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Impact of Spores on the Comparative Efficacies of Five Antibiotics for Treatment of *Bacillus anthracis* in an *In Vitro* Hollow Fiber Pharmacodynamic Model

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Bacillus anthracis*, the bacterium that causes anthrax, is an agent of bioterrorism. The most effective antimicrobial therapy for *B. anthracis* infections is unknown. An *in vitro* pharmacodynamic model of *B. anthracis* was used to compare the efficacies of simulated clinically prescribed regimens of moxifloxacin, linezolid, and meropenem with the "gold standards," doxycycline and ciprofloxacin. Treatment outcomes for isogenic spore-forming and non-spore-forming strains of *B. anthracis* were compared. Against spore-forming *B. anthracis*, ciprofloxacin, moxifloxacin, linezolid, and meropenem reduced the *B. anthracis* population by 4 $\log_{10}$ CFU/ml over 10 days. Doxycycline reduced the population of this *B. anthracis* strain by 5 $\log_{10}$ CFU/ml (analysis of variance [ANOVA] $P = 0.01$ versus other drugs). Against an isogenic non-spore-forming strain, meropenem killed the vegetative *B. anthracis* the fastest, followed by moxifloxacin and ciprofloxacin and then doxycycline. Linezolid offered the lowest bacterial kill rate. Heat shock studies using the spore-producing *B. anthracis* strain showed that with moxifloxacin, ciprofloxacin, and meropenem therapies the total population was mostly spores, while the population was primarily vegetative bacteria with linezolid and doxycycline therapies. Spores have a profound impact on the rate and extent of killing of *B. anthracis*. Against spore-forming *B. anthracis*, the five antibiotics killed the total (spore and vegetative) bacterial population at similar rates (within 1 $\log_{10}$ CFU/ml of each other). However, bactericidal antibiotics killed vegetative *B. anthracis* faster than bacteriostatic drugs. Since only vegetative-phase *B. anthracis* produces the toxins that may kill the infected host, the rate and mechanism of killing of an antibiotic may determine its overall *in vivo* efficacy. Further studies are needed to examine this important observation.

Bacillus anthracis is a Gram-positive bacillus that causes cutaneous, gastrointestinal, and inhalational anthrax (10). This microbe exists as two forms: vegetative bacteria and spores. In the bodies of mammals and in fluids that are rich in nutrients, such as glucose, inositol, and L-alanine, spores germinate into vegetative bacteria (2, 30, 31) using macromolecules that are prepackaged within the spores (4, 30). The vegetative bacteria reproduce and manufacture the toxins that are responsible for the morbidity and mortality associated with the diseases caused by this pathogen (17). Vegetative bacteria form spores when they are exposed to arid or high-oxygen-containing environments and environments where nutrients are sparse (30, 31). Spores can survive in their dormant state for years (17). Vegetative *B. anthracis* is killed by disinfectants, including alcohol and quaternary ammonium. It is also killed when it is heated at 65°C for at least 30 min, a process known as "heat shocking" (15, 35). Spores are resistant to these disinfectants but are killed with 10% bleach and when autoclaved (17, 29). It is believed that spores are not killed by antibiotics. The effect of antibiotics on the formation of spores by vegetative *B. anthracis* has not been fully explored.

Humans are infected with *B. anthracis* by inhalation or ingestion of its spores and by cutaneous inoculation of spores and/or vegetative bacteria (10). With the inhalation route of infection, *B. anthracis* spores are phagocytosed by alveolar macrophages and are transported to pulmonary hilar lymph nodes. In the hilar lymph nodes the spores germinate into vegetative bacteria, which enter the bloodstream and disseminate throughout the body (17, 26). Vegetative *B. anthracis* produces toxins which cause the rapid death associated with severe anthrax infections.

The inhalation route has been used in acts of bioterrorism and biowarfare. In 2001, *B. anthracis* spores were sent in envelopes through the U.S. postal service as an agent of bioterrorism. Twenty-two people developed cutaneous or inhalational anthrax, leading to five deaths (17). Over 10,000 people who may have been exposed to anthrax spores were prescribed 60- to 100-day courses of antibiotics for postexposure prophylaxis. Forty percent of these individuals did not complete their antibiotic regimens because of noncompliance or drug toxicity (36).

Ciprofloxacin and doxycycline are the standards for treatment of anthrax infections (17). These antibiotics proved effective in the 2001 anthrax attacks, as reported in small clinical case reports, and in studies conducted in animals (6, 7, 13, 15, 18, 19, 42). However, the relative efficacies of ciprofloxacin and doxycycline have never been examined in humans and have not been examined in animals using dosages that simulate the serum drug exposures measured in humans. Yet this information is crucial since the administration of the most rapidly active drug may have a life-saving advantage in

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patients who are critically ill from disease due to this microbe. Furthermore, since *B. anthracis* isolates resistant to ciprofloxacin and doxycycline have been described (1, 3, 5, 9, 12), identification of additional antimicrobial agents with efficacies against this pathogen is needed.

In this study we used an *in vitro* hollow fiber pharmacodynamic model of *B. anthracis* infection to compare the efficacies of simulated clinically prescribed regimens of ciprofloxacin and doxycycline with each other and with those of three candidate antibiotics for the killing of *B. anthracis* and for prevention of emergence of resistance during therapy. Using spore-forming and non-spore-forming isogenic strains of *B. anthracis*, we also characterized the degree to which the spore form had an impact on the observed rate of bacterial killing. Finally, using heat shock studies we characterized the effect of each antibiotic on the balance between spore formation and spore germination and how an alteration in cycling of *B. anthracis* between spore and vegetative phases may contribute to the relative efficacy of each drug versus those of the others.

MATERIALS AND METHODS

Microorganisms. The spore-forming ΔSterne strain can exist in both the spore and vegetative phases, while its nonsporulating isogenic mutant, the CR4 strain (43), exists solely in the vegetative phase. Both strains were supplied by Henry Heine (United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD). These isolates are biosafety level 2 (BSL-2) microbes. The ΔSterne strain lacks the pX01 and pX02 plasmids and therefore does not produce lethal and edema toxins and does not have a capsule. CR4 also lacks the pX01 and pX02 plasmids but does carry a plasmid for the protective antigen (one component of lethal and edema toxins) and kanamycin resistance (43). The isolates were stored at −80°C. For each study, the microbes were streaked onto blood agar plates and incubated overnight at 35°C. Bacterial colonies were directly suspended in Mueller-Hinton II broth (MHB; BBL, Sparks, MD). The bacterial suspensions were diluted to the desired concentrations with medium and were used immediately. The concentrations of bacteria in the suspensions were confirmed by quantitative cultures.

Quantitative cultures conducted on aliquots of the starting bacterial inoculum, some of which were heat shocked (incubation of the bacterial suspension at 65°C for 30 min to kill the vegetative-phase *B. anthracis*) and some of which were not (15, 35), showed that approximately 85% of the starting inoculum consisted of vegetative bacteria. Strain CR4 consists solely of vegetative bacteria (43). Cultures of the entire bacterial suspension of this non-spore-forming strain after it is heat shocked were negative for growth, showing that vegetative *B. anthracis* is killed when heat shocked. In contrast, a spore suspension that was generated by incubating a broth culture of the spore-forming ΔSterne strain with 10 mg/liter of ciprofloxacin for 4 days (to kill the vegetative population but not the spore population) yielded similar quantitative culture results before and after it was heat shocked. Phase-contrast microscopy of the pre-heat-shocked specimen confirmed that the ciprofloxacin-treated ΔSterne suspension consisted solely of spores. The data for the studies using the CR4 and ΔSterne strains showed that heat shocking killed the vegetative *B. anthracis* population but did not affect the viability of the spore population.

