Borrelia burgdorferi, the causative agent of Lyme disease, forms drug-tolerant persister cells.

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Running title: Persisters of Borrelia burgdorferi

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**ABSTRACT**

*Borrelia burgdorferi* is the causative agent of Lyme disease, which affects an estimated 300,000 people annually in the US. When treated early, the disease usually resolves, but left untreated, can result in symptoms such as arthritis and encephalopathy. Treatment of the late stage disease may require multiple courses of antibiotic therapy. Given that antibiotic resistance has not been observed for *B. burgdorferi*, the reason for the recalcitrance of late stage disease to antibiotics is unclear. In other chronic infections, the presence of drug-tolerant persisters has been linked to recalcitrance of the disease. In this study, we examined the ability of *B. burgdorferi* to form persisters. Killing of growing cultures of *B. burgdorferi* with antibiotics used to treat the disease was distinctly biphasic, with a small subpopulation of surviving cells. Upon regrowth, these cells formed a new subpopulation of antibiotic-tolerant cells, indicating that these are persisters rather than resistant mutants. The level of persisters increased sharply as the culture transitioned from exponential to stationary phase. Combinations of antibiotics did not improve killing. Daptomycin, a membrane-active bactericidal antibiotic, killed stationary phase cells, but not persisters. Mitomycin C, an anti-cancer agent that forms adducts with DNA, killed persisters and eradicated both growing and stationary cultures of *B. burgdorferi*. Finally, we examined the ability of pulse-dosing an antibiotic to eliminate persisters. After addition of ceftriaxone, the antibiotic was washed away, surviving persisters were allowed to resuscitate, and antibiotic was added again. Four pulse-doses of ceftriaxone killed persisters, eradicating all live bacteria in the culture.
Introduction

All pathogens studied to date form persisters, dormant variants of regular cells which are tolerant to killing by antibiotics. The ability to produce persisters explains the puzzling recalcitrance of chronic infections to antibiotics that are effective against the same pathogen *in vitro*. Indeed, many chronic infections are caused by drug-susceptible pathogens [1, 2]. The immune system can effectively remove sessile cells from the blood and many of the tissues, and this accounts for the efficacy of antibiotics, including bacteriostatic compounds, in treating uncomplicated infections. When the immune response is limited, the result is often a chronic infection [2]. Biofilms are a well-studied case of immune evasion and serve as a paradigm for understanding chronic infections.

In biofilms, cells are protected from the large components of the immune system by a surface exopolymer [3-5]. Antibiotics kill the regular cells, but dormant persisters survive, and when the concentration of antibiotic drops, they resuscitate and repopulate the biofilm [2]. This scenario is supported by our finding of high-persister (hip) *Pseudomonas aeruginosa* selected in the course of prolonged antibiotic treatment [6]. Isolated from patients with late-stage cystic fibrosis, hip mutants can produce 1000 times more persisters than the parent strain; this indicates that selection for increased tolerance (rather than resistance) provided the pathogen with a survival advantage.

Similarly, hip mutants are selected during treatment of oral thrush caused by *Candida albicans* [7]. In *Salmonella typhimurium*, entrance of pathogens into human cells where they are protected from the immune system is accompanied by a sharp increase in persister formation and tolerance to killing by antibiotics [8]. In tuberculosis, dormant cells are likely responsible for the need of a lengthy treatment of the acute stage and for
the latent form of the disease. *Mycobacterium tuberculosis* hides from the immune system in macrophages or in granulomas [9].

*B. burgdorferi* causes Lyme disease with 300,000 estimated cases annually in the United States alone [10]. When treated early with antibiotics, the disease usually resolves [11, 12]. If treatment is delayed, the pathogen spreads throughout the body and can cause meningitis, arthritis, and carditis. Meningitis and carditis are mostly self-limited, but Lyme arthritis can persist for years [13, 14]. A substantial proportion of patients receiving their first course of antibiotics for Lyme arthritis do not respond fully to a 28 day course of treatment. In such cases, retreatment with additional courses of antibiotics is recommended [13, 15, 16]. *B. burgdorferi* avoids immune attack by antigenic variation of surface components and by decreasing exposure of antigens [17-19]. In this regard, Lyme disease resembles other chronic infections where the pathogen is protected from the immune system, and persister cells may enable it to survive treatment with antibiotics. In *Escherichia coli*, the model organism for the study of persisters, dormant cells are formed primarily through expression of toxin/antitoxin (TA) modules. Toxins confer dormancy by either inhibiting protein synthesis or by decreasing the energy level of cells [20-22]. TA modules are widely spread among bacteria, and are copiously present in some pathogens. *E. coli* has more than 30 TA modules and *M. tuberculosis* over 75 [23, 24]. Interestingly, there are apparently no TA modules in the genome of *B. burgdorferi* [25]. Virtually nothing is known about persisters in this species. In this study, we report formation of drug-tolerant persisters in *B. burgdorferi* and describe possible approaches to their elimination.
Materials and methods

