, at least four transformants were picked for MIC determination.

DNA sequencing. In order to assess the presence of the expected substitutions, fragments encompassing the QRDRs of *parC* and/or *gyrA* were generated by PCR from all transformants studied. Purified PCR fragments were sequenced with a PCR sequencing kit from Promega according to the manufacturers' recommendations by using primer PNC10 for *parC* and PNC7 for *gyrA*.

RESULTS AND DISCUSSION

Roles of ParC and GyrA in FQ resistance. Different mutants that had been characterized previously (7) were chosen as DNA donors for the transformation experiments. Their main characteristics are listed in Table 1. Using these donor strains, we examined the impact on resistance of various combinations of the following mutations: Ser79Tyr, Ser79Phe, Asp83Tyr, and Asp83Gly in ParC and Ser84Tyr, Ser84Phe, and Glu88Lys in GyrA. These mutations have previously been reported in pneumococci (5, 7, 13, 15, 16, 23). The mutations were introduced into R6 by transformation with amplified QRDR fragments of *parC* or *gyrA* (transformants Tr1 through Tr8), with *parC* being introduced first when successive transformations were performed (transformants Tr9 through Tr20). To rule out the presence of mutations other than those expected in the transformants with a single ParC or GyrA mutation, we sequenced the QRDRs of GyrA, GyrB, and ParE of one of the ParC transformants (transformant Tr4) as well as the QRDRs of GyrB, ParC, and ParE of one of the GyrA transformants (transformant Tr8).

Mutations at positions 79 and/or 83 in ParC. The transformation of R6 with *parC* fragments from the different mutants yielded the low-level FQ-resistant transformants Tr1, Tr2, Tr3, and Tr4 (Table 2), which were selected with pefloxacin (20 μg/ml) at a relatively high frequency (2×10^{-3}). The MICs of pefloxacin and ciprofloxacin for these transformants were increased four- to eightfold, while the MICs of sparfloxacin, grepafloxacin, trovafloxacin, Bay y3118, and moxifloxacin, with the exception of the MIC of trovafloxacin for transformant Tr3 with the Asp83Gly change, were repeatedly increased two- to fourfold. Similar increases in the MIC of sparfloxacin for ParC mutants have previously been observed by Tankovic et al. (23). The single Ser79Tyr, Ser79Phe, or Asp83Gly mutation in ParC, as well as the double mutation Ser79Tyr and Asp83Tyr in ParC, resulted in similar increases in the MICs of each compound.

Mutations at positions 84 and/or 88 in GyrA. First attempts to select resistant transformants of R6 with *gyrA* fragments from resistant donors were unsuccessful with pefloxacin, ciprofloxacin, or even sparfloxacin as the selecting agents. However, by optimizing our selection conditions, using concentrations of sparfloxacin (0.30 to 0.40 μg/ml) very close to the MIC for recipient strain R6, we succeeded in selecting such transformants (transformants Tr5 through Tr8) at frequencies of about 5×10^{-4}. No changes in the MICs of pefloxacin or trovafloxacin were observed. The MICs of ciprofloxacin were not clearly 1 or 2 μg/ml. We therefore used plates containing intermediate concentrations and found that the MICs of ciprofloxacin were 1, 1.5, 2, 1.5, and 1.75 μg/ml for R6, Tr5, Tr6, Tr7, and Tr8, respectively. Thus, at most a twofold increase in the MICs of ciprofloxacin was repeatedly seen; this increase was, however, twofold less than that observed for transformants which had one or two ParC mutations (transformants Tr1 through Tr4). The MICs of sparfloxacin (used as the selecting agent in these transformation experiments) were increased four- to eightfold, while those of grepafloxacin, Bay y3118, and moxifloxacin were increased two- to fourfold. One or two GyrA mutations at positions 84 and/or 88 similarly affected the MICs of each compound for the different transformants.

When the two sets of transformants harboring a mutation(s) in either ParC or GyrA are considered, the question remains whether ParC or GyrA is the preferential target of the FQs tested. Judging from the MICs presented in Table 2 (for transformants Tr1 through Tr8), the answer should be relatively easy to obtain when the MIC is clearly increased after the introduction of one but not the other modified target. As previously suggested by Tankovic et al. (23), Gootz et al. (5), and Janoir et al. (7), ParC appears to be the preferential target of pefloxacin and trovafloxacin, which is in agreement with the present observation that their MICs were increased only when the transformants harbored a mutation(s) in ParC.