Antibiotics. Pharmaceutical grade linezolid, moxifloxacin, and meropenem were purchased from CuraScript, Inc. (Orlando, FL). Ciprofloxacin and doxycycline were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Stock solutions of each antibiotic were stored at −80°C. For each study, aliquots of antibiotic stocks were thawed and diluted to the desired concentrations. The drug solutions were used immediately.

Antibiotic susceptibility and mutation frequency studies. Broth microdilution and agar dilution MICs for the ΔSterne and CR4 strains of the study drugs were determined simultaneously in cation-adjusted MHB according to the methods described by CLSI (8) and on Mueller-Hinton II agar (MHA). These susceptibility studies were conducted in duplicate on three different days. The MICs were read after 24 h of incubation. Minimum bactericidal concentrations (MBCs) were determined by standard methods (32). Mutation frequencies in response to 1.5 times the MIC of linezolid and 2.5 times the MICs of the other antibiotics were determined in at least three separate trials. For the mutation frequency studies, the colonies on MHA that was supplemented with ciprofloxacin, moxifloxacin, linezolid, or doxycycline were counted after the cultures were incubated at 35°C for 4 days. Since meropenem rapidly degraded to below the MICs of the bacterial strains after 24 h of incubation at 35°C (data not shown), the mutation frequency plates that contained this drug were read after 20 h of incubation. A subset of colonies that grew on drug-supplemented agars was subjected to susceptibility testing to confirm that these isolates were less susceptible to the evaluated drug.

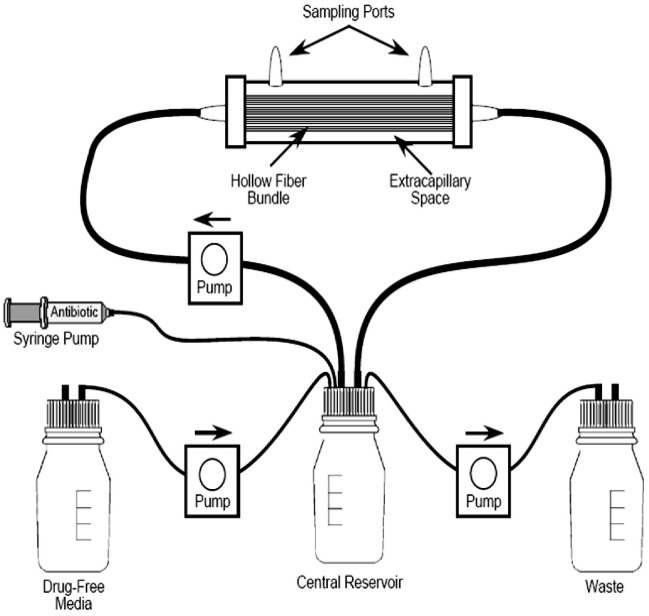

FIG 1 Schematic of a hollow fiber system experimental arm.

Comparative efficacies of five antibiotics against the spore-producing ΔSterne strain. The *in vitro* hollow fiber systems have been described previously (9, 11, 22–24). Bacteria inoculated into the extracapillary space of a hollow fiber cartridge (FiberCell Systems, Inc., Frederick, MD) containing MHB can be exposed to fluctuating concentrations of a drug that simulate the non-protein-bound (free) serum concentration-time profiles reported for clinically prescribed antibiotic regimens that are used in humans (Fig. 1). The antimicrobial effects of these antibiotic regimens can be assessed by conducting serial quantitative cultures of bacterial samples that were collected from the hollow fiber systems over the course of an experiment.

The efficacies of five antibiotics against the spore-producing ΔSterne strain were compared in four separate trials. For each trial, six hollow fiber experimental arms containing MHB were inoculated with 15 ml of a 10^7-CFU/ml concentration of the ΔSterne strain of *B. anthracis*. Heat shock studies demonstrated that approximately 85% of the inoculated suspension consisted of vegetative bacteria and 15% consisted of spores. The first hollow fiber system received no antibiotic and served as the nontreated control arm. The mean steady-state human free serum concentration-time profiles for the following clinically prescribed antibiotic regimens were simulated in the remaining five hollow fiber infection systems: 500 mg ciprofloxacin given every 12 h, 400 mg moxifloxacin given every 24 h, 100 mg doxycycline given every 12 h after a 200-mg loading dose, 600 mg linezolid given every 12 h, and 1 g meropenem given every 8 h. The simulations were for the non-protein-bound (free) fraction of each drug. The

TABLE 1 Targeted steady-state pharmacokinetic values of clinical antibiotic regimens for simulation in hollow fiber systems[a]

Antibiotic regimen[b]	Half-life (h)	Protein binding rate (%)	C_{max} (mg/liter)	C_{min} (mg/liter)	AUC (mg · h/liter)
Ciprofloxacin, 500 mg q12h	4.2	30	2.4	0.39	15.028
Moxifloxacin, 400 mg q24h	12	50	2.3	0.58	30.489
Meropenem, 1 g q8h	1	5	60	0.047	122.105
Doxycycline, 100 mg q12h	18	90	0.32	0.21	6.068
Linezolid, 600 mg q12h	5.5	31	10.4	2.60	134.590

[a] The values are for the non-protein-bound (free) fraction of the drug; they represent levels reported in reference 33 to be in the serum of humans for the regimens specified. C_{max}, maximum concentration of drug in serum; C_{min}, minimum concentration of drug in serum; AUC, area under the concentration-time curve.
[b] q12h, every 12 h.

pharmacokinetic parameters for the drug regimens that were simulated within the hollow fiber systems are shown in Table 1 (33). Treatment continued for 10 days.

Antibiotic therapy was initiated within 1 h after the bacteria were inoculated into the hollow fiber systems. Over the course of the 10-day studies, aliquots of the bacterial suspensions were collected from each system. The suspensions were washed twice to prevent drug carryover and were quantitatively cultured onto drug-free blood agar to determine the effect of antibiotic treatment of the total bacterial population. An aliquot of each bacterial suspension was also quantitatively cultured onto agar supplemented with 2.5 times the MIC of the treatment antibiotic (1.5 times the MIC for linezolid) to characterize the effect of each antibiotic regimen on the amplification of resistant mutants. The lower multiple of MIC for linezolid was used because preliminary studies demonstrated that a ≥1.5-fold increase in MIC was seen in mutants selected with linezolid compared with the MIC for the parent isolate while a ≥2.5-fold increase in MICs was seen in mutants with decreased susceptibilities to the other investigated antibiotics.

Over the first 48 h of each 10-day experiment, 10 to 14 samples of medium were collected from each hollow fiber system and the concentration of antibiotic in each sample was measured by liquid chromatography-tandem mass spectrometry (LC/MS/MS) (ciprofloxacin, moxifloxacin, doxycycline, and meropenem) or LC/MS (linezolid) (11, 12, 22, 23) to confirm that the targeted serum concentration-time profiles reported in Table 1 were simulated. The hollow fiber experiment using the spore-forming ΔSterne strain was conducted four times, and the results were combined. Means and standard deviations for the quantitative culture results were calculated. The significance of differences in total bacterial densities associated with each drug regimen was analyzed by analysis of variance (ANOVA) at the day 1, 2, and 4 time points to check for differences in rapidity of killing for the antibiotic regimens and on day 10 to check for overall differences in treatment efficacies. If differences were identified, multiple pairwise comparisons were made with alpha decay by Bonferroni's adjustment.