Bacterial strains and growth conditions: *Borrelia burgdorferi* B31 5A19 that had been passaged five times *in vitro* was kindly provided by Dr. Monica Embers [26]. *B. burgdorferi* was grown in BSK-II liquid media in a microaerophilic chamber (34°C, 3% O₂, 5% CO₂). Cultures were started by thawing -80°C glycerol stocks of *B. burgdorferi* (titer approximately 10⁷ cfu/mL) and diluting 1:20 into fresh BSK-II media.

BSK-II liquid medium was prepared according to protocol received from Monica Embers' lab by adding the following ingredients to 400 ml of deionized water and mixing thoroughly: 20 g bovine serum albumin (Sigma), 2 g neopeptone (Fluka), 0.8 g yeastolate (BD), 4 g HEPES sodium salt (Sigma), 2.4 g 10X CMRL (US Biologicals), 0.28 g sodium citrate (Fisher), 0.32 g sodium pyruvate (Sigma), 2 g glucose (Fisher), 0.16 g N-acetyl-glucoasamine (Sigma), 0.88 g sodium pyruvate (Sigma). The pH of the medium was adjusted to 7.6 and 24 ml of rabbit serum (Sigma) was added to the media. The medium was then filtered through a 0.22 µm filter.

Semi-solid plating was used to obtain cfu counts [27]. First, BSK 1.5X medium for semi-solid plating was prepared as in Samuels 1995 [27]. The following ingredients were added to 1 L of deionized water (LabChem, Inc) and mixed thoroughly: 8.33 g neopeptone (Fluka), 4.22 g yeastolate (BD), 9.99 g HEPES acid (Fisher), 8.33 g glucose (Fisher), 1.22 g sodium citrate (Fisher), 1.33 g sodium pyruvate (Sigma), 0.670 g N-acetyl-glucosamine (Sigma), 7.66 g sodium bicarbonate (Sigma). The pH of the media was adjusted to 7.5 and then 83.25 g of bovine serum albumin (Sigma) was added. The medium was stirred for one hour then filtered using a 0.22 µm filter. 1.5X BSK-II was stored at 4°C and used within 7 days of preparation. On the day of plating,
125 ml of 1.5X BSK was mixed with 6 ml rabbit serum and 19 ml 1X CMRL (97.89 mg/mL) and equilibrated to 55°C. 1.7% agarose (Lonza) was melted and equilibrated to 55°C. When all ingredients had equilibrated to 55°C, 1.7% agarose was added to 1.5X BSK at a ratio of 2:1 (BSK:agarose) to create BSK agarose. 8 ml of BSK agarose was dispensed into 60mm Petri dishes as bottom agar and allowed to solidify. For top agar, 100 µl of the given dilution of *B. burgdorferi* was mixed with 5 ml of 55°C BSK agarose and poured onto the bottom agar plates and allowed to solidify. The plates were incubated in zip lock bags in microaerophilic chamber (34°C, 3% O₂, 5% CO₂) for at least 21 days to obtain visible colonies.

**Antimicrobial agents:** Amoxicillin (Sigma), doxycycline hydrochloride (MP Biomedicals), ceftriaxone disodium salt hemi (heptahydrate) (Sigma), and vancomycin hydrochloride (Sigma) were dissolved in water. Mitomycin C (Sigma), gemifloxacin mesylate (Tecoland Corporation), and spectinomycin dihydrochloride pentahydrate (RPI) were dissolved in DMSO. Daptomycin cyclic lipopeptide (Sigma) was dissolved in a 5 µg/ml solution of calcium chloride. Stock solutions of antibiotics were aliquotted and stored at -20°C until use. Antibiotics did not undergo freeze-thaw cycles.

