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Activities of Different Fluoroquinolones against *Bacillus anthracis* Mutants Selected In Vitro and Harboring Topoisomerase Mutations

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Three sets of mutants of *Bacillus anthracis* resistant to fluoroquinolones were selected on ciprofloxacin and moxifloxacin in a stepwise manner from a nalidixic acid-resistant but fluoroquinolone-susceptible plasmidless strain harboring a Ser85Leu GyrA mutation. A high level of resistance to fluoroquinolones could be obtained in four or five selection steps. In each case, ParC was the secondary target. However, in addition to the GyrA mutation, expression of high-level resistance required (i) in the first set of mutants, active drug efflux associated with a mutation in the QRDR of ParC; (ii) in the second set, two mutations in the QRDR of ParC associated with a mutation in GyrB; and (iii) in the third set, two QRDR mutations, one in ParC and one in GyrA. Interestingly, several selection steps occurred without obvious mutations in the QRDR of any topoisomerase, thereby implying the existence of other resistance mechanisms. Among the fluoroquinolones tested, garenoxacin showed the best activity.

Bacillus anthracis, the etiological agent of anthrax, is a potential threat and could be used in bioterrorism and biological warfare. While different therapeutic regimens may be used for the treatment of anthrax (3, 4, 10, 15, 18), ciprofloxacin is one of the drugs that have been recommended for first-line therapy (4, 18). Recent reports have shown that all *B. anthracis* strains tested were also susceptible to other fluoroquinolones (2, 17). It is very likely that many of them are potential alternatives to ciprofloxacin. In vitro selection of resistant mutants of the *B. anthracis* Sterne strain in the presence of different quinolones, i.e., ciprofloxacin, alofloxacin, and gatifloxacin, was previously reported by one team (1, 5), but the resistance mechanism was not described until recently by Price et al. using the *B. anthracis* Ames strain (21). The present study was carried out in order to identify, in a *B. anthracis* Sterne derivative, the number of steps necessary to obtain high-level resistance to different fluoroquinolones, to characterize the underlying mechanism at the different steps of the selection process, and to determine the activity of different fluoroquinolones against the selected mutants.

MATERIALS AND METHODS

Antimicrobial agents. The antimicrobial agents were obtained from their respective manufacturers: norfloxacin from Merck Sharp and Dohme-Chibret, Paris, France; pefloxacin, ofloxacin, and levofloxacin from Aventis, Vitry-sur-Seine, France; ciprofloxacin and moxifloxacin from Bayer Pharma, Puteaux, France; garenoxacin from Bristol Myers Squibb Laboratories, Wallingford, Conn.; gemifloxacin from SmithKline Beecham Laboratories, Harlow, United Kingdom, and norfloxacin from Sigma, St Louis, Mo.

Bacterial strains and MIC determinations. *B. anthracis* strain 9131 (7) (obtained from Michèle Mock, Pasteur Institute, France), used in this study, is a derivative of the Sterne strain which became plamidless during the selection for nalidixic acid resistance. It harbors a Ser85Leu mutation (this study) compared to the GyrA sequence of the *B. anthracis* Ames strain present in the National Center for Biotechnology Information database (NC003997), but it remains susceptible to the new fluoroquinolones. Resistant mutants were selected stepwise on Mueller-Hinton (MH; Difco) medium containing increasing concentrations of either ciprofloxacin or moxifloxacin (one- to eightfold the MIC for each selected mutant). About 10^9 cells were spread on MH agar containing the antibiotic. At each step, one colony was used for subsequent selection steps at the concentration of fluoroquinolone specified in Table 1. MICs were determined in duplicate by the standard agar dilution method as described previously (19): 10^4 CFU was spotted on MH agar plates containing various concentrations of the antibiotic tested, and MICs were read after 18 h of incubation at 37°C. MICs were also determined in the presence of reserpine (10 μg/ml) (Sigma) to detect an eventual active efflux mechanism of resistance (11); active fluoroquinolone efflux was considered to be present if at least a fourfold decrease in the MIC was observed (11).

PCR experiments and DNA sequencing. Chromosomal DNA was prepared with the InstaGene Matrix kit (Bio-Rad). Amplification of the regions encompassing the topoisomerase II quinolone resistance-determining regions (QRDR) was carried out as previously described (16) using the following oligonucleotide primer pairs: *parC*F (5′-CGTGACGGCTTAAAACCAGTA-3′) and *parC*R (5′-TTCCGTATAACGCATTGCTG-3′), *gyrA*F (5′-CGGCTCTCTTTCAGAACCAT-3′) and *gyrA*R (5′-AAAACCTGTGCATCGTAGGG-3′), *parE*F (5′-ACTTACTTTGTATAAAGGTGGAAGT-3′) and *parE*R (5′-TGGTAAGTTAACACCCGCACA-3′), and *gyrB*F (5′-GCTCTTCAAAAGATCCAGCAA-3′) and *gyrB*R (5′-CGGTGGCTGTGCAATATAGA-3′). Direct sequencing, on both strands, was performed with the oligonucleotides used for amplification by the BigDye Terminator method and an automated 377 DNA sequencer (Applied Biosystems).