Effect of spores on the killing of *B. anthracis* by the five evaluated drugs. In the hollow fiber studies described above, the effect of each antibiotic regimen on the killing of the total bacterial population of the ΔSterne strain was assessed. To characterize the role of spores in defining the rate of killing of *B. anthracis* by each of the five study drugs, a separate set of hollow fiber experiments, described in this section, were used to characterize the effect of antibiotic therapy on the differential rate of killing of the spore-forming ΔSterne strain and the nonsporulation isogenic mutant, CR4. Six hollow fiber systems containing MHB were inoculated with 10^7 CFU/ml of the ΔSterne strain, and another six hollow fiber systems were inoculated with the non-spore-forming CR4 strain. The first hollow fiber system that was inoculated with each of the *B. anthracis* strains served as the nontreatment control arm. The remaining five arms that were inoculated with each of the *B. anthracis* strains were treated with the five antibiotic regimens that were described in the previous experiment and in Table 1. The sampling times for measuring the concentrations of drugs in medium and for conducting quantitative cultures of bacterial samples that were collected from each of the hollow fiber systems over the course of the 10-day studies were identical to those employed in the section "Comparative efficacies of five antibiotics against the spore-producing ΔSterne strain."

These studies were conducted on three separate occasions. Differences in killing of the total bacterial populations of the ΔSterne strain and the CR4 isolate by the antibiotics were statistically assessed on days 1, 2, 4, and 10 of the study by ANOVA. A P value <0.05 was considered significant.

Heat shock studies using the spore-forming ΔSterne strain. To determine the effect of antibiotic therapy on the total and spore populations of *B. anthracis*, another set of hollow fiber experiments were conducted. Hollow fiber system arms were inoculated with 10^7 CFU/ml of the spore-producing ΔSterne strain. The hollow fiber system arms containing MHB were treated with fluctuating concentrations of ciprofloxacin, moxifloxacin, meropenem, linezolid, and doxycycline that simulated the free concentration-time profiles for these drugs, as specified previously (Table 1). These experiments were extended from 10 to 14 days to better characterize the effect of each antibiotic on the spore and vegetative populations. Another hollow fiber system served as the no-treatment control arm. Samples of bacteria that were collected from each hollow fiber system over the duration of the study were divided into two aliquots. One of the two aliquots was heat shocked by incubating the sample in a water bath at 65°C for 30 min to kill the vegetative-phase *B. anthracis* without affecting the viability of spores. Thus, the non-heat-shocked sample quantifies the total (vegetative and spore) population and the heat-shocked sample enumerates the spore population. The arithmetic difference between the total and heat-shocked samples provides an estimate of the size of the vegetative population within that *B. anthracis* suspension. The bacterial samples that were heat shocked and those that were not were washed twice to prevent drug carryover before they were quantitatively cultured. Differences in quantitative culture results on days 1, 2, 4, and 14 of treatment were assessed by ANOVA. A P value of <0.05 was considered significant. The study was conducted twice with the ΔSterne strain.

Effect of candidate antibiotics on viability of *B. anthracis* spores in time-kill studies. Since spores are resistant to harsh chemicals and extremes of environmental temperatures, it is often assumed that the viability of spores is not affected by antibiotics. To investigate whether this assumption is true for the antibiotics examined in this investigation, colonies of the ΔSterne *B. anthracis* strain that were grown overnight at 35°C on a blood agar plate were suspended in medium and heat shocked at 65°C for 30 min to kill the vegetative population. Quantitative cultures of the resultant suspension before and after heat shock were similar, demonstrating that the suspension consisted of spores. The spore suspension was centrifuged to form a pellet, and the pellet was then resuspended to approximately 10^7 CFU/ml using MHB that was supplemented with 0.15 mM D-alanine (Sigma-Aldrich, St Louis, MO). This concentration of D-alanine suppresses the germination of spores into vegetative bacteria by approximately 90% (27). Doxycycline, linezolid, ciprofloxacin, moxifloxacin, and meropenem were added to separate Erlenmeyer flasks of the spore suspensions to achieve the peak concentrations for the respective drugs that were simulated in the hollow fiber experiments. Based on the degradation rate of the antibiotics when incubated at 35°C (data not shown), meropenem was added to the respective flask once daily to reestablish the initial drug concentration. Doxycycline was added to the respective flask once every 4 days. A separate flask of MHB supplemented

TABLE 2 MICs, MBCs, and MBC/MIC ratios for *B. anthracis* ΔSterne and CR4 strains[a]

Antibiotic	ΔSterne strain				Non-spore-forming CR4 strain			
	MIC (mg/liter) by:		MBC[b] in MHB	MBC/MIC ratio	MIC (mg/liter) by:		MBC in MHB	MBC/MIC ratio
	Broth microdilution in MHB	Agar dilution on MHA			Broth microdilution in MHB	Agar dilution on MHA		
Ciprofloxacin	0.06	0.06	1	16	0.06	0.06	0.125	2
Moxifloxacin	0.06	0.06	1	16	0.06	0.06	0.06	1
Meropenem	0.03	0.03	0.5	16	0.06	0.03	0.06	1
Doxycycline	≤0.003	0.06	>64	>1,000	≤0.006	0.125	>64	>1,000
Linezolid	2	2	>64	>32	2	2	>64	>32

[a] Testing was conducted in Mueller-Hinton II broth (MHB) and on Mueller-Hinton agar (MHA). An 80% reduction in growth was used as the MIC endpoint. Results for 3 studies, conducted in duplicate, are reported.

[b] The MBCs were the same for bacterial suspensions of the ΔSterne strain that were heat shocked, a procedure that kills the vegetative-phase microbes.

with D-alanine and the spore suspension was not treated with antibiotics and served as a control arm. The bacterial suspensions were incubated at 35°C in ambient air on a platform shaker. At 0, 5, and 12 h and then on days 1, 2, 3, 4, 7, and 10, aliquots of bacterial suspension were washed to prevent drug carryover. The suspensions were quantitatively cultured before and after heat shock to determine the viability of the spore population with antibiotic exposure. These studies were conducted twice.

A separate experiment using the CR4 strain was conducted to compare the effects of 0.15 mM D-alanine on the growth of vegetative bacilli and on the susceptibility of vegetative bacilli to heat shocking. In the same experiment we also examined the effect of D-alanine on the growth of the vegetative and spore forms of the ΔSterne strain when a spore suspension of the ΔSterne strain was incubated in MHB that was supplemented with this amino acid and one that was not. Briefly, the CR4 and ΔSterne strains were grown overnight on agar at 35°C. Colonies of each strain was suspended in fresh D-alanine-free MHB. The suspension of the ΔSterne strain was heat shocked to produce a spore suspension (confirmed by quantitative cultures of aliquots collected before and after heat shock). Then aliquots of the CR4 and ΔSterne suspensions were incubated in MHB that was supplemented with 0.15 mM D-alanine and in MHB that was not. Samples were taken from each flask over a 10-day period. Half of each sample was heat shocked before both samples were quantitatively cultured to determine the effect of this concentration of D-alanine on the evaluated study endpoints. Antibiotics were not used in this set of studies. These experiments were conducted twice.

RESULTS

MIC, MBC, and mutation frequency values. The MICs and MBCs of the five investigated antibiotics for the spore-forming ΔSterne and the isogenic isolate that exists only as vegetative bacteria (strain CR4) are shown in Table 2. The MICs of ciprofloxacin, moxifloxacin, linezolid, and meropenem individually were similar when testing was conducted in MHB and on agar and did not differ between the two *B. anthracis* strains. The MICs of doxycycline, determined on agar, were manyfold higher than the broth MIC.