**Killing experiments:** *B. burgdorferi* was cultured in liquid BSK-II media for 3 days to late-exponential growth phase or for 5 days to stationary phase. Antibiotics were then added to the culture. The cultures were incubated in the microaerophilic chamber (34°C, 3% O₂, 5% CO₂). At a given time point, an aliquot of the culture was washed twice by centrifuging the culture at 13.2k rpm for 5 minutes and resuspending the pellet in an
equal volume of fresh BSK-II medium. The cultures were then serially diluted in fresh BSK-II media. 100 µl of the appropriate dilution was mixed with 5 ml of BSK agarose and poured as top agar. Plates were incubated in the microaerophilic chamber until visible colonies appeared (at least 21 days).

**Growth-persister experiments:** Cultures of *B. burgdorferi* were started as described above. At each time point, an aliquot of a growing culture was removed, diluted, and plated for cfu counts to generate the growth curve. A second aliquot (1 mL or 3 mL) was removed at the same time and challenged for five days with the indicated antibiotic. After five days, an aliquot of challenged culture was removed, washed twice, diluted, and plated for cfu counts to generate the persister curve.

**Minimum Inhibitory Concentration (MIC) testing:** A slightly modified version of the broth microdilution [28] was used. *B. burgdorferi* was grown in liquid culture for three days to reach exponential phase and then back diluted 1:10 into fresh BSK-II media to make the inoculum solution. All antibiotics were prepared as stock solutions in solvent (water or DMSO) based on the concentration to be tested and diluted in two fold increments in a 96 well stock plate. 2 µl per well of the antibiotic stock solution was transferred to the 96 well MIC plate to which 198 µl of the *B. burgdorferi* inoculum solution was added (final inoculum of approximately $10^6$ cells/well). Media, growth, and vehicle controls were included on each plate. The MIC plate was covered with Breatheasy Film (Diversified Biotech) and incubated in the microaerophilic chamber (34°C, 3% O₂, 5% CO₂) for 72 hours. The lowest concentration of antibiotics that
showed inhibition of growth was interpreted as the MIC. All MIC assays were repeated at least twice in triplicate.

Results

Characterization of B. burgdorferi Persisters

The presence of persisters is indicated by a biphasic killing pattern in a time-dependent killing experiment. The bulk of the population is rapidly killed, followed by a slower rate of death in a subpopulation of tolerant cells [29, 30]. In order to determine whether B. burgdorferi forms persisters, time-dependent killing experiments were performed with antibiotics commonly prescribed to patients with Lyme disease.

Doxycycline is a bacteriostatic protein synthesis inhibitor; amoxicillin and ceftriaxone inhibit bacterial cell wall synthesis and are bactericidal for many bacteria. Minimum inhibitory concentrations (MICs) of doxycycline, amoxicillin, and ceftriaxone were determined (Table 1). Levels of antibiotics close to what is achievable with standard clinically prescribed treatment dosing were chosen to evaluate persister formation in B. burgdorferi, and we used colony forming unit (cfu) count to determine viability.

Amoxicillin (6 µg/ml, 100X MIC) and ceftriaxone (3 µg/ml, 300X MIC) at clinically achievable levels killed the majority of cells in the first day, after which a slow phase of death followed for the next 6 days (Figure 1a). This characteristic biphasic pattern of killing is consistent with the presence of drug-tolerant persister cells.

Previous studies have shown that the persister fraction in other bacteria remains relatively unchanged even as the antibiotic level increases. We sought to determine if B. burgdorferi persisters behaved similarly in a dose-dependent killing experiment. As the...
concentration of amoxicillin and ceftriaxone increased, the fraction of surviving cells remained largely unchanged (Figures 1b-c). Doxycycline is a bacteriostatic antibiotic, but at higher concentrations appeared to effectively kill *B. burgdorferi* (Figure 1d). Again, the fraction of surviving cells did not change significantly with increasing levels of the compound. Thus, *B. burgdorferi* forms persisters capable of surviving very high concentrations of antibiotics, which exceed what is clinically achievable.

