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Persister Development by *B. burgdorferi* Populations In Vitro

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Abstract

Doxycycline is a commonly used antibiotic to treat Lyme disease and other bacterial infections. The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) for *Borrelia burgdorferi* have been investigated by different groups, but are experimentally established here as a function of input cell density. We demonstrate that *B. burgdorferi* treated in the stationary phase have a higher probability of regrowth following removal of antibiotic. In addition, we determine experimentally and mathematically that the spirochetes which persist post-treatment do not have a longer lag phase, but exhibit a slower growth rate than untreated spirochetes. Finally, we demonstrate that treating the spirochetes by pulse-dosing was not found to eliminate growth or reduce the persister population *in vitro.* From these data, we propose that *B. burgdorferi* persister development is stochastic and driven by slowed growth.

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47 **Introduction**

48

49 Bacteria and humans have been in a constant "arms race" for survival. The advent of
50 antibiotics has only been another weapon in the arsenal that we use to protect ourselves, and
51 shortly after penicillin's discovery, it was found that bacteria could become tolerant and resistant
52 to this new class of weapons (1, 2). Antibiotic tolerance, or the capability of bacteria to enter a
53 non-dividing, dormant state in response antibiotics, is not a new phenomenon, and has been well
54 studied in *Escherichia coli* and *Pseudomonas aeruginosa* (3-9). Similar studies have shown that
55 the spirochete *Borrelia burgdorferi* (*B. burgdorferi*) can persist in low-nutrient stressful
56 conditions (10, 11), and a similar mechanism may allow persistence in the presence of the
57 bacteriostatic antibiotic doxycycline (12).

58 Recent work has suggested that a genetically homogeneous bacterial population can
59 dynamically alter gene expression to adapt to changing environmental conditions, resulting in a
60 phenotypically heterogeneous population (13). These dynamic changes in gene expression can
61 result both from different individual bacterial responses to external stimuli, and from pre-existing
62 variations within individual bacteria causing differing responses (6, 14). The term "stochastic" is
63 used to describe these changes to denote the randomness in which the changes can occur (6). As
64 a concrete example, if an external environmental stimulus affects a bacterial population, the
65 resulting changes may not be uniform, since bacterial subpopulations of the larger population
66 will respond to the environmental triggers at different rates (13, 15). A unique challenge,
67 therefore, is reconciling changes in gene expression with the emergence of unique
68 subpopulations with phenotypic differences from the larger population.

If external stimuli on a bacterial subpopulation randomly trigger expression of genes that induce a state of dormancy, then a persister subpopulation can emerge. If the number of surviving subpopulations is known, then these data can be applied to predict the capability of a subpopulation of bacteria to survive an antibiotic treatment, and therefore, for the population as a whole to persist after antibiotic therapy. Here, the ability of subpopulations to transition from a persister to a non-persister state can be used to indirectly model the chance of finding persister subpopulations within a larger population. *B. burgdorferi* has been shown to persist when treated with tetracycline antibiotics (16, 17), but as of yet, the mechanism of *B. burgdorferi* persistence has not been described.

To establish parameters for antibiotic efficacy, the MIC, or Minimum Inhibitory Concentration, and the MBC, or Minimum Bactericidal Concentration of an antibiotic, are two quantification values of antibiotics that are used. The MIC of a particular antibiotic denotes the minimum concentration that is necessary to inhibit growth. In the context of resistant mutants, the resistant population would grow readily in the presence of the MIC, whereas tolerant populations would stagnate in an established MIC, but regrow after the removal of an antibiotic (6). The Minimum Bactericidal Concentration (MBC) is the lowest concentration of an antibiotic that is necessary to either eradicate bacteria, or prevent the recovery of a bacterial population. Growth and recovery of the culture can be reliably indicated by the presence of motile spirochetes (18).