RESULTS AND DISCUSSION

Two fluoroquinolones, ciprofloxacin and moxifloxacin, were chosen as selectors. The reason for this choice was that *B. anthracis* strain 9131 was still suceptible to these quinolones and that these compounds might target different topoisomerases preferentially, as they do in *Streptococcus pneumoniae* (20, 22). Since a mutation in *GyrA* was present and affected the suceptibility to nalidixic acid (MIC, 64 μg/ml), we cannot infer which target in this strain would preferentially have been targeted at first by ciprofloxacin or moxifloxacin. However, from

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TABLE 1. MICs of 10 fluoroquinolones against the original strain and mutants of *B. anthracis* BA9131

Strain	Selection[a] (μg/ml)	Amino acid at indicated position[b]					MIC[c] (μg/ml) of:												
		GyrA		ParC		GyrB, 470	CIP	CIP+R	MOX	MOX+R	NOR	NOR+R	GAR	GAR+R	PEF	OFL	LEV	GAT	GEM
		85	89	81	82														
BA9131[d]		L (TTA)	E (GAA)	S (TCC)	S (TCT)	N (AAC)	0.12	0.12	0.12	0.12	0.25	0.25	0.015	0.015	0.25	0.25	0.12	0.06	0.015
BAM1[e]	0.12	L					1	1	1	1	4	4	0.25	0.25	4	4	2	0.5	0.25
BAM2	2	L					2	2	2	2	8	4	0.5	0.5	8	4	2	1	0.25
BAM3	4	L		F (TTC)			8	4	8	8	32	4	0.5	0.5	64	16	8	1	0.5
BAM4	16	L	G (GGA)	F (TTC)			32	32	32	32	64	32	4	4	128	128	64	8	2
BAC1[f]	1	L					2	1	2	2	4	2	0.5	0.5	4	4	1	0.5	0.25
BAC2	4	L			P (CCT)		8	4	4	2	32	16	0.5	0.5	16	8	4	1	0.5
BAC3	8	L			P (CCT)		16	8	4	2	64	32	0.5	0.5	16	8	4	2	0.5
BAC4	16	L		F (TTC)	P (CCT)		32	16	8	8	128	128	1	1	64	32	8	2	1
BAC5	64	L		F (TTC)	P (CCT)	D (GAC)	128	64	8	8	≥256	≥256	2	2	128	128	32	8	8
BAC11[f]	1	L					8	2	2	1	16	4	0.5	0.5	4	4	2	1	1
BAC12	8	L					16	4	2	2	64	16	0.5	0.5	4	4	2	2	2
BAC13	16	L					32	8	4	2	128	32	0.5	0.5	4	8	4	4	2
BAC14	32	L					64	16	4	2	≥256	64	1	1	8	8	4	8	8
BAC15	256	L		Y (TAC)			256	64	16	8	≥256	≥256	2	1	128	64	32	16	16

[a] Concentration of antibiotic used for selection.
[b] No mutation was found in the ParE QRDR.
[c] CIP, ciprofloxacin; MOX, moxifloxacin; NOR, norfloxacin; GAR, garenoxacin; PEF, pefloxacin; OFL, ofloxacin; LEV, levofloxacin; GAT, gatifloxacin; GEM, gemifloxacin; R, reserpine (10 μg/ml).
[d] Plasmidless Sterne derivative strain harboring a Ser85Leu substitution.
[e] Mutants obtained from BA9131 on moxifloxacin.
[f] Mutants obtained from BA9131 on ciprofloxacin.

a recent study using the suceptible *B. anthracis* Ames strain and ciprofloxacin as selector, it was shown that GyrA with the same Ser85Leu mutation was the first subunit to be affected during the selection process (21).

At each step, a single colony was purified and used for selection of the subsequent mutant. Mutation frequencies ranged between 10^{-7} and 10^{-9}. When moxifloxacin was used as the selector (Table 1), all first-step mutants were phenotypically homogenous (BAM1; Table 1). In contrast, when ciprofloxacin was used as the selector, two resistance phenotypes were observed among the first-step mutants (BAC1 and BAC11; Table 1). Each of those three types of mutants was then used for subsequent selection steps with the same quinolone: moxifloxacin for the BAM series and ciprofloxacin for the BAC series.