Density-dependent formation is a common feature of persisters reported for all pathogens examined so far, including *E. coli*, *Staphylococcus aureus*, *P. aeruginosa*, and *M. tuberculosis* [6, 31-33]. In order to test this property in *B. burgdorferi*, samples from a growing culture were removed over time, exposed to a lethal dose of antibiotic for 5 days, and then plated for cfu. There was a characteristic dip in persister levels in the early log phase, which is probably due to the resuscitation of dormant cells carried over from the inoculum (Figure 2). At mid-log phase, there is a sharp increase in persister levels, which continues as the density of the culture rises. In *E. coli*, once the culture reaches stationary state, complete tolerance is achieved for β-lactams that only kill growing cells [34]. In *B. burgdorferi*, we observe a very different picture – both amoxicillin and ceftriaxone kill stationary cells fairly well, yet the fraction of persisters continues to increase. One possibility is that this “stationary” culture actually represents a steady state where some cells die and others grow.

Next, we tested whether the *B. burgdorferi* cells surviving antibiotic treatment are drug-tolerant persisters or resistant mutants. For this, colonies produced by the surviving cells were regrown and tested for MIC. The amoxicillin and ceftriaxone MIC remained unchanged, showing that surviving cells had not acquired or developed a
genetic mechanism for antibiotic resistance. The population grown from the surviving cells produced the same level of persisters as the original population (Figure 3). These experiments show that \textit{B. burgdorferi} forms typical persister cells.

\textbf{Eradication of \textit{B. burgdorferi} Persisters}

\textbf{Drug combinations.} Some antibiotics act synergistically, such as sulfonamide and trimethoprim, polymixin and gentamicin, aminoglycosides and \(\beta\)-lactams [35], and we wanted to see if a combination of compounds known to be active against \textit{B. burgdorferi} will increase efficiency of killing both regular and persister cells.

All possible two-drug combinations of amoxicillin, ceftriaxone, and doxycycline were tested with a late-exponential phase culture in a time-dependent killing experiment and found to be no more effective than the drugs used individually in killing of \textit{B. burgdorferi} (Figure 4a). Doxycycline actually inhibited the action of amoxicillin. We have shown previously that fluoroquinolones and aminoglycosides can kill non-growing cells [36, 37], and we next tested these compounds against \textit{B. burgdorferi}. The pathogen is generally poorly susceptible to compounds from these classes. However, the \textit{B. burgdorferi} MICs for gemifloxacin (fluoroquinolone) and spectinomycin (aminoglycoside) are within achievable human dosing levels so we chose to test them [38-41] (Table 1). Both gemifloxacin and spectinomycin were ineffective in killing \textit{B. burgdorferi} at tested concentrations (Figure 4b). Combining these compounds also did not improve killing (Figure 4b).
Experimental compounds. Having shown that combinations of clinically prescribed antibiotics for Lyme disease are unable to effectively kill persister bacteria, we sought to examine some novel potential antimicrobial agents. We recently showed that acyldepsipeptide (ADEXP4), an activator of the ClpP protease, effectively kills persisters in *S. aureus* [42]. In the presence of ADEXP4, the protease cleaves mature proteins, forcing the cell to self-digest. However, ADEXP4 did not have significant activity against *B. burgdorferi* (not shown), which may be due to poor penetration.

We then considered whether knowledge of *B. burgdorferi* biology might be exploited to predict vulnerability to existing approved compounds. *B. burgdorferi* lives under microaerophilic conditions, where the capacity for energy generation is limited by comparison to aerobic organisms. Daptomycin is the only approved membrane-acting antibiotic that disrupts the proton motive force. The *B. burgdorferi* MIC to daptomycin was fairly high, 12-25 µg/ml (Table 1), in accordance with published data [43].

Daptomycin was highly bactericidal against *B. burgdorferi*, but a remaining subpopulation of persisters survived (Figure 5), suggesting that *B. burgdorferi* persisters can tolerate a drop in the energy level. Next, we tested vancomycin. This large glycopeptide antibiotic binds to lipid II, precursor of peptidoglycan, on the outside of the cytoplasmic membrane. Vancomycin is highly effective against Gram-positive bacteria, but does not penetrate across the outer membrane of Gram-negative species.

Surprisingly, the vancomycin MIC with *B. burgdorferi* is low, 0.25 µg/ml, similarly to Gram-positive species. *B. burgdorferi* has an outer membrane; the basis for this anomaly is unclear. Vancomycin effectively killed growing cells of *B. burgdorferi*, but not persisters, and was comparable to ceftriaxone (not shown). We also tested teixobactin,
a compound we recently discovered, which also binds lipid II [44]. At 1.2 kDa, teixobactin is considerably smaller than vancomycin (1.8 kDa), but it did not exhibit good activity in killing *B. burgdorferi* (not shown).