Importantly, determinations of MIC that are used to establish dosages in humans and animals are determined by *in vitro* culture of bacteria, unlikely to mimic the growth characteristics of the pathogen *in vivo*. Alterations in nutrients, oxygen and cell density affect

91 growth, which in turn will affect the efficacy of antibiotics such as doxycycline which target
92 actively-dividing cells.

93 Two of the most commonly prescribed antibiotics that are used to treat Lyme disease
94 have different metabolic activities. Doxycycline acts on the bacterial 30S ribosomal subunit
95 (19), while ceftriaxone can generally be described as having beta-lactam activity (20). Although
96 previous clinical studies showed that doxycycline can be as effective as third-generation
97 cephalosporin antibiotics like ceftriaxone (21, 22), recent *in vitro* work suggests that doxycycline
98 may not be as effective as ceftriaxone against stationary-phase bacteria (23). Importantly, the
99 role of immune responses to infection is integral to the treatment mode for microbiostatic
100 antibiotics like doxycycline such that results from *in vitro* studies should not be over interpreted
101 to the *in vivo* situation.

102 Different values have been obtained by several labs for the MIC and MBC of antibiotics
103 used against *B. burgdorferi*i (16, 24-27), likely as a result of differences in methodology, strain
104 variation, and cell density. Therefore, we performed MIC and MBC assays for doxycycline
105 using a well-characterized strain of *B. burgdorferi* with varying input cell densities. We then
106 followed these assays with a probability assay, specifically designed to quantify the emergence
107 of persister populations after treatment with doxycycline. Our results indicate that *B.*
108 *burgdorferi* growth dynamics affect the response to doxycycline treatment and prompt
109 consideration of this factor when determining effective concentrations.
110
111 **Methods**
112 ***Borrelia burgdorferi***. Low passage (p4 or p5) *Borrelia burgdorferi sensu stricto strain* B31
113 clonal isolate 5A19 (28) was grown at 34^{o} C in BSK-II media (29), as described previously (30).

Because the spirochetes are microaerophilic and gene expression is affected by oxygen levels (31) they were grown in a tri-gas incubator set at 5% CO_2, 3% O_2, and the rest N_2. The *B. burgdorferi* were seeded at low concentration from a frozen glycerol stock, and then grown to the necessary cell density.

MIC Assays. To determine the effect of growth phase on the minimum inhibitory concentration (MIC) of doxycycline, the following experiments were performed. *B. burgdorferi* were grown to early log phase at 5 x 10^5 cells/mL, early log phase at 4-7 x 10^6 cells/mL, mid log phase at 4-9 x 10^7 cells/mL, and late log phase at 1.25 x 10^8 cells/mL. The groups of cells were treated with increasing concentrations of doxycycline, ranging from 0, 0.1, 0.25, 0.5, 1.0, and 2.5 µg/mL for 5 days. Previous studies have used not less than 72 hours of treatment for MIC determination (27, 32), but based on the time-dependent mechanism of doxycycline, we extended it to 5 days, as has also been reported (23, 33). The MIC was quantified as the lowest concentration necessary to inhibit growth over a five-day period.

MBC Assays. To determine the effect of growth phase on the minimum bactericidal concentration (MBC) of doxycycline, the assay was performed as follows. Low passage B31.5A19 were grown in BSK-II media in either 15 mL, or 5 mL tubes, to 10^8 cells/mL, then diluted to either 1 x 10^7 or 1 x 10^6 cells/mL. Similarly, a B31.5A19 low passage isolate was grown to 10^7 cells/mL and diluted to either 2 x 10^5 or 1 x 10^6 cells/mL. All cultures were then treated with doxycycline hyclate for 5 days at concentrations of 0, 0.1, 1.0, 2.5, 5.0, 10.0, 25.0, and 50.0 µg/mL. After 5 days, the number of motile spirochetes in cultures was counted, as an indicator of viability (18). A subculture of the *B. burgdorferi* was then taken by removing half