The first-step mutants selected on either moxifloxacin or ciprofloxacin showed 8- to 64-fold increased MICs of these compounds and no new mutation in the QRDRs of *gyrA*, *gyrB*, *parC*, or *parE*. In the presence of reserpine, one type of mutant selected on ciprofloxacin (BAC11) showed a fourfold decrease in the MIC of ciprofloxacin and norfloxacin but not of moxifloxacin or garenoxacin. It was, nevertheless, not sufficient to reverse the resistance phenotype to that of the parental strain. Therefore, it was considered that an increased efflux was present in the BAC11 mutant and that, similarly to the other first-step mutants (BAM1 and BAC1) an additional, unexplained resistance mechanism, not related to a change in the QRDR, was also present.

In the stepwise resistant mutants of the BAM series (selected on moxifloxacin), those with a moxifloxacin MIC of 8 μg/ml and a Ser81Phe substitution in the QRDR of ParC were selected only at the third step. At the fourth step, mutant BAM4, with a moxofloxacin MIC of 32 μg/ml, was selected. It harbored, in addition to the previous substitution, a Glu89Gly substitution in the QRDR of GyrA.

Using ciprofloxacin, the second-step mutant BAC2 showed a fourfold increased MIC of ciprofloxacin compared to that of BAC1, which could be explained by a Ser82Pro substitution in the QRDR of ParC. The fourth-step mutant, BAC4 showed a ciprofloxacin MIC of 32 μg/ml and a Ser81Phe in addition to the previous Ser82Pro substitution in the QRDR of ParC, with no new mutation in the QRDR of GyrA. This resulted in an unusual association of mutations in ParC. The next step of selection raised the MIC of ciprofloxacin to 128 μg/ml and was associated with an Asn470Asp substitution 15 amino acids downstream of the conserved PLRGK motif in the GyrB QRDR (23). Interestingly, the same mutation was previously described at an equivalent position (Asn470Asp) in ParE of *Staphylococcus aureus* and was associated with increase resistance to fluoroquinolone and increased suceptibility to novobiocin (8, 14). The stepwise mutants generated from strain BAC11 showing increased efflux had no mutation in the QRDR of any topoisomerase subunit until the fifth step of selection, where mutant BAC15 showed a ciprofloxacin MIC of 256 μg/ml and a Ser81 Tyr substitution in the QRDR of ParC.

A cross-resistance to all other quinolones tested was found (Table 1). Interestingly, the MIC of chloramphenicol, a nonquinolone agent, was the same (4 μg/ml) for BA9131 and all its derivatives. The lowest MICs, not higher than 4 μg/ml; were observed for garenoxacin. They were 2- to 8-fold and 4- to 16-fold lower than those observed for gatifloxacin and moxifloxacin, respectively. Thus, the mutants would be within the suceptibility range for garenoxacin, in contrast to most of the quinolones tested (9). It was previously shown that garenoxacin was very active against gram-positive organisms and, in particular, was still active against some strains of *S. aureus* and *S. pneumoniae* harboring mutations in both the topoisomerase IV and the gyrase (6, 12, 13). The size and growth rate of the first- and second-step mutants selected on moxifloxacin (BAM1 and BAM2) were similar to those of the parental strain. However, BAM3 to BAM5 were smaller (two to threefold) and grew at about 50% of the rate at which the parent grew. For the ciprofloxacin mutants, only BAC15 was smaller and grew similar to BAM3. No reversion was observed after 20 generations. This study demonstrates that in neither of the three sets of mutants was the sequence of events similar as far as the order of mutations or the association of substitutions are concerned. In all cases, a significant increase in the MICs was observed at the first step of selection for the three series of mutants (BAM1, BAC1, and BAC11) and no mutation other than that present in the GyrA subunit of the parental strain were detected in the QRDR of either topoisomerase. However, one of the mutants (BAC11) expressed, in addition to an unknown mechanism of resistance, an increased efflux, as inferred from the increased fluoroquinolone susceptibility in the presence of reserpine. In the BAM1 and BAC11 series, apart from the Ser85Leu mutation present in the parental strain, at many steps of selection there were no mutations in the QRDRs of the topoisomerases and there was no increased reserpine-sensitive drug efflux. Obviously, mutations outside of the QRDR, as well as increased efflux through other systems, may have been selected for. Finally, the highest level of resistance was associated with at least two to four mutations in the QRDR of the different subunits of the topoisomerases. Taken together, our results show that high-level resistance to fluoroquinolones in *B. anthracis* may occur via several routes, that it results from different associations of mutations, and that garenoxacin showed the best activity among the different fluorquinolones tested.

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