**Prodrugs.** Growth under microaerophilic conditions suggests vulnerability to compounds whose action depends specifically on a low oxygen environment. Nitroaromatic compounds such as metronidazole are prodrugs that are converted into reactive drugs by bacterial nitroreductases. These enzymes are expressed under anaerobic or microaerophilic conditions, and target pathogens living in these environments (i.e. *Helicobacter pylori, Clostridium difficile, E. coli*). We found that some nitroaromatic compounds like nitrofurantoin are effective in killing *E. coli* persisters [45]. However, we did not detect homologs of nitroreductases in the genome of *B. burgdorferi*. The MIC for nitroaromatic compounds (nitrofurantoin, nitrofurazone, and metronidazole) was too high to make them useful agents for killing *B. burgdorferi* persisters (data not shown).

Another compound that depends on a reductive environment for action is the prodrug mitomycin C. Upon entering the cell, mitomycin C is reduced into an active drug which then forms covalent adducts with DNA [46]. Originally discovered in a screen for antibiotics, mitomycin C is now used as an anticancer agent. Cancers often create a microaerophilic environment, which, together with rapid cell division, accounts for the relatively selective action of mitomycin C against them. Functional RecBC and RecFOR pathways are required to repair DNA damaged by mitomycin C in *E. coli* [46]. Interestingly, according to genomic data, *B. burgdorferi* lacks the genes of the RecFOR pathway [47], further suggesting vulnerability to this compound.
Mitomycin C eradicated a late exponential culture of *B. burgdorferi* within 24 hours, with no detectable persisters remaining (Figure 6a). This was observed with a low, clinically achievable dose of the compound - 1.6 µg/ml, or 8X MIC. In a dose-dependent experiment, eradication of a late exponential culture was achieved within 5 days with a 0.8 µg/ml (4X MIC) dose of the compounds (Figure 6b). Finally, mitomycin C was tested against a stationary culture of *B. burgdorferi*. Surprisingly, eradication was achieved with a low dose of 4X MIC within 24 hours (Figure 6c). It appears that a stationary population is more susceptible to this compound than an exponentially growing one.

**Pulse-dosing.** Apart from identifying compounds capable of killing persisters, it may also be possible to eliminate them with conventional bactericidal antibiotics using pulse-dosing. Based on our results, the level of persisters is lowest during early exponential growth (Figure 2). We reasoned that allowing growth to resume and then re-treating them as they enter exponential phase could kill persisters surviving an antibiotic challenge. Eradication of the culture could then be achieved after several rounds of killing and regrowth. To test this, a culture of *B. burgdorferi* was exposed to amoxicillin or ceftriaxone. The surviving persisters were allowed to resuscitate for a short period of time in fresh media, and then exposed to antibiotic again for a second round of killing. Persisters were substantially diminished after four rounds of killing with amoxicillin, and were eradicated below the limit of detection after four rounds of killing with ceftriaxone (Figure 7). Additionally, we found that a ceftriaxone solution stored under experimental conditions (in BSK-II media at 34°C, 3% O₂, 5% CO₂) does not lose activity, as
measured by MIC against *B. burgdorferi*, for up to 20 days. The activity of amoxicillin measured similarly, however, dropped 20-fold over 20 days which suggests degradation over time. The resulting MIC was still lower than the concentration used in killing experiments. This pulse-dosing experiment shows that a population of the pathogen can be eradicated with conventional antibiotics commonly used to treat the disease.

**Discussion**

The presence of drug-tolerant persisters can explain the recalcitrance of chronic infections to antimicrobial therapy, especially in cases when the disease is caused by a susceptible pathogen. While some chronic infections are ancient – leprosy, syphilis, tuberculosis – many cases in developed countries are consequences of otherwise successful medical intervention. Various indwelling devices (catheters, prostheses, heart valves) provide a substratum for biofilms that protect persisters from the immune system [2]. Even in bacterial infections that are routinely successfully treated with antibiotics, there is dependence upon the host immune system to control persisting bacteria that are not eradicated by antibiotics. The role of the immune system becomes evident when these same infections involve immunocompromised hosts and antibiotic eradication of the infection becomes much more difficult.