137 of the culture on day 5, gently pelleting at 2700x g for 20 minutes at room temperature, and
138 resuspending in doxycycline-free media. The cultures were checked to verify the pellet was not
139 lost after spinning. At day 5 after resuspending in doxycycline-free media, the culture where no
140 motile spirochetes were detected, that came from the lowest concentration of antibiotic, was
141 indicated as the MBC for doxycycline with *B. burgdorferi.* The assay was performed with 4
142 tubes per doxycycline concentration and the MBC, assessed at 5 days post-treatment, was found
143 to be 50 µg/ml or lower, depending on the bacterial cell density. This concentration was
144 therefore used in the probability assays (below).
145
146 ***Probability Assays***. To quantify the amount of *B. burgdorferi* that would persist *in vitro* after
147 treatment with the MBC of doxycycline, an assay was set up as follows. For the first part of the
148 assay, cells were diluted to one of the following test concentrations. *B. burgdorferi* cells were
149 grown to 2-3 x 10^8 cells/mL, then diluted to a concentration of early (2 x 10^6 cells/mL), mid (2 x
150 10^7 cells/mL), and late (1 x 10^8 cells/mL) log density in 15 mL Eppendorf tubes. The *B.*
151 *burgdorferi* cultures were then treated with the MBC of doxycycline (50 µg/ml) for 5 days. On
152 day 5, the *B. burgdorferi* were pelleted and resuspended in doxycycline-free media, and allowed
153 to regrow for 11 days. The end time point was established at 11 days after performing a test
154 assay, which demonstrated that when regrowth occurred, it was most frequently seen within the
155 first 11 days. Multiple tubes per treatment group were grown (see Table 2). At day 11, the
156 cultures were checked, and the presence or absence of motile spirochetes was documented. If
157 motile *B. burgdorferi* were present, the sample was marked as (+) growth; otherwise, it was
158 scored as (-) growth and a count of # of tubes with motile spirochetes/total was obtained.

159 For the second part of the assay, *B. burgdorferi* cells were seeded from a glycerol stock,
160 and grown in BSK-II in15 mL conical tubes to concentrations of 2-3 x 10^6 cells/mL, 2-3 x 10^7
161 cells/mL, and 1 x 10^8 cells/mL, then treated with doxycycline for 5 days. The cultures were then
162 resuspended in doxycycline-free media as described previously. The *B. burgdorferi* cultures
163 were allowed to regrow for 11 days, and evaluated as (+) for growth, and (-) for no growth as
164 described above.
165
166 ***Pulse Dose Assay.*** Cultures of *B. burgdorferi* were grown to 3 x 10^7 cells/mL in 5 mL snap-cap
167 tubes, treated with 50 µg/mL of doxycycline for 5 days, then pelleted and resuspended in
168 doxycycline-free media. The culture tubes were incubated and monitored each day for growth.
169 When the growth resulting from surviving bacteria reached early-log phase ($\simeq$ 5 x 10^6 cells/mL),
170 50 µg/mL of doxycycline was added before the *B. burgdorferi* cell density could grow to the
171 initial assay concentration (3 x 10^7cells/mL). After 5 days of doxycycline treatment, the
172 doxycycline was removed, and the *B. burgdorferi* cultures were monitored again. The cycle of
173 treating and removing the doxycycline was repeated 3 times, similar to that described by Lewis
174 et al (6). Thirty-four samples were used in the pulse dose assays, and approximately 30% of the
175 total number of samples regrew after the first treatment. Successive treatments had a regrowth
176 rate of approximately 50%. The assay was fully completed (three treatments and regrowth
177 phases) one time. At the pulse dose assay endpoint, a glycerol stock was made from the *B.*
178 *burgdorferi* culture for analysis and comparison with the Probability Assay. See Figure 3a for a
179 representative diagram.
180