The MBCs of ciprofloxacin, moxifloxacin, and meropenem were 16-fold higher than the MIC values for the ΔSterne strain of *B. anthracis*, while the MBC and MIC values for these antibiotics for the CR4 isolate were similar. The MBCs for these drugs for the ΔSterne strain did not change after it was heat shocked, showing that the increased MBCs seen with this strain were due to the spores that are produced by this *B. anthracis* strain.

The MBC/MIC ratios were 1 to 2 for ciprofloxacin, moxifloxacin, and meropenem for the CR4 strain, while the ratios were >32 for doxycycline and linezolid. Antibiotics with MBC/MIC ratios of ≤2 are deemed bactericidal, while those with MBC/MIC ratios of >4 are bacteriostatic. Thus, against CR4 ciprofloxacin, moxifloxacin, and meropenem were bactericidal while linezolid and doxycycline were bacteriostatic. However, against the spore-forming ΔSterne strain, all five antibiotics had MBC/MIC ratios of ≥16. Thus, the five antibiotics were bacteriostatic against the spore-forming ΔSterne strain by the foregoing definition.

The mutation frequencies for each of the five study antibiotics are shown in Table 3 for the ΔSterne *B. anthracis* strain. The mutation frequencies for the isogenic ΔSterne and CR4 strains in response to the antibiotics were similar (data not shown).

Comparative efficacies of five antibiotics against the spore-producing ΔSterne strain of *B. anthracis*. These experiments measure the effect of the different antibiotic regimens on the total (spore plus vegetative) ΔSterne population. In four trials, the controls grew well in the hollow fiber systems, increasing from an average of 7.2 log CFU/ml to approximately 8.5 log CFU/ml within 3 days. The simulated regimens for meropenem, ciprofloxacin, moxifloxacin, and linezolid produced similar overall kill rates for the total ΔSterne population. Doxycycline therapy resulted in an overall lower concentration of bacteria starting on day 4 of therapy ($P = 0.01$), which persisted for the remainder of the 10-day study ($P = 0.01$) (Fig. 2). After 24 h of treatment the five antibiotic regimens reduced the bacterial density of the ΔSterne strain by 0.82 (linezolid) to 1.65 log CFU/ml compared to the 0-h values. The highest rates of antimicrobial killing were seen in the first 4 days of treatment, where a 3.10 to 4.23 log CFU/ml reduction in the bacterial population was seen compared to the bacterial densities that were measured at the start of therapy (Fig. 2). Thereafter, the rates of kill of *B. anthracis* were lower. Emergence of resistance was not observed with any of the antibiotic regimens examined.

The measured concentration-time profiles for each of the antibiotics were within 15% of the targeted profiles (data not shown).

TABLE 3 Mutation frequencies of the *B. anthracis* ΔSterne strain in response to five antibiotics (ranges from three trials)

Antibiotic	Mutation frequency at:		
	1.5× MIC	2× MIC	3× MIC
Ciprofloxacin	—[a]	−7.18 to −7.51	−7.44 to −7.88
Moxifloxacin	—	−8.08 to −8.43	−8.58 to −8.64
Meropenem	—	<−8.54	<−8.54
Doxycycline	—	−7.38 to −8.11	−7.55 to −8.28
Linezolid	−6.46 to −7.24	—	—

[a] —, not done.

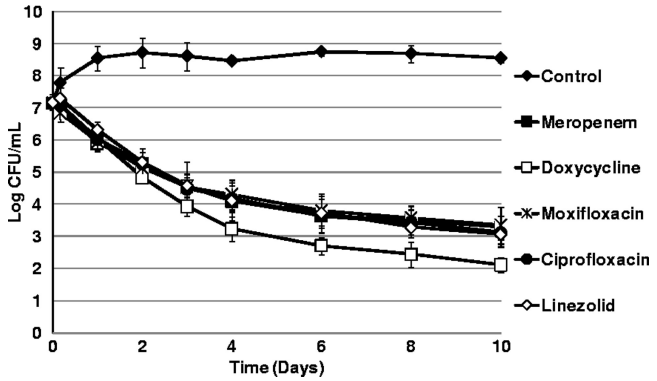

FIG 2 Hollow fiber experiments comparing the effects of five antibiotics against the ΔSterne strain of *B. anthracis*. The bacterial suspension for this spore-forming strain consists of both spores and vegetative bacilli. The data represent the results of four trials. The error bars represent 1 standard deviation (SD).

Comparative killing of the spore-producing ΔSterne and the isogenic non-spore-producing CR4 strains of *B. anthracis*. The ΔSterne strain produces both spores and vegetative bacilli, while the CR4 strain exists only as vegetative bacteria. Figure 3 shows the averaged results of three trials, with Fig. 3B through D showing subsets of the data to highlight some important findings. Figure 3A shows that the spore-forming ΔSterne and nonsporulating CR4 strains grew well within the hollow fiber systems, entering stationary-phase growth in approximately 24 h. Similar to results of the first comparative treatment study (Fig. 2), the rates with which meropenem, ciprofloxacin, moxifloxacin, and linezolid killed the spore-forming ΔSterne strain were similar (Fig. 3A). Doxycycline tended to provide a better kill rate than the other antibiotics, although the difference was not statistically different in these trials.

CR4 exists only as vegetative bacteria. The five antibiotics killed the vegetative bacilli at substantially greater rates than those for killing the spore-forming ΔSterne strain (Fig. 3A and B). Meropenem provided the highest rate of killing of vegetative *B. anthracis*, followed by moxifloxacin and ciprofloxacin and then doxycycline. Linezolid generated the lowest bacterial kill rate.

Figure 3C and D separate the effects for the antibiotics so that the comparative effect of each of the bactericidal and bacteriostatic antibiotics against the ΔSterne and CR4 strains can be clearly visualized. Meropenem, ciprofloxacin, and moxifloxacin killed the CR4 strain faster than the ΔSterne strain by day 1 of therapy, and the better antimicrobial effect against the CR4 strain continued for the remainder of the experiments (Fig. 3C). In con-