TABLE 2. Susceptibilities of R6-derived transformants to seven fluoroquinolones

Strain	Recipient[a]	Amino acid at the indicated positions in the following protein:				Active efflux[b]	MIC (μg/ml)[c]						
		ParC[d]		GyrA[e]									
		79	83	84	88		PEF	CIP	SPX	TRO	GRP	BAY	MOF
R6 (WT[f])		S	D	S	E	−	8	1	0.25	0.25	0.25	0.03	0.12
Tr1	R6*parC*/R6p16	Y	—[g]	—	—	−	64	4	0.5	0.5	0.5	0.12	0.5
Tr2	R6*parC*/30201	F	—	—	—	−	64	4	0.5	0.5	0.5	0.06	0.5
Tr3	R6*parC*/5456	—	G	—	—	−	64	4	0.5	0.25	0.5	0.06	0.25
Tr4	R6*parC*/R6p16b1b4	Y	Y	—	—	−	64	4	0.5	0.5	0.5	0.06	0.25
Tr5	R6*gyrA*/5456	—	—	Y	—	−	8	1–2[h]	1	0.25	1	0.06	0.5
Tr6	R6*gyrA*/30201	—	—	F	—	−	8	2	1	0.25	1	0.12	0.5
Tr7	R6*gyrA*/R6p16	—	—	—	K	−	8	1–2[h]	2	0.25	1	0.12	0.5
Tr8	R6*gyrA*/R6p16b1b4	—	—	Y	K	−	8	1–2[h]	2	0.25	1	0.12	0.5
Tr9	Tr1*gyrA*/5456	Y	—	Y	—	−	128	32	16	4	16	1	4
Tr10	Tr1*gyrA*/30201	Y	—	F	—	−	128	32	16	4	16	1	8
Tr11	Tr2*gyrA*/30201	F	—	F	—	−	128	32	16	8	8	0.5	4
Tr12	Tr1*gyrA*/R6p16	Y	—	—	K	−	128	32	32	8	8	1	8
Tr13	Tr3*gyrA*/5456	—	G	Y	—	−	128	32	8	2	8	0.5	2
Tr14	Tr3*gyrA*/R6p16	—	G	—	K	−	128	32	16	1	4	0.25	2
Tr15	Tr3*gyrA*/R6p16b1b4	—	G	Y	K	−	128	32	16	1	8	0.5	4
Tr16	Tr1*gyrA*/R6p16b1b4	Y	—	Y	K	−	128	32	64	16	16	2	16
Tr17	Tr4*gyrA*/5456	Y	Y	Y	—	−	128	64	16	8	16	0.5	4
Tr18	Tr4*gyrA*/30201	Y	Y	F	—	−	128	32	16	16	16	0.5	4
Tr19	Tr4*gyrA*/R6p16	Y	Y	—	K	−	128	32	64	16	16	1	16
Tr20	Tr4*gyrA*/R6p16b1b4	Y	Y	Y	K	−	128	64	128	16	16	32	>64
R6tr5929	R6^{Spn5929}	—	—	—	—	+	8	4	0.25	0.25	0.25	0.03	ND[i]
$Tr1_{Fqf}$[g]	R6tr5929*parC*/R6p16	Y	—	—	—	+	64	16	0.5	0.5	0.5	0.06	ND
$Tr2_{Fqf}$	R6tr5929*parC*/30201	F	—	—	—	+	32	16	0.25	0.5	0.5	0.03	ND
$Tr3_{Fqf}$	R6tr5929*parC*/5456	—	G	—	—	+	32	16	0.5	0.25	0.5	0.06	ND
$Tr4_{Fqf}$	R6tr5929*parC*/R6p16b1b4	Y	Y	—	—	+	64	16	0.5	0.5	0.5	0.06	ND
$Tr11_{Fqf}$	Tr11^{R6tr5929}	F	—	F	—	+	128	128	16	8	8	0.5	ND
$Tr12_{Fqf}$	Tr12^{R6tr5929}	Y	—	—	K	+	128	128	16	8	8	1	ND
$Tr20_{Fqf}$	Tr20^{R6tr5929}	Y	Y	Y	K	+	128	128	128	8	32	32	>64

[a] The superscripts indicate the PCR-generated fragment or genomic DNA donor strain.
[b] − and +, absence and presence of increased active efflux, respectively.
[c] PEF, pefloxacin; CIP, ciprofloxacin; SPX, sparfloxacin; TRO, trovafloxacin; GRP, grepafloxacin; BAY, Bay y3118; MOF, moxifloxacin.
[d] Amino acid position according to the *S. pneumoniae* ParC coordinates (16).
[e] Amino acid position according to the *S. aureus* GyrA coordinates (12).
[f] WT, wild type.
[g] No difference from wild-type strain R6.
[h] The MICs of ciprofloxacin for transformants Tr5, Tr7, and Tr8 were found to be 1.5, 1.5, and 1.75, respectively.
[i] ND, not done.
[j] Fqf, fluoroquinolone active efflux.