Mathematical Modeling. To determine a mathematical model for the growth of persister subpopulations in *B. burgdorferi*, data from the Probability Assay and Pulse Dose Assay were analyzed. In a given assay with *B. burgdorferi*, for calculation purposes, a tube was considered a population. Logarithmic growth in bacterial cultures can be defined by the equation $P = P_0e^{kt}$, where P = final population, P_0 = initial population, k = growth constant, and t = time. To create a model that could ascertain the quantity of persisters in any given population , the time to regrowth using both the probability assay and the pulse dose assay was used to determine *t* (time to regrowth for the subpopulation P_0 that regrows after doxycycline treatment). The final population P is simply defined as the quantity of a population that regrew after treatment, and the growth constant *k* can be derived from an average growth rate of populations over a defined period of time. While a logistic curve model is commonly used for bacterial batch cultures (34), applying this to our data did not result in a good fit; the *k* value derived was much higher than what was observed over several experiments (data not shown).

A given population of *B. burgdorferi* is also composed of subpopulations $p_1, p_2,\ldots p_n$, described as $P = \sum p_n$. During antibiotic treatment, a bacterial subpopulation that is not killed by antibiotic therapy, and does not grow in the presence of antibiotics, can be described as a subpopulation of dormant persisters. As observed in the pulse dose assay and the probability assay, this subpopulation will grow and form a new population after the removal of doxycycline, meaning the initial subpopulation will be P_0, and the regrown population after removal of antibiotic at time *t* will be P.

Limiting dilution analysis. A mid-log culture of untreated *B. burgdorferi* spirochetes were diluted to 10 spirochetes per mL, mixed thoroughly, and plated by adding 100 µl (average of 1

spirochete per well) of the culture to each well of a 96-well plate. An additional 80 µl of media was added to each well to prevent drying. After growth was visible by change in media color and a small pellet at bottom of well, four random wells were resuspended and counted.

Statistical Analysis. Analysis of Variance (ANOVA), student's t-test, and Fisher's exact test were performed with GraphPad Prism® and with R (http://www.R-project.org). Error bars for Figure 3b were calculated using Standard Deviation, while error bars for Figures 1 and 2 were calculated using Standard Error of the Mean.

Results

The effect of cell density on MIC. Several papers have been published on the MIC for doxycycline. The concentrations of *B. burgdorferi* that were used in the assays were either log 5 or log 6, and the MIC ranged from 0.25 µg/mL to 0.5 µg/mL, based on the criteria of inhibiting growth over a 3-5 day period (16, 23, 35). The results from the doxycycline MIC assays suggested that at early log phase, 0.25 µg/mL is a sufficient MIC (Table 1, Figure S1). At mid log phase, 1.0 µg/mL is required, while at late log phase, 2.5 µg/mL is the necessary MIC. Furthermore, if the number of motile spirochetes on day 5 is plotted against the lowest concentration that stopped growth, there is a linear correlation between decreasing cell density and doxycycline concentration (Figure 1).

The effect of cell density on MBC. We reasoned that although doxycycline is bacteriostatic, it acts on the 30S ribosomal subunit (36) to inhibit protein synthesis, so it could effectively eliminate a bacterial population if the concentration and duration were sufficient. This has been shown by other groups as well (26, 27). Therefore, an MBC assay was set up to