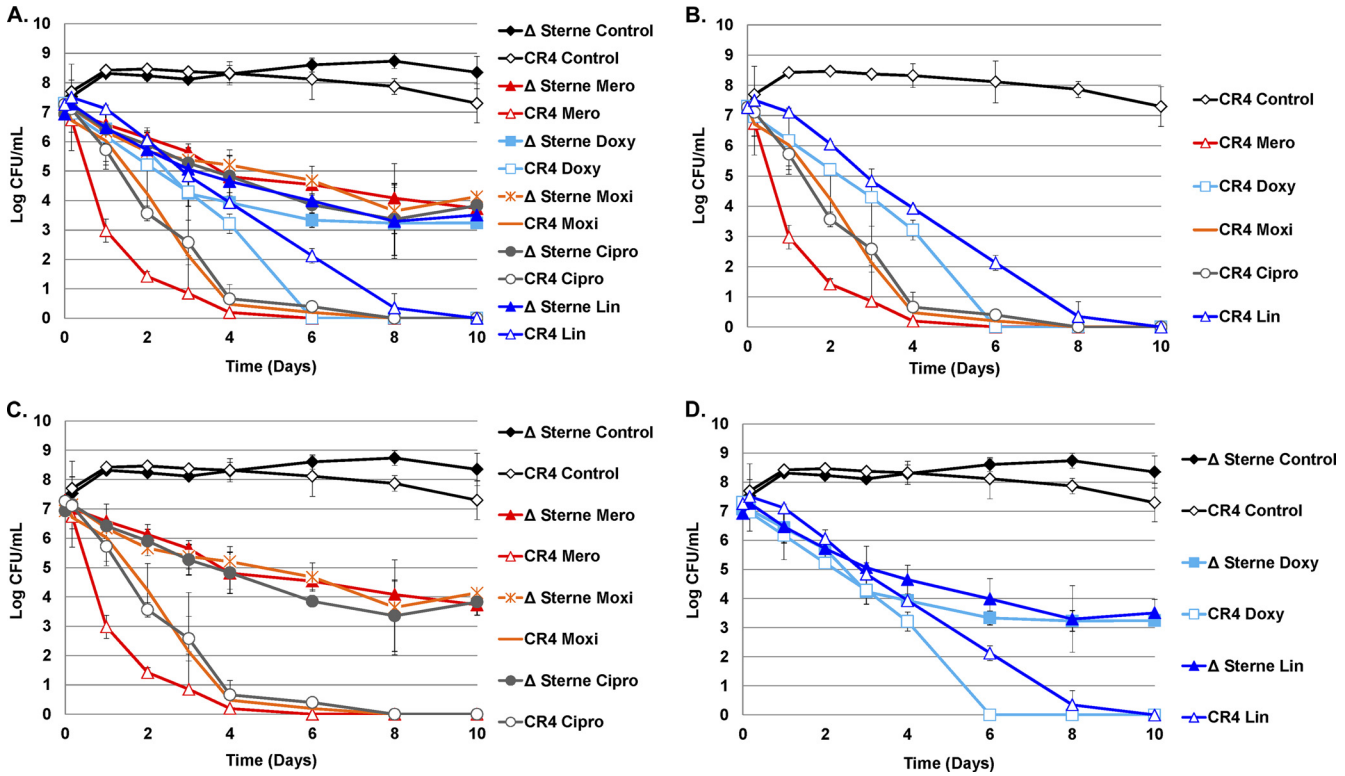

FIG 3 Antimicrobial effects of simulated clinical regimens of linezolid (Lin), moxifloxacin (Moxi), ciprofloxacin (Cipro), meropenem (Mero), and doxycycline (Doxy) on the spore-forming (ΔSterne) strain of *B. anthracis* and a non-spore-forming isogenic mutant (CR4) within a hollow fiber pharmacodynamic model. The ΔSterne strain exists as spore and vegetative-phase bacteria, while the isogenic CR4 strain exists only as vegetative bacteria. The data represent the results of three trials. (A) Effect of the antibiotics on the total populations of the ΔSterne and CR4 strains. (B to D) Subsets of the data presented in panel A for clarity or for highlighting important concepts. Panel B shows only the results of the antimicrobial effect of the five antibiotic regimens on the non-spore-forming strain, CR4. Panel C shows the comparative antimicrobial effects of simulated clinical regimens of meropenem, ciprofloxacin, or moxifloxacin against the ΔSterne and CR4 strains of *B. anthracis*, and panel D shows the comparative efficacies of doxycycline and linezolid for the two *B. anthracis* strains. In all panels, the results for the ΔSterne strain are shown by solid symbols and results for the CR4 strain are shown using the same symbols and color of curves as the antibiotic regimens that were evaluated with the ΔSterne strain except that open symbols were used. The error bars represent 1 SD.

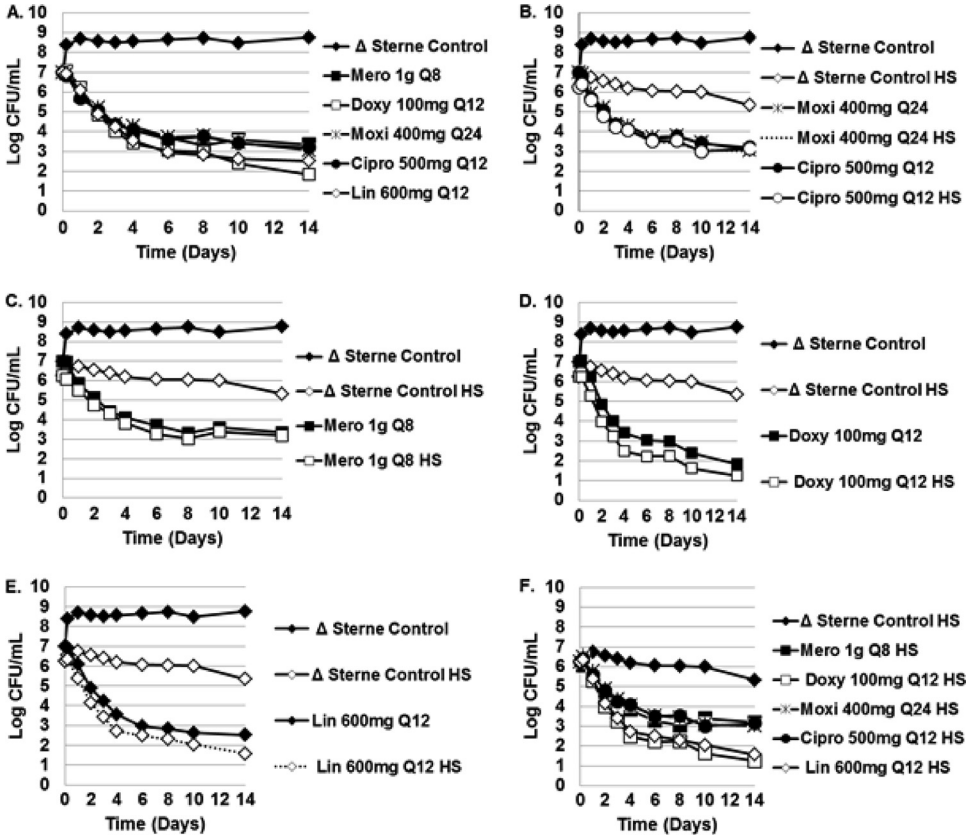

FIG 4 Effect of each antibiotic regimen on the total (spore plus vegetative) and spore populations of the ΔSterne strain of *B. anthracis* elucidated by heat shocking a portion of the bacterial suspensions collected from hollow fiber systems. Panels A through F show different subsets of data derived from one of two experiments that performed similarly in order to highlight specific concepts. (A) Effect of the five antibiotic regimens on the total (spore and vegetative) *B. anthracis* population. (B to E) Antimicrobial effect of moxifloxacin and ciprofloxacin (B), meropenem (C), doxycycline (D), and linezolid (E) on the total (non-heat-shocked) and spore (heat-shocked) populations. Total and spore populations of ΔSterne in the no-treatment control arm are also shown. In panels A to E the total populations are shown using curves with solid symbols and the HS populations are shown using curves with open symbols. HS, heat shock. (F) Effect of the antibiotics on the spore population.

trast, over the first 4 days of treatment, doxycycline and linezolid killed the ΔSterne and CR4 strains at similar rates (Fig. 3D). Thereafter, the rates of killing of vegetative-phase *B. anthracis* (strain CR4) by doxycycline and linezolid persisted, while the rates of killing of the ΔSterne strain by the same antibiotics decreased. On day 6 of the study doxycycline eradicated the CR4 strain. Linezolid eradicated the CR4 strain by day 10 of therapy. In contrast, more than 10^3 CFU/ml of the spore-forming ΔSterne strain was present within the hollow fiber cartridge after 10 days of therapy with any of the 5 investigated antibiotics (Fig. 3C and D).