*B. burgdorferi* is a pathogen that can affect immunocompetent hosts. It establishes long term infections of years to lifelong in both its natural (i.e. mice) and incidental (i.e. humans) hosts in the absence of antibiotic therapy [14, 48]. Treatment in the early stages of disease results in good outcomes. Delays in diagnosis and treatment lead to sequelae that may require additional treatment. For example, patients who
develop arthritis, which typically begins after 1 month of untreated infection, often do not respond fully to a first course of 28 days of antibiotics [49]. The majority of these patients have evidence of *B. burgdorferi* DNA in their synovial fluid and will respond to additional one or two month courses of antibiotics [13, 16]. A smaller minority of patients referred to as “antibiotic resistant Lyme arthritis” will continue to have arthritis with synovial fluid that is PCR negative for *B. burgdorferi* DNA. These patients typically respond to anti-inflammatory agents such as methotrexate or TNF-inhibitors. Both these groups of patients should be distinguished from the highly controversial group of patients with “chronic Lyme disease” that exhibit fatigue, myalgias and arthralgias without objective evidence of disease. For the first group of Lyme arthritis patients responsive to antibiotics, given that there is no reported resistance to clinically used tetracyclines, β-lactams, and cephalosporins in the pathogen, the need for lengthy courses of therapy is unclear. The presence of persister cells is one possible explanation and this is a pattern that is seen in other infections where persister cells are thought to be relevant for disease *in vivo.*

We found that similar to other pathogens, the pattern of killing of *B. burgdorferi* by bactericidal antibiotics is biphasic, with a small subpopulation of surviving persisters. These surviving clones are not resistant mutants; upon regrowth they form a new persister subpopulation. Also similar to *E. coli, S. aureus,* and other pathogens, the density of persisters increases as the culture deviates from strictly exponential growth, reaching a maximum at stationary state. This is probably due to a deterioration of growth conditions resulting in increasing numbers of dormant cells. However, of note, the stationary state in *B. burgdorferi* is atypical, as amoxicillin and ceftriaxone continue...
to kill the majority of cells despite an increase in the level of persisters in the population. Cell-wall acting antibiotics do not normally kill non-growing cells; one possibility is that stationary state *B. burgdorferi* cultures represent a steady state of growing and dying cells. The ability of β lactams to kill non-growing cells has also been observed in *M. tuberculosis* where a combination of meropenem and a β lactamase inhibitor was able to kill viable but non-replicative cells [50]. The authors speculate that peptidoglycan remodeling continues in these non-replicating cells allowing for the activity of the β lactam. This is another possible explanation of the killing we observe of stationary phase *B. burgdorferi* with amoxicillin and ceftriaxone.

In a recently published study, Iyer, et al. [51] treated two different strains of *B. burgdorferi* with ceftriaxone and were unable to detect live *B. burgdorferi* by subculture in liquid medium. However, the cell density in that study was $10^7$ cells/ml, and according to our data, persister levels in this early exponential culture are low. In some of the biological replicates treated with ceftriaxone, we have not been able to recover live cells. At higher cell densities, the presence of persisters is unambiguous.

One common strategy for improving elimination of infective agents is to combine existing compounds. For example, β lactams and aminoglycosides are known to synergize with each other to achieve effective killing of *Enterococci* [52]. We tested combinations of standard antibiotics used in treatment of Lyme disease as well as a combination of a fluoroquinolone and an aminoglycoside, compounds that often synergize and are capable of killing non-growing cells. However, there was no synergy in killing *B. burgdorferi* with any of the tested combinations.
We recently described efficient killing of persisters in *S. aureus* [42] and in *E. coli* [45], and tested these compounds against *B. burgdorferi*. ADEP4, an activator of the Clp protease, causes massive protein degradation in *S. aureus*, killing regular cells and persisters. However, ADEP4 was not active against *B. burgdorferi*. We also reported that nitrofuran prodrugs are effective in killing *E. coli* persisters. Nitrofurans are reduced by bacterial nitroreductases into generally reactive compounds, explaining their activity against persisters. Nitroreductases are expressed under anaerobic or microaerophilic conditions. *B. burgdorferi* is a microaerophilic organism, but does not have obvious homologs of a nitroreductase, and nitrofurans we tested were fairly inactive.