227 determine what concentration of doxycycline would prevent growth and motility after 5 days.
228 After 5 days, the MBC for the sub-cultures was determined by the number of motile spirochetes
229 (Figure 2). There was no motility observed on day 5 for any culture or cell density that was
230 treated with 50 µg/mL, suggesting a doxycycline MBC value of 50 µg/mL for *B. burgdorferi*.
231 Next, we conducted a probability assay to determine whether regrowth would occur after
232 a set time in early log, mid-log, and stationary phase populations. Following a 5-day treatment
233 with 50 µg/mL doxycycline, the cultures were examined after 11 days. If motile *B. burgdorferi*
234 were not observed by day 11, they were not positive after two more weeks, so we used day 11 as
235 the time point. The assay was first performed by growing *B. burgdorferi* from a glycerol stock
236 to stationary phase, and then diluting the cultures to lower concentrations of early log, mid-log,
237 and early stationary phase. There was a significant difference in the number of cultures with
238 motile *B. burgdorferi* in early, mid, and stationary phase groups (Table 2a). When the assay was
239 repeated by growing the cultures from a glycerol stock to the desired concentrations, there was
240 no regrowth or motility in any group except the stationary phase (Table 2b). A significant
241 difference ($p < 0.01$) between the assays where spirochetes were diluted to the desired
242 concentration versus those grown to the desired concentration was observed, which further
243 suggested that population density and growth phase differentially affect the ability of *B.*
244 *burgdorferi* to form persister cells in the presence of doxycycline.
245 **The effect of pulse-dosing on *B. burgdorferi* regrowth**. To explore the possibility that
246 a small number of persister cells survive post-treatment, a pulse-dose assay similar to the model
247 used by Kim Lewis (6, 7, 37) with *E. coli* and *P. aeruginosa* was established. We expected that
248 the persister population would be reduced by each subsequent treatment, and this would be
249 evidenced by lack of regrowth, or slower regrowth. Our results show that the treatment was

unable to kill the persisters, which regrew after each treatment (Figure 3). Furthermore, the rate of regrowth did not significantly decline after the first treatment cycle (Figure 3b). Interestingly, while the first treatment was applied to a dense culture, subsequent treatments were applied to early log phase spirochetes, which would not have been expected to produce persisters.

By examining growth after each pulse, we determined that the time to regrowth did not increase (Figure 3) with subsequent pulses (and after the 2^{nd} and 4^{th} pulses actually decreased). The calculated initial cell density increased slightly as well. This indicates that the persister population was not reduced in proportion with multiple treatments.

Exploring the mechanism of persister development. Given these results, we hypothesized that repeated administration of antibiotics (or pulses) could produce a population selected for *B. burgdorferi* persisters. A glycerol stock was made from the culture of *B. burgdorferi* that regrew after 3 treatments in the pulse-dose assay. This was designated as the "persister regrowth (PR)" stock and was used to examine the mechanism of persister development. We reasoned that if the PR stock consisted of a population that has been selected for the persister phenotype, then a higher proportion of this population has the potential to form persisters. Thus, if we subjected it to our probability assay, treatment at all growth phases should result in persister development. Conversely, if the PR stock consisted of bacteria which all have the same potential for persister development, the results should be the same as those shown in Tables 2a and b. The results are shown in Table 3. The PR stock of *B. burgdorferi* did regrow after treatment, albeit at a lower frequency than for the untreated stock for each growth phase. These data indicate that the PR population is not selected for persister development and the process is likely stochastic.

Mathematical model of persister growth. If the results of the probability assay and the pulse dose assay are analyzed together, several useful data points can be obtained. As already described, if a population of *B. burgdorferi* in stationary phase is treated with doxycycline, it is more likely to adopt a persister phenotype. To describe it another way: as cell density increases, so does the tendency for each bacterial cell to transition into a survival state upon the introduction of the antibiotic doxycycline. At earlier growth phases, a persister population may exist, but the time *t* to regrowth is much greater, and the chance of regrowth is much less than 50% (Table 2). To create a predictive model, which could ascertain in any given population the likelihood of such an occurrence, we must first assume that growth rate is constant within the cycle of regrowth, and if growth occurs, it will be exponential and follow the exponential growth rate $P = P_0e^{kt}$.

Bacterial populations do not follow simple logarithmic growth, but proceed through lag, log and stationary phases. In our analysis of growth following doxycycline treatment, we had difficulty applying the $P = P_0e^{kt}$ equation because *k* was variable and *t* was a large enough number that P_0 was calculated to be <<1. In essence, we assumed that the lag phase was longer than for untreated cells and determined that the growth constant varied, depending on the cell density and period of growth. For example, cell population densities < 10^5 cells/mL do not appear to be in logarithmic growth (Figure S1), so to determine the growth rate constant *k*, the values used were time $t = 2$, $P_0 = 4 \times 10^5$ cells/mL, and $P = 7.2 \times 10^7$ cells/mL (Figure S1), which provide $k = 2.596$. However, if this constant is used for the equation $P = P_0e^{kt}$ to determine the initial population of persisters following cessation of treatment in pulse I of our pulse-dose assay, the culture reached 1×10^7(P) after 18 days (*t*), so solving for P_0 gives 0.345 (less than 1 bacterial cell).