Heat shock *in vitro* hollow fiber experiments with the spore-forming ΔSterne strain. The ΔSterne strain can exist as spores and vegetative bacilli. At predetermined time points, bacterial samples collected from each hollow fiber system were divided into two aliquots. One aliquot was immediately subjected to quantitative culture to delineate the effect of a treatment regimen on the total (spore plus vegetative) *B. anthracis* population, while the second aliquot was heat shocked before it was quantitatively cultured to define the effect of the same regimens on the spore populations. In this experiment the total population in the control arm increased from approximately 10^7 CFU/ml to 10^8-10^9 CFU/ml and remained within this range for the duration of the experiment (Fig. 4A). Heat shock studies demonstrated that the spore population in the control arm slowly decreased from a value of 6.2 log CFU/ml on day 0 to 5.2 log CFU/ml on day 14 of the experiment (Fig. 4B).

As shown in Fig. 4A, the five antibiotics reduced the total ΔSterne *B. anthracis* populations at similar rates over the first 3 days of treatment. From days 4 to 14, linezolid and doxycycline therapies reduced the concentrations of the total *B. anthracis* populations at a higher rate than ciprofloxacin, moxifloxacin, and meropenem (~0.5 and 1 log CFU/ml differences by day 14 for linezolid and doxycycline, respectively, compared to the other three drugs).

Comparison of the quantitative culture results for bacterial samples that were heat shocked with results for those that were not showed that, with simulated clinical regimens for ciprofloxacin, moxifloxacin, and meropenem, the total (spore plus vegetative) population and the spore-only populations were similar over the 14-day experiment (Fig. 4B and C). In contrast, with linezolid and doxycycline therapies, the total (spore plus vegetative) population was greater than the spore population by approximately 0.75 log CFU/ml (Fig. 4D and E) between days 4 and 14 of treatment. A 0.75 log CFU/ml difference between the total and spore populations means that vegetative bacteria comprised approximately 83% of the total population (% vegetative population = [total

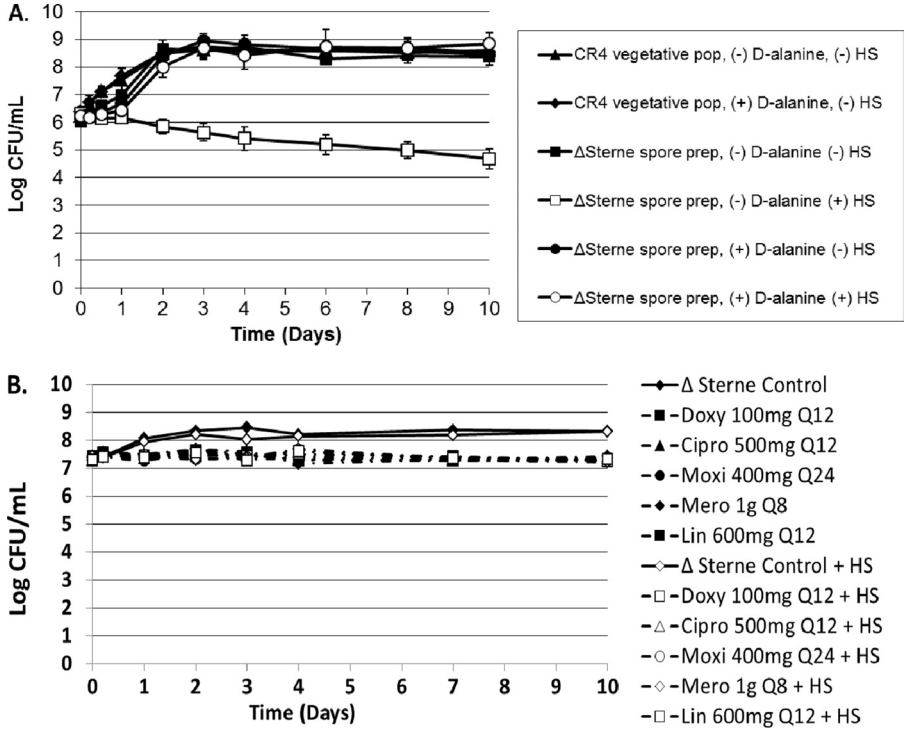

FIG 5 (A) Comparison of increases in the ΔSterne spore and vegetative bacillus populations from an initial spore suspension that was incubated in MHB that was supplemented with 0.15 mM D-alanine, a concentration that reduced the rate in which spores germinate to vegetative bacilli by approximately 90% (27), and one incubated in MHB that was not supplemented. Quantitative cultures were conducted on samples taken from flasks that were not heat shocked (solid symbols) and flasks that were heat shocked (open symbols) to evaluate the effect of D-alanine on the spore and vegetative populations. Growth of CR4, the strain that exists only as vegetative bacilli, was used as a comparator. Error bars, 1 SD. (B) Viability of ΔSterne spores that are exposed to ciprofloxacin, moxifloxacin, meropenem, linezolid, and doxycycline. The MHB was supplemented with D-alanine. Solid curves represent the non-antibiotic-treated control arms that were or were not heat shocked. The broken curves represent the antibiotic-treated arms (total and heat-shocked arms). This experiment was conducted twice. For clarity, the error bars are not shown.

population − spore population]/total population = [non-heat-shocked population − heat-shocked population]/non-heat-shocked population). Figure 4F shows the effect of the different antibiotic therapies on the heat-shocked (spore) population. The spore population decreased faster with doxycycline and linezolid therapies than with moxifloxacin, ciprofloxacin, and meropenem.

Viability of spores incubated with antibiotics. The levels of growth of CR4 in antibiotic-free MHB that was supplemented with 0.15 mM D-alanine and in MHB that was not supplemented were similar, showing that D-alanine did not affect the replication of vegetative *B. anthracis* (Fig. 5A).

For the ΔSterne spore suspension that was incubated in MHB without D-alanine supplementation, there was a delay in the growth of the ΔSterne strain relative to CR4 (Fig. 5A). Since only vegetative bacteria can replicate, the delay reflected the time needed for the spores to germinate into vegetative bacteria before they could start multiplying. Incubation of the initial ΔSterne spore suspension in MHB containing 0.15 mM D-alanine resulted in a further delay in the increase in the total *B. anthracis* population (Fig. 5A). It is likely that the additional delay was due to the lower rate of germination of spores to vegetative forms in the presence of this amino acid. The heat shock studies showed that D-alanine reduced the rate at which spores germinated into vegetative bacilli since the total and spore populations increased when this bacterium was incubated in MHB supplemented with D-alanine but decreased when the ΔSterne strain was incubated in medium that was free of D-alanine (Fig. 5A).

In a separate set of experiments, a spore suspension of the ΔSterne strain was produced by heat shock. The spore suspension was then incubated in medium containing 0.15 mM D-alanine and one of the five antibiotics. Phase-contrast microscopy confirmed that the ΔSterne suspensions consisted of spores. Over the next 10-days, quantitative cultures of the spore suspensions before and after heat shocking yielded similar results, showing that none of the five antibiotics affected the viability of the spores (Fig. 5B).

DISCUSSION

Since inhalational anthrax is a rare disease in humans and since it is unethical to intentionally infect people with *B. anthracis*, randomized controlled, double-blinded clinical trials cannot be conducted to define the relative efficacies of different antibiotic regimens for the treatment of infections due to this pathogen. Yet, identification of the antibiotic regimen that kills *B. anthracis* at the highest rate may improve treatment outcomes for this rapidly progressive, often fatal disease. Moreover, the pharmacokinetics of drugs frequently differ between animals and humans (9, 19). Thus, studies in animals may not predict the relative efficacies of clinically prescribed antibiotic regimens in people (9, 21).