We also tested daptomycin, a lipopeptide that acts by increasing K⁺ permeability of the membrane. Being in a low-energy (microaerophilic) environment, the pathogen may be vulnerable to membrane-acting compounds. Daptomycin killed the majority of cells in a stationary culture, but the level of surviving persisters was comparable to that of a stationary culture treated with ceftriaxone. In a recent publication daptomycin was reported to kill *B. burgdorferi* persisters more effectively than regular cells [43]. This conclusion was based on equating stationary cells with persisters. As follows from our experiments, a stationary culture harbors a small subpopulation of persisters. The actual level of stationary cells apparently surviving treatment by daptomycin in that study was very high, 28%, as determined by live/dead staining. Under similar conditions, we detect about $10^3$ (0.002%) surviving persisters by cfu count. It appears that live/dead staining may be over reporting the level of live *B. burgdorferi* cells.

Another weakness of the pathogen is its apparently limited ability for DNA repair. Based on the genome, *B. burgdorferi* lacks recFOR. In *E. coli*, both RecBC and
RecFOR are required for repair of DNA damage caused by mitomycin C, an anticancer drug. Mitomycin C at a low, clinically achievable dose (8X MIC), eradicated *B. burgdorferi* persisters in both exponential and stationary cultures within 24 hours. A highly reduced environment activates mitomycin C, and this contributes to its selective action in microaerophilic tumors. While the killing of persisters by mitomycin C is impressive, given the toxicity of this drug, this is more of a proof-of-principle for a compound exploiting the weaknesses of this pathogen rather than a clinically useful agent. Treatment with mitomycin C can result in serious negative side effects and it should not be used for treatment of Lyme Disease. This agent will be useful to examine the possible contribution of persisters to the disease in an animal model of infection.

Another peculiar feature of *B. burgdorferi* and a weakness of the pathogen is the lack of development of resistance to any antibiotic used to treat Lyme disease. Even attempts to raise mutants resistant to amoxicillin and ceftriaxone *in vitro* have been unsuccessful. Joseph Bigger proposed an interesting strategy for elimination of persisters in 1944, in the first publication describing these cells [53]. The rationale is to add antibiotic to kill off regular cells; wash it away; allow the culture to start regrowing, at which point persisters will resuscitate. Reintroducing antibiotics will kill the regrowing bacteria. The argument against pulse dosing is that this protocol invites resistance development. Given that this is not a concern for *B. burgdorferi*, pulse dosing may be an effective strategy and we performed pulse dosing with amoxicillin and ceftriaxone. Persisters were eradicated with ceftriaxone in four pulses. These experiments form the basis for testing pulse dosing in an animal model, and if successful, in humans.
While we have identified the presence of *B. burgdorferi* persisters in cultures of the organism, the mechanisms by which they are able to survive remain unknown.

There are multiple pathways of persister formation in other bacteria. The study of persisters so far identified redundant TA modules as a main component responsible for persister formation in *E. coli* and *S. typhimurium* [8, 20, 22]. TA modules are widely spread among bacteria, but are surprisingly absent from the genome of *B. burgdorferi*.

Other components leading to persister formation in *E. coli* have been detected as well - the stringent response [54], various metabolic processes [55, 56], global regulators, and protein stabilizing chaperones [56]. Future work will determine if these or other processes are involved in persister formation in *B. burgdorferi* and if persisters play a role in the pathogenesis of Lyme disease in humans.

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References


Figure Legends

FIG 1
Killing of *B. burgdorferi* by antibiotics. (a) Time-dependent killing. Antibiotics were added to an exponentially growing culture, samples were taken over time, washed, diluted, and plated in semi-solid BSK-II media for cfu counts. The culture was treated with amoxicillin (Amox) (6 µg/ml) or ceftriaxone (Cef) (3 µg/ml) (n=9). (b-d) Dose-dependent killing. A late-exponential culture of *B. burgdorferi* culture was exposed to antibiotics for 5 days, and surviving cells were determined by cfu count. The culture was treated with amoxicillin (b), ceftriaxone (c), or doxycycline (Dox) (d) (n=6). Error bars represent standard error.