In order to estimate the regrowth mathematically, we therefore attempted to calculate the time it would take 1 persister cell to begin regrowth, in essence the lag phase, which is prolonged following treatment. We determined the growth rate (which is not constant) for periods of growth between log4-log 6, log5-log6 and log6-log7 and determined the average to be 1.335 (Table 4), which was used for *k*. In the pulse-dose assay, the longest period to regrowth (after pulse I) was 18 days. To determine the lag period, we set P_o=1, calculated *t*, and subtracted the calculated t from the actual *t*. This resulted in an extrapolated lag phase of 6 days.

To experimentally determine the time to regrowth of a single bacterial cell, we performed a limiting dilution analysis. Here, a mid-log culture of untreated *B. burgdorferi* spirochetes were plated at an average of 1 spirochete per well in a 96-well plate. After growth was visible, four random wells were resuspended and counted, giving 1.08, 1.153, 1.88 and 1.28 x 10^8 after 12 days +21hrs. The average of 1.351 x 10^8 was used to calculate *t* using the untreated cell growth rate of 2.596, with a result of 7.211 days. Subtracting this from the actual growth duration also provided a lag phase of 6 days. Thus, it appears that the lag phase for a single cell is about 6 days, identical to the extrapolated lag phase for persister cells, and it is the growth rate that is slower for antibiotic-treated *B. burgdorferi*.

Discussion

The addition of any substance which causes alterations in the individual bacterium or small subpopulations of bacteria can have profound effects on the bacterial population as a whole. These subpopulations, although genetically identical to the bulk population, will uniquely respond to external stimuli. The data suggest that one such external stimulus is the antibiotic doxycycline. These different responses can confer an advantage to survival of the

bacterial population as a whole, when the population is subjected to adverse conditions (6, 38-40). Since the subpopulations are genetically identical, and have no specific mechanism to inactivate antibiotics, this is fundamentally different from resistance. The bacterial subpopulations which correctly induce the genes that activate dormancy, while the larger population may get killed by an antibiotic, are termed persisters, a term coined by Dr. Joseph Bigger (1).

Mechanisms of persistence in other bacterial species have been well studied. *Escherichia coli* can be induced into a persisting state using Toxin/Antitoxin (TA) modules such as *tisB* (7, 41, 42). The *tisB* gene induces a decrease in proton motive force and ATP, but is normally repressed. Subpopulations which induce it under normal growth conditions do not survive (7), such as if it is accidentally induced during SOS repair. In cases where a DNA topoisomerase inhibitor such as ciprofloxacin is present, bacterial subpopulations which induce *tisB* have a better chance of survival, while other subpopulations which attempt SOS repair will most likely be killed off (7).

The regrowth of persisters after antibiotic treatment may also be influenced by environmental conditions, and not by a predetermined subpopulation number (43). Stationary phase *E. coli* cultures were diluted and added to minimal or LB media, with norfloxacin and ampicillin. When colony-forming units (CFU) were plotted against time for all cultures, minimal media produced a delay in the bacteria exiting from a dormant, persister state (43). Interestingly, a different experiment whose purpose was to look for quorum-sensing in *E. coli* found that if old media from stationary phase cells was added to growing cultures, there was no effect on persister formation in growing cell populations (6). This result could suggest that multiple pathways can trigger dormancy and persistence, as has been well published (7, 44).