In this study we simulated the mean human serum concentration-time profiles for clinically prescribed regimens of

the "gold standards" for anthrax therapy, ciprofloxacin and doxycycline, and three candidate antibiotics in an *in vitro* hollow fiber pharmacodynamic model to determine which drug regimen offered the best antimicrobial effect against the spore-producing ΔSterne *B. anthracis* strain. The comparative studies showed that ciprofloxacin, moxifloxacin, meropenem, and linezolid produced similar rates and extents of bacterial killing of the total *B. anthracis* population (Fig. 2). Each resulted in a 4 log CFU/ml reduction in the bacterial density after 10 days of therapy. However, the simulated clinical regimen for doxycycline was superior to these four antibiotics since doxycycline produced a higher rate of killing of *B. anthracis*, resulting in a 5 log CFU/ml reduction in the bacterial density with 10 days of treatment ($P < 0.01$) (Fig. 2).

The finding that linezolid was equivalent to ciprofloxacin, moxifloxacin, and meropenem and that doxycycline killed better than these antibiotics was unexpected since linezolid and doxycycline are bacteriostatic against other bacterial species, including *Staphylococcus aureus* and coagulase-negative staphylococci, while ciprofloxacin, moxifloxacin, and meropenem are bactericidal against these pathogens (14, 20). Based on the effects of these antibiotics against staphylococci, these antibiotics were expected to be more effective than doxycycline and linezolid against *B. anthracis*.

The expectation that doxycycline and linezolid would perform as well as or be less effective than ciprofloxacin, moxifloxacin, and meropenem was also suggested by the MBC/MIC ratios of these drugs for the CR4 strain, which exists only as vegetative bacilli (Table 2). Bactericidal agents typically have MBC/MIC ratios of 1 or 2, while bacteriostatic agents have MBC/MIC ratios of ≥4 (41). For *B. anthracis* CR4, the MBC/MIC ratios were ≤2 for ciprofloxacin, moxifloxacin, and meropenem and >32 for doxycycline and linezolid, suggesting that ciprofloxacin, moxifloxacin, and meropenem were bactericidal against vegetative *B. anthracis* while doxycycline and linezolid were bacteriostatic. Against the ΔSterne strain (which produces spores and vegetative bacteria) the MBC/MIC ratios were ≥16 for all five antibiotics, suggesting these drugs would have a bacteriostatic and, hence, a slow killing effect against the spore-producing *B. anthracis* isolate. However, the MBC/MIC ratios were >32 for linezolid and >1,000 for doxycycline, suggesting that these antibiotics would be the least effective of all the antibiotics evaluated. Application of heat shock to the ΔSterne bacterial suspension to kill the vegetative bacterial subpopulation (but not the spore population) before it was used for MBC determinations did not alter the MBCs of the antibiotics for this *B. anthracis* strain (data not shown), suggesting that bacterial spores were responsible for the higher MBC/MIC ratio seen with the ΔSterne strain.

The simultaneous comparison of the rates of killing of the ΔSterne and CR4 strains by the five antibiotic regimens in hollow fiber studies confirmed that spores heavily influenced the rate and extent of killing of *B. anthracis* by these drug regimens (Fig. 3A). In these comparative experiments the five antibiotics killed the spore-producing ΔSterne strain at similar rates, resulting in total bacterial densities that were within 1 log CFU/ml of each other after 10 days of treatment (with final total bacterial counts of 3 to 4 log CFU/ml). All the antibiotics tested killed the CR4 strain, which exists solely as vegetative bacteria, faster than the ΔSterne strain. Further, against the CR4 strain, there was a clear superiority of effect among the antibiotics examined. Meropenem provided the highest rate of killing of vegetative *B. anthracis*, followed by ciprofloxacin and moxifloxacin and then doxycycline. Linezolid generated the lowest rate of killing. Meropenem reduced the CR4 vegetative phase bacteria to undetectable levels (<50 CFU/ml) after 6 days of treatment. Ciprofloxacin, moxifloxacin, and doxycycline achieved this endpoint with 6 to 8 days of therapy, and linezolid did so with 10 days of treatment (Fig. 3B). Thus, these studies showed that spores significantly influenced the overall rate and extent of killing of the ΔSterne strain for all of the antibiotics evaluated.

But why do the "bacteriostatic" drugs doxycycline and linezolid perform better than or as well as the "bactericidal" agents ciprofloxacin, moxifloxacin, and meropenem in the killing of the spore-forming ΔSterne strain? Insights are provided by the heat shock studies employing the ΔSterne strain, which suggest that differences in the mechanisms by which the bacteriostatic and bactericidal antibiotics affect the cycling of *B. anthracis* between spore and vegetative forms determine the overall efficacies of these two groups of antibiotics.

Heat shocking of a ΔSterne *B. anthracis* suspension kills the vegetative population without affecting the viability of the spores (35; this study). Thus, quantitative culturing of a ΔSterne suspension before and after it is heat shocked delineates the effect of an antibiotic on its total and spore populations, respectively. The arithmetic difference between the two populations provides an estimate of the size of the vegetative population in that *B. anthracis* suspension.

In the current project we show that 0.15 mM D-alanine does not alter the rate of growth of vegetative *B. anthracis* strain CR4 (Fig. 5A). The delay in the rise in the ΔSterne population relative to CR4 is likely due to the time required for spores to germinate into vegetative bacteria since only vegetative bacteria can replicate. The heat shock studies show that D-alanine reduces the rate in which ΔSterne spores germinate into vegetative *B. anthracis* (16, 27) (Fig. 5A) since the spore populations in the ΔSterne growth control arms increase in antibiotic-free MHB that is supplemented with D-alanine but decrease in antibiotic-free medium without D-alanine (Fig. 5A). Further, the addition of D-alanine to the MHB does not protect vegetative *B. anthracis* from being killed by the heat shock procedure since CR4 suspensions grown in MHB that is supplemented with D-alanine and those grown in MHB that is not both yield sterile cultures after heat shocking (data not shown).

The emergence of a vegetative population from the ΔSterne spore suspension shows that spores do germinate into vegetative bacteria *in vitro* (Fig. 5B). Importantly, since spores do not replicate, the rise in the spore population in MHB supplemented with D-alanine demonstrates that vegetative bacilli do sporulate *in vitro*, hence completing the spore↔vegetative *B. anthracis* cycle (Fig. 5B).

Incubation of the ΔSterne strain in MHB containing D-alanine and any of the five antibiotics examined in this project generated a bacterial suspension that consists of spores (i.e., the quantitative cultures of these suspensions before and after heat shocking yielded similar results [Fig. 5B]). Since the concentrations of spores incubated with antibiotic and D-alanine were stable over the 10-day study, it is clear that none of the antibiotics used in this investigation kill spores (Fig. 5B).

Treatment of the ΔSterne strain with the bacteriostatic antibiotic linezolid or doxycycline reduces the total bacterial population by an extent similar to the reduction achieved by treatment with

the bactericidal drugs ciprofloxacin, moxifloxacin, and meropenem or to a greater extent (Fig. 2 and 4A). The heat shock studies reveal that the total ΔSterne populations that are treated with ciprofloxacin, moxifloxacin, or meropenem consist primarily of spores (Fig. 4B and C). To our surprise, the total ΔSterne populations that are treated with linezolid or doxycycline consist primarily of vegetative bacilli (Fig. 4D and E). Furthermore, with linezolid and doxycycline therapies the spore populations decrease at higher rates than are observed with ciprofloxacin, moxifloxacin, or meropenem (Fig. 4F). This suggests that linezolid and doxycycline reduce the vegetative and spore populations through a different mechanism than ciprofloxacin, moxifloxacin, and meropenem.