**FIG 2** Growth dependent persister formation in *B. burgdorferi*. Growth in BSK-II medium was determined by cfu count. Persister levels were determined by taking samples from the growing culture, exposing to antibiotic for 5 days, and counting cfu. (a), amoxicillin (Amox) (6 µg/ml); (b), ceftriaxone (Cef) (3 µg/ml). N=6. Error bars represent standard error.

**FIG 3** Persister formation is not heritable. Colonies recovered from a persister experiment before and after antibiotic treatment were used to inoculate fresh BSK-II media. The colonies were allowed to grow for 3 days and treated with the same antibiotic used in the original persister experiment for 5 days. Persister levels of the colonies recovered after antibiotic treatment (Persisters) were not significantly different than the colonies recovered before antibiotic treatment (Control). N=5. Error bars represent standard error. Amox = amoxicillin, Cef = ceftriaxone.
FIG 4 Killing of *B. burgdorferi* with drug combinations. (a) Time-dependent killing of late exponential *B. burgdorferi* cultures exposed to the indicated antibiotics in combination. Amoxicillin (Amox) (6 µg/ml), ceftriaxone (Cef) (3 µg/ml), and doxycycline (Dox) (2.5 µg/ml) (n=6). (b) Killing of late exponential *B. burgdorferi* exposed to gemifloxacin (Gemi) (1.5 µg/ml) and/or spectinomycin (Spec) (160 µg/ml) singly or in combination (n=6). An aliquot was taken at indicated time points, washed, diluted, and plated on semi-solid BSK-II media for cfu counts. Error bars represent standard error.

FIG 5 Killing of *B. burgdorferi* by daptomycin. Time dependent killing of stationary phase *B. burgdorferi* exposed to daptomycin (81 µg/mL) (n=3). Error bars represent standard error.

FIG 6 Killing of *B. burgdorferi* by mitomycin C (MMC). (a, c) Time-dependent killing of *B. burgdorferi*. Three independent cultures of *B. burgdorferi* either at late exponential phase (a) or stationary phase (c) of growth were treated with MMC: 0.8 µg/ml (4X MIC) or 1.6 µg/ml (8X MIC). (b) Dose-dependent killing of late exponential cultures of *B. burgdorferi* culture after 5-day exposure to increasing concentrations of MMC. An aliquot was taken at indicated time points, washed, diluted, and plated on semi-solid BSK-II media for cfu counts. N=6. The x-axis is the limit of detection. An asterisk represents eradication to the limit of detection.

FIG 7 Pulse dosing results in effective killing of *B. burgdorferi* persisters. Late exponential cultures of *B. burgdorferi* were treated with (a) ceftriaxone (Cef) (3 µg/ml) or
(b) amoxicillin (Amox) (6 µg/ml) for 5 days. This represents the first round of killing. The cultures were then washed and allowed to recover in fresh BSK-II media for 24 hours. They were then treated again with amoxicillin (6 µg/ml) or ceftriaxone (3 µg/ml) for a further 5 days to give the second round of killing. This was repeated for a total of four rounds of killing with a 24 hours period of growth in fresh media between each round. Bars represent the average of at least three independent cultures (n=3-6) and error bars represent standard error. The x-axis is the limit of detection. An asterisk represents eradication to the limit of detection.
Table 1: Selected antibiotics tested against *B. burgdorferi*.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Class</th>
<th>MIC (µg/ml)</th>
<th>Max. serum concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin</td>
<td>β-lactam</td>
<td>0.06</td>
<td>7.6 [57]</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>Cephalosporin</td>
<td>0.01</td>
<td>256.9 [58]</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>Tetracycline</td>
<td>0.25</td>
<td>2.6-5.9 [59]</td>
</tr>
<tr>
<td>Gemifloxacin</td>
<td>Fluoroquinolone</td>
<td>0.125</td>
<td>2.33 [38]</td>
</tr>
<tr>
<td>Spectinomycin</td>
<td>Aminoglycoside</td>
<td>2</td>
<td>140-160 [41]</td>
</tr>
<tr>
<td>Daptomycin</td>
<td>Lipopeptide</td>
<td>12.5-25</td>
<td>55-133 [60]</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>Glycopeptide</td>
<td>0.25</td>
<td>40 [61]</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>Antitumor antibiotic</td>
<td>0.2</td>
<td>3.2 [62]</td>
</tr>
</tbody>
</table>

The MIC was determined by broth microdilution method.