For the other FQs studied (ciprofloxacin, sparfloxacin, grepafloxacin, Bay y3118, and moxifloxacin), the situation is less clear as far as the primary target is concerned, since the introduction of either modified *parC* or modified *gyrA* resulted in a decrease in susceptibility; the decrease was more noticeable, however, with ciprofloxacin after transformation with mutated *parC* and with sparfloxacin and grepafloxacin after transformation with mutated *gyrA*. The latter results do not contradict those of Pan et al. (15) and Pan and Fisher (17), who suggested that ParC is the primary target of ciprofloxacin and that GyrA is the primary target of sparfloxacin. In their experiments, it was probably easier to select one-step mutants with higher levels of resistance when ciprofloxacin was used for the selection of ParC mutants or when sparfloxacin was used for the selection of GyrA mutants. On the other hand, in this study, it would have been difficult to differentiate ParC transformants selected with sparfloxacin or grepafloxacin or GyrA transformants selected with ciprofloxacin from the nontransformant background, since only small increases in MICs were observed for these transformants. Therefore, since for both ParC and GyrA transformants the MICs of these FQs were increased, one could hypothesize that both topoisomerases might function as simultaneous "preferential" or, rather, "initial" targets. This is best illustrated with Bay y3118 and moxifloxacin, the MICs of which were increased to the same degree by the introduction of either the ParC or the GyrA mutation(s). In this matter, it is interesting that, using sparfloxacin (0.5 to 2 μg/ml) as the selecting agent, we were able to isolate after one round of selection mutants from the susceptible clinical strain 5714, which harbored only either the GyrA Ser84Tyr or the ParC Ser79Phe mutation. These mutations resulted in two- and fourfold increases in the MIC of sparfloxacin, respectively (8a). Finally, this raises the question of whether the concept of a primary FQ target in *S. pneumoniae* still holds.

Mutations at positions 79 and/or 83 in ParC, combined with mutations at positions 84 and/or 88 in GyrA. The second round of transformation of transformants Tr1, Tr2, Tr3, and Tr4 with *gyrA* fragments from the different donors (Table 1) led to high-level-resistant transformants (transformants Tr9 through Tr20) at frequencies of about 5×10^{-4}. Compared to the mutations in ParC or GyrA only, the association of both ParC and GyrA mutations resulted in 2- to 512-fold increases

in the MICs depending upon the compound and the mutations. The MICs of pefloxacin and ciprofloxacin were almost identical, independent of the number and nature of the mutations in ParC and GyrA. Considering the other FQs tested, when the Asp83Gly mutation in ParC was associated with one or two GyrA mutations (transformants Tr13, Tr14, and Tr15), the MICs were 2- to 16-fold lower than those observed when the Ser79Tyr mutation in ParC was associated with the same GyrA mutations (transformants Tr9, Tr12, and Tr16).

For Bay y3118 and moxifloxacin, the highest level of FQ resistance was seen with transformant Tr20, which was obtained after the introduction of the GyrA double mutation Ser84Tyr and Glu88Lys into transformant Tr4, which already has two ParC mutations. The MICs for Tr20 were similar to those for the in vitro-selected donor strain R6p16b1b4 (Table 1; data not shown), suggesting that no other mutations were responsible for the highest level of resistance to Bay y3118 and moxifloxacin in this strain.

Contribution of the active efflux to FQ resistance. In a second set of transformation experiments, we tested the contribution of active efflux to topoisomerase IV- and gyrase-mediated FQ resistance in *S. pneumoniae*. The genetic determinant of active FQ efflux (*fqfA*) had previously been transferred to susceptible strain R6 in a single-step transformation, yielding transformant R6tr5929 (24). We used this transformant as a recipient of different PCR-generated fragments of *parC* and *gyrA*. The comparison of the MICs of FQs for the R6tr5929-derived transformants (transformants $Tr1_{Fqf}$ through $Tr20_{Fqf}$) and the R6-derived transformants with the corresponding ParC and GyrA mutations but lacking active efflux showed that the *fqfA* determinant was responsible for a two- to fourfold increase in the MICs of ciprofloxacin only and that its effect was independent of the number and nature of the topoisomerase mutations (Table 2). Similar results were obtained in reverse experiments, by transformation of transformants Tr1 and Tr4 with total DNA of strain R6tr5929 (data not shown). Unexpectedly, for some FQs, as exemplified by Tr20 and $Tr20_{Fqf}$, MICs decreased twofold in the presence of increased efflux. These results were repeatedly seen, and we have no explanation for this phenomenon.

We conclude, first, that the contribution of the increased active efflux to FQ resistance is modest whether it is combined with mutations in ParC or in both ParC and GyrA. Second, the first level of resistance to FQ is apparently reached when one or two mutations occur in either ParC or GyrA, while a further level of resistance is reached when both targets are modified. This is in agreement with previous observations (5, 7, 13, 15, 17, 23). Also, as far as most FQs tested are concerned, it seems that the increases in the MICs for the transformants with altered ParC and GyrA were greater than those that would be expected if one added the increase observed for transformants each containing one altered topoisomerase only. Interestingly, the highest level of resistance to some of the FQs tested is seen when two ParC mutations are combined with two GyrA mutations.

It was previously suggested that for gram-positive bacteria quinolones had preferential targets, either ParC or GyrA, depending upon their structure as well as that of the topoisomerases (4, 15, 17, 21). The MICs of quinolones are thought to result essentially from the inhibition of the preferential target. From the present study, it seems possible that for *S. pneumoniae*, some FQs, such as ciprofloxacin, sparfloxacin, grepafloxacin, Bay y3118, and moxifloxacin, might target both ParC and GyrA, as recently suggested for DU6859a and staphylococci (4, 21). If our hypothesis is valid, it is likely that when either ParC or GyrA is modified, the inhibition of pneumococcal growth should result from the inhibition of both topoisomerases, the wild type and the modified one.

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