In Vitro Selection and Characterization of *Bacillus anthracis* Mutants with High-Level Resistance to Ciprofloxacin

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Mutants of attenuated *Bacillus anthracis* with high-level ciprofloxacin resistance were isolated using a three-step in vitro selection. Ciprofloxacin MICs were 0.5 µg/ml for first-step mutants, which had one of two *gyrA* quinolone resistance-determining region (QRDR) mutations. Ciprofloxacin MICs were 8 and 16 µg/ml for second-step mutants, which had one of three *parC* QRDR mutations. Ciprofloxacin MICs for third-step mutants were 32 and 64 µg/ml. Mutants for which MICs were 64 µg/ml had one of two additional mutations within the *gyrA* QRDR or one of two mutations within the *gyrB* QRDR. Mutants for which MICs were 32 µg/ml had no additional target modifications but showed evidence of enhanced ciprofloxacin efflux.

Bacillus anthracis is a spore-forming zoonotic pathogen that occasionally infects humans, causing cutaneous, intestinal, or pulmonary forms of anthrax (9, 11). Although all three human disease forms are rare, the potential for using *B. anthracis* as a biological weapon makes development of antimicrobial resistance a particularly relevant concern. The two recommended therapeutics for human anthrax are doxycycline (DOX) and ciprofloxacin (CIP) (3, 4, 11). Natural resistance to CIP and DOX is undocumented (4, 5, 6); however, resistance to each has been obtained in vitro (2, 22). DOX resistance was conferred on *B. anthracis* by transfection with the pBC16 plasmid carrying the tetracycline resistance gene, *tet* (22). CIP resistance was selected through serial passage, though the genotypic determinants eliciting resistance were not characterized (2).

The bactericidal action of CIP results from the binding of gyrase-DNA and topoisomerase IV-DNA complexes and the release of DNA with double-stranded breaks (for review, see references 7 and 21). Most fluoroquinolone-resistant mutants have amino acid changes in quinolone resistance-determining regions (QRDRs) of the GyrA subunit of gyrase and the ParC subunit of topoisomerase IV. However, resistance can also arise from the overexpression of multidrug transporters and residue changes in the QRDRs of the GyrB and ParE subunits of gyrase and topoisomerase IV. Low-level resistance can be acquired with a single missense mutation within a QRDR or a point mutation in the regulatory region of an efflux pump, but high-level resistance requires a combination of mutations. The stepwise accumulation of QRDR mutations required for high-level CIP resistance follows a species-specific pathway in vitro (8, 19). However, the utility of these in vitro studies for predicting the progression of a given species to a high-level fluoroquinolone-resistant phenotype in vivo is not clear (12, 25).

In order to characterize CIP resistance determinants and

mutation rates in *B. anthracis*, we used an in vitro stepwise selection procedure (8) to develop high-level CIP-resistant mutants. Mutation rates were determined using a Luria-Delbrück model (15), and genotypes of resistant strains were determined at each step. CIP MICs were determined for each unique mutant. Finally, multidrug efflux inhibitors were used to test for enhanced CIP efflux.

Stepwise selection and isolation of high-level CIP-resistant mutants. Spontaneous CIP-resistant mutants of the attenuated *B. anthracis* ΔANR strain (plasmid-cured Ames strain) were selected in a three-step process using standard microbiological techniques. Colonies from blood agar plates were used to inoculate culture tubes containing 5 ml of Mueller-Hinton (MH) broth (Difco). Cultures were incubated overnight at 37°C and then transferred to 0.45-µm-pore-size nitrocellulose membrane filters (Millipore). Membranes were placed cell side up onto MH agar containing CIP at three to four times the MIC for the parent strain (8) and incubated for ~40 h. A single colony from each positive plate was cataloged (either as a frozen stock or as a spore stock) and analyzed for topoisomerase mutations in the *gyrA* and *parC* QRDRs by fluorescent DNA sequence analysis (*gyrB* and *parE* QRDRs were analyzed at the end of the study). QRDRs were amplified by PCR using primers designed from the incomplete *B. anthracis* genome sequence (Table 1; The Institute for Genomic Research, Rockville, Md.). Fifty-microliter PCRs were prepared in PCR buffer (20 mM Tris [pH 8.4], 50 mM KCl) (Gibco) using 0.10 mM deoxynucleoside triphosphates, 2 mM MgCl₂, ~5 ng of template DNA prepared as described previously (14), 0.04 U of *Taq* DNA polymerase/µl (Gibco), and 0.2 µM forward and reverse primers. Reactions were heated to 94°C for 5 min and then cycled 35 times at 94, 60, and 72°C for 20 s at each temperature. PCR products were sequenced on either an ABI377 or an ABI3100 fluorescent sequencer using the ABI PRISM Ready Reaction BigDye terminator cycle sequencing kit according to the manufacturer's specifications (Applied Biosystems). The most common unique genotype from each selection step was used for subsequent selections until high-level CIP-resistant strains were identified.