In a previous project we derived a mathematical model from hollow fiber study data which predicted that the ΔSterne spores incubated in antibiotic-free medium and medium supplemented with ciprofloxacin germinate into vegetative bacilli at similar rates (11). In the current investigation, we show that none of the five antibiotics examined kill *B. anthracis* spores (Fig. 5B). These findings suggest that the reduction in the spore population with ciprofloxacin, moxifloxacin, and meropenem therapies is due to the loss of the spores as they germinate into vegetative bacilli. Further, since these three antibiotics do kill vegetative bacteria (Fig. 3B and 4B and C), they also decrease the pool of vegetative bacteria that are available to form new spores. Consistent with this hypothesis is the observation that the spores decline faster in the antibiotic-treated arms than in the control arm of the hollow fiber systems (Fig. 4B to E). Since antibiotics do not kill spores and do not accelerate the speed at which spores germinate (which results in loss of spores), the higher rate of reduction of the spore population in the treatment arms versus the control group suggests that vegetative bacilli spontaneously cycle between spore and vegetative phases in the control arm and that antibiotic therapy diminishes the rate at which vegetative bacilli form new spores.

With linezolid and doxycycline treatments the rates of reduction of the total (primarily vegetative) populations are similar to or higher than the rates associated with ciprofloxacin, moxifloxacin, and meropenem therapies, although the rates of reduction in the spore population are higher with linezolid and doxycycline than with the other antibiotics (Fig. 4A and F). Linezolid and doxycycline do not affect the viability of *B. anthracis* spores (Fig. 5B). Thus, similar to results for ciprofloxacin, moxifloxacin, and meropenem, the decline in the spore population with linezolid and doxycycline therapies for the ΔSterne strain is due in part to the loss of spores as they germinate into vegetative bacteria. Our experiments with the CR4 strain shows that linezolid and doxycycline kill vegetative bacteria at a lower rate than ciprofloxacin, moxifloxacin, and meropenem (Fig. 3A). The lower rate of killing also may contribute to the large vegetative population that is seen in the linezolid and doxycycline arms compared to that for the other antibiotics tested.

However, one would expect that the larger vegetative population that is observed with linezolid and doxycycline therapies would result in a lower rate in reduction of the spore population than is seen with ciprofloxacin, moxifloxacin, and meropenem because more vegetative bacilli are available to form new spores. The faster decline in the spore population together with the large size of the vegetative populations associated with linezolid and doxycycline therapies suggests that these two protein synthesis-inhibiting antibiotics (34, 40) interrupt the formation of new spores by the vegetative population, perhaps by stopping the manufacture of enzymes and other molecules that are needed for vegetative bacteria to produce new spores. Since ciprofloxacin, moxifloxacin, and meropenem are not protein synthesis inhibitors, these antibiotics are not expected to block the synthesis of the materials that are needed for spore formation.

Importantly, interrupting the cycling of vegetative *B. anthracis* back into spores with linezolid and doxycycline compensates for the slower killing of the vegetative population by these bacteriostatic antibiotics. Spores are not killed by antibiotics. The inability of vegetative bacilli that are exposed to linezolid and doxycycline to form spores provides these slower-killing antibiotics with ample time to kill the vegetative population. The net effect is that the efficacies of the bacteriostatic drugs linezolid and doxycycline are similar to or better than the efficacies of the bactericidal agents ciprofloxacin, moxifloxacin, and meropenem. It is possible that doxycycline clears the *B. anthracis* population faster than linezolid because these antibiotics inhibit protein synthesis by different mechanisms (34, 40).

One potential concern is that the higher proportion of vegetative bacilli observed with linezolid and doxycycline therapies may result in inferior treatment outcomes *in vivo* compared with ciprofloxacin, moxifloxacin, and meropenem since it is the vegetative bacilli that produce lethal toxin and edema toxin (10, 28). These toxins cause the morbidity and mortality associated with anthrax infections. However, in a separate *in vitro* hollow fiber study we demonstrated that linezolid completely inhibited the production of protective antigen, a key component of both lethal toxin and edema toxin, in the Sterne strain of *B. anthracis* (25). Consistent with our finding are the reports that linezolid and clindamycin, another protein synthesis inhibitor, stopped the production of toxins by *S. aureus* and streptococci (37–39, 44). In contrast, penicillin (a cell wall-active drug) kills *S. aureus*, resulting in an increase in toxin concentrations in medium due to release of intracellular toxins (39). Ciprofloxacin is not a protein synthesis inhibitor. This drug kills vegetative *B. anthracis* but does not directly stop toxin synthesis. Thus, with ciprofloxacin therapy protective antigen was detected in the medium for 8 to 24 h after treatment was initiated (25). Ciprofloxacin and meropenem performed similarly (our unpublished data).

The effect of doxycycline on the production of toxins by *B. anthracis* was not examined. However, it is likely that doxycycline would also stop the production of *B. anthracis* toxins. Since toxins contribute to the morbidity and mortality of anthrax infections, it is possible that the protein synthesis inhibitors doxycycline and linezolid may be as effective, if not more effective, than ciprofloxacin, moxifloxacin, and meropenem in the treatment of severe *B. anthracis* infections *in vivo*.

In summary, by simulating human serum concentration-time profiles for five antibiotics within an *in vitro* hollow fiber pharmacodynamic model, we demonstrated that the bacteriostatic antibiotics linezolid and doxycycline are as effective or more effective than the bactericidal agents ciprofloxacin, moxifloxacin, and meropenem in reducing the total ΔSterne *B. anthracis* population, even though the three bactericidal agents kill vegetative bacteria faster than the two bacteriostatic drugs. None of the antibiotics examined in this project kill spores, nor do they appear to alter the ability of spores to germinate into vegetative bacilli. The heat shock studies show that ciprofloxacin, moxifloxacin, and meropenem rapidly kill vegetative bacilli, resulting in ΔSterne popula-

tions that consist primarily of spores. Thus, for these bacteriostatic antibiotics the rate of clearance of the ΔSterne strain from the hollow fiber systems is dependent on the rate at which the spores germinate into vegetative bacilli and, to a lesser extent, the efficiency with which these antibiotics kill vegetative bacteria before they have the opportunity to form new spores. In contrast, the heat shock studies suggest that the bacteriostatic drugs (linezolid and doxycycline) interrupt the ability of vegetative bacteria to cycle back into spores, resulting in a ΔSterne population that consists primarily of vegetative bacteria. Thus, the clearance of *B. anthracis* by linezolid and doxycycline is dependent on the rate at which these bacteriostatic drugs kill the vegetative bacilli. Although linezolid and doxycycline kill vegetative *B. anthracis* slower than the three bactericidal drugs examined in this project, it is clear that the inhibition of spore formation by doxycycline exposes the antibiotic-vulnerable vegetative bacteria to the antimicrobial effect of linezolid and doxycycline for a longer duration, resulting in equivalent or greater rates of clearance of the total *B. anthracis* population from the hollow fiber systems. Since the toxins are responsible for the morbidity and mortality associated with anthrax infections, *in vivo* studies are warranted to determine if the differences in spore and vegetative populations seen with bacteriostatic and bactericidal agents are also seen *in vivo* and to determine whether or not toxin production is altered *in vivo* by protein synthesis-inhibiting bacteriostatic drugs, as these effects may affect outcomes in ways that cannot be predicted by *in vitro* infection models.

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