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TABLE 1. Primers used in this study

Name	Sequence (5' → 3')	QRDR gene
ParCQRDR1F	GTGTTAGGTGACCGCTTTGCACGTTATAGTAAATA	<i>parC</i>
ParCQRDR1R	GTAAACAACCGGTTCTTCACTCGTATCATC	<i>parC</i>
GyrAQDR1F	ACGTATTAATTCATAGAGATTTAGACATTCTTGCTTCTGTATA	<i>gyrA</i>
GyrAQDR1R	CATTTTTAGATTACGCAATGAGTGTTATCGTATCTCG	<i>gyrA</i>
gyrAQDR1 - 03F	ACGCAATGAGTGTTATCGTATCT	<i>gyrA</i>
gyrAQDR1 - 03R	AATATCGGGATTATGACTTA	<i>gyrA</i>
parCQRDR1 - 03F	CTCCCGCTTGAAGACGTGTTAGGT	<i>parC</i>
parCQRDR1 - 03R	AACCGGTTCTTCACTCGTAT	<i>parC</i>
parEQDR1 - 03F	GCCATTGTATCAGAGCATTTAGCTTACTTTT	<i>parE</i>
parEQDR1 - 03R	ATCGGTCATGATTACAACCTTTATCGTA	<i>parE</i>
gyrBQRDR1 - 03F	AGAGGCATTTGAAAAGTTCTTACTA	<i>gyrB</i>
gyrBQRDR1 - 03R	CAATAATTTGACGCATATAACGATAGA	<i>gyrB</i>

Susceptibility testing. MICs were determined by the agar dilution method. Cultures were grown overnight on MH agar supplemented with 5% sheep blood. Colonies were suspended in MH broth to a density equivalent of a 0.5 McFarland standard. Two microliters of this suspension was dropped onto agar dilution plates. Once the spots had been absorbed, the plates were inverted and incubated for 24 h at 35°C. *B. anthracis* ΔANR was used as the reference strain for determinations of MICs for mutants.

Mutation rate estimation. Mutation rates for first-, second-, and third-step CIP-resistant mutants were estimated with 96 independent cultures of *B. anthracis* ΔANR, S1-1, and S2-1, respectively (Table 2). A single colony of the starting isolate was suspended in Luria-Bertani (LB) broth and used to inoculate each of the independent cultures with approximately 1,000 cells. For first- and third-step mutants, 96 1-ml cultures were grown in LB broth in four 24-well plates (Costar). For step 2, 96 100-μl cultures were grown in LB broth in a single 96-well plate (Costar). All plates were incubated overnight at 37°C in a G24 environmental incubator shaker (New Brunswick Scientific) with shaking at 225 rpm. Six cultures were chosen at random for each step and used to determine the

average total number of cells present in each culture. The remaining 90 cultures were plated onto MH plates with CIP concentrations of 0.25, 1.5, and 24 μg/ml for steps 1, 2, and 3, respectively. For step 2, the 100-μl cultures were plated directly. For steps 1 and 3, the 1-ml cultures were transferred to sterile 1.5-ml microcentrifuge tubes and centrifuged at 3,000 × g for 5 min. Approximately 850 μl of the supernatant was removed, and the pellet was resuspended in the remaining broth and plated. All of the plates were incubated at 37°C for ~48 h. Up to four putative resistant colonies from each positive plate were transferred to fresh selective medium and incubated at 37°C for ~48 h to confirm resistance. The number of plates devoid of resistant mutants represents zero mutational events. This value was used with the cell count in the Poisson distribution to estimate the mutation rate for each step (15).

Mutations and mutation rates. The sequential selection of *B. anthracis* on increasing CIP concentrations resulted in a stepwise accumulation of mutations, leading to mutants for which CIP MICs were as high as 64 μg/ml (~1,000-fold higher than that for the wild type) (Table 2). First-step mutants developed at a rate of 6.6×10^{-10} and had one of two mutations

TABLE 2. Mutants identified in this study

Strain ^a	CIP MIC (μg/ml)	Mutation rate	Frequency (%)	<i>gyrA</i> mutation ^b		<i>parC</i> mutation ^b		<i>gyrB</i> mutation	
				Nucleotide	Amino acid	Nucleotide	Amino acid	Nucleotide	Amino acid
ΔANR	0.06	— ^d	—	—	—	—	—	—	—
S1-1	0.5	5.3×10^{-10}	80	<u>C254→T</u>	<u>S85→L</u>	—	—	—	—
S1-2	0.5	1.3×10^{-10}	20	<u>G265→A</u>	<u>E89→K</u>	—	—	—	—
S2-1	8	7.3×10^{-9}	73	C254→T	S85→L	C242→T	S81→F	—	—
S2-2	8	1.6×10^{-9}	16	C254→T	S85→L	<u>C242→A</u>	<u>S81→Y</u>	—	—
S2-3	16	1.1×10^{-9}	11	C254→T	S85→L	<u>G253→A</u>	<u>E85→K</u>	—	—
S3-1 ^c	32	3.1×10^{-10}	65	C254→T	S85→L	C242→T	S81→F	—	—
S3-6	64	6.7×10^{-11}	14	C254→T	S85→L	C242→T	S81→F	<u>A1423→G</u>	<u>N475→D</u>
S3-8	64	3.4×10^{-11}	7	C254→T	S85→L	C242→T	S81→F	<u>G1309→A</u>	<u>D437→N</u>
S3-11	64	3.4×10^{-11}	7	C254→T	S85→L	C242→T	S81→F	—	—
S3-16	64	3.4×10^{-11}	7	<u>G265→A</u>	<u>E89→K</u>	C242→T	S81→F	—	—
				C254→T	S85→L				
				<u>A266→C</u>	<u>E89→A</u>				

^a Strain designations are based upon selection step, CIP MIC, and DNA sequence analysis of the QRDRs. The first part of the designation represents the step at which the strain was identified (e.g., S1-1 was isolated during the first selection step). The second part of the designation is a unique numerical identifier indicating genotype and phenotype. In some cases this designation represents more than one isolate with identical phenotypic and genotypic characteristics.

^b Mutations not underlined are those that were gained in the previous step(s).

^c S3-1 isolates are genotypically identical to the parent strain (S2-1) at *gyrA*, *gyrB*, *parC*, and *parE* QRDRs but are phenotypically distinct based on CIP MIC and INF271 susceptibility.

^d —, not applicable (ΔANR) or no mutation (all other strains).

FIG. 1. Effect of INF271 on the susceptibility of third-step, high-level CIP-resistant *B. anthracis* mutants. S2-1 is the parent strain for the third-step mutants; S3-1, -3, -4, -5, -7, -12, -14, -15, and -17 are third-step mutants with no additional gyrase or topoisomerase mutations. S3-6, -8, -11, and -16 are third-step mutants with additional gyrase mutations. $\Delta\Delta$, *Bacillus subtilis* with inactivated *blt* and *bmr* transporter genes; $\Delta\Delta$ NA, *B. subtilis* strain $\Delta\Delta$ with a functional NorA transporter from the plasmid expression vector pBEV.

within the *gyrA* QRDR (Table 2). Eighty percent of these mutants possessed a C254→T missense mutation in *gyrA* (Table 2). (Note that other, rarer *gyrA* mutations were identified; however, these strains died during the cataloging process due to an asporogenic state and remain unconfirmed.) Second-step mutants developed at a rate of 1.0×10^{-8} and possessed one of three mutations within the *parC* QRDR (Table 2). As with the S1 mutants, one transition, C242→T, was overrepresented (73%) (Table 2). Third-step mutants developed at a rate of 4.8×10^{-10} . In total, fourteen S3 mutants were isolated, nine for which CIP MICs were 32 $\mu\text{g}/\text{ml}$ and five for which MICs were 64 $\mu\text{g}/\text{ml}$. Mutants for which MICs were 64 $\mu\text{g}/\text{ml}$ had one of two additional mutations within the *gyrA* QRDR or one of two mutations within the *gyrB* QRDR (Table 2). Mutants for which MICs were 32 $\mu\text{g}/\text{ml}$ were devoid of additional mutations within any of the topoisomerase QRDRs, *gyrA*, *gyrB*, *parC* and *parE*.

Evidence of multidrug efflux. The absence of additional missense mutations in the QRDRs of many (65%) third-step mutants led to investigations into multidrug transport. Blast searches of the partial *B. anthracis* genome sequence with the gene sequences of the *B. subtilis* multidrug efflux pumps *bmr* (gb L25604) and *blt* (gb L32599) (1) revealed homologues of each. Both of these pumps are members of the major facilitator superfamily and can externalize a diverse group of chemicals, including fluoroquinolones, ethidium bromide, rhodamine dyes, and chloramphenicol (1, 17, 18). Interestingly, ethidium bromide MICs were not increased for any of the CIP-resistant mutants. However, susceptibility to CIP in the presence of the potent efflux inhibitor INF271 (20 $\mu\text{g}/\text{ml}$) was increased eightfold in the nine S3 mutants devoid of additional QRDR mutations (Fig. 1) (16). The susceptibility of the S2 parent strain and the other five S3 mutants was increased only twofold (a one- to twofold increase in CIP susceptibility was observed for all S1 and S2 strains [data not shown]). Disruption of the high-level resistant phenotype among the nine putative efflux mutants was confirmed with another structurally unrelated multidrug transport inhibitor, reserpine (Sigma) (16; data not shown).

The spontaneous stepwise resistance rates calculated in this study (6.6×10^{-10} , 1.0×10^{-8} , and 4.8×10^{-10}) are similar to the rate calculated for rifampin resistance in *B. anthracis* (1.6×10^{-9}) (24) and to those reported for fluoroquinolone resistance in other species (20, 23). These data confirm a normal mutation rate in *B. anthracis* and argue against a reduced mutation rate as a potential explanation for the lack of genetic diversity among *B. anthracis* isolates (10, 13, 14). The targeted stepwise accumulation of mutations (S1 *gyrA* → S2 *parC*) in *B. anthracis* indicates that the primary target of CIP is GyrA and that the secondary target is ParC.

This is the first reported evidence of efflux-based CIP resistance in *B. anthracis*. Further characterization of this phenomenon will provide deeper insight into the different fluoroquinolone resistance mechanisms in this important pathogen.

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