Additionally, the study (43) proposed that instead of rigidly describing a population as "persister" and "non-persister", populations and subpopulations of bacteria should be more dynamically defined. Subpopulations of persisters can change depending on the environment, and the activation of the correct metabolic pathway can prompt individual cells to risk growth and awake from dormancy (45), similar to stochastic responses. As bacteria respond to stimuli, the responses can be modeled as a transition from one state to another during a time interval (15, 38). Although numerous different genes and pathways in a bacterium may be expressed in response to a stimulus, a simplified biphasic model can be used to examine specific criteria, such as a growth or death response to a given stimulus. In addition, predictions about the probability of changes in state can be made (13, 46).

While persister formation is not unique to *B. burgdorferi*, certain characteristics of this pathogen may influence or affect its entrance into dormancy. These spirochetes traverse between commensal inhabitance of ticks, survival and proliferation in reservoir hosts and pathogenic persistence in incidental hosts. Entry into slow growth or dormancy is necessitated by prolonged periods of nutrient deprivation within the unfed tick. In addition, the spirochetes may occupy niches in the mammalian host that receive lower levels of oxygen and blood flow/nutrients (47). Therefore, the ability to form persisters, while yet stochastic in nature, may be more advantageous for *B. burgdorferi* than for other bacterial pathogens. Indeed the frequency of *B. burgdorferi* persisters was observed to be higher than *E. coli* in a recent report (48).

In addition, the MIC and MBC values for multiple antibiotics has been shown to vary with different strains and clinical isolates of *B. burgdorferi*, though no direct resistance mechanism has been identified (27, 32). In particular, a study of erythromycin resistance showed dramatic differences in susceptibility among several strains, along with increased

tolerance induced with pre-exposure (49). While we only used one strain in our study, our pulse-dose assay combined with the probability assay indicated that pre-exposure did not enhance tolerance, indicating that different mechanisms may govern the responses to antibiotics. Importantly, the impact of host-adaptation may be significant. Notably, clinical isolates were shown to be more tolerant of erythromycin, possibly due to the adoption of a slow-growing phenotype *in vivo*.

This work was performed entirely *in vitro* and therefore does not take into account the influence of host adaptation, immune responses or the tissue penetration of antibiotic on doxycycline treatment of a *B. burgdorferi* infection. However, multiple studies in animals have shown that *B. burgdorferi* is not fully eradicated with antibiotic treatment (16, 17, 26, 50-52). The development of slow-growing or dormant persisters in the presence of doxycycline (23, 33), a microbiostatic antibiotic would indicate that reliance on immune control coincident with treatment would be necessary for efficacy of antimicrobial therapy. Given the multiple strategies that *B. burgdorferi* utilizes to evade the immune response (53), the survival of persisters post-treatment with doxycycline is a reasonable possibility. If persisters do develop *in vivo*, then prolonged antibiotic therapy may not offer significant improvement of clinical outcome (54), should this be the result of dormant persisters (perhaps also attenuated (51)) continuing to elicit inflammatory responses. Our pulse-dose assay results also indicated that pulsing with doxycycline may not be effective. This is in contrast to a recent report, but it is important to note that different antibiotics with different mechanisms were used in those studies (33).

These studies imply that a portion of *B. burgdorferi* may be tolerant to doxycycline following their entry into a state of dormancy. The results presented here further indicate that the development of *B. burgdorferi* persisters is governed by stochasticity and that dormancy may be

387 related to both growth phase and cell density. It has been shown with other bacteria (55-57) that
388 oxidative stress or nutrient deprivation can induce a persister state. It is possible that a lack of
389 readily available nutrients for some *B. burgdorferi* subpopulations, similar to what would occur
390 at stationary phase or in the unfed tick (58), can induce persister development and dormancy, and
391 enable survival after the introduction of an antibiotic like doxycycline. Follow-up studies using
392 RNASeq will aim to identify the specific genes induced among *B. burgdorferi* survivors of
393 antibiotic treatment that are associated with a persister state.
394

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410 **Tables**

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Table 1. Results of MIC Assay	
Input Cell Density	**Concentration of Doxycycline that Inhibited Growth, Day 0 to Day 5**
2 x 10^5 cells/mL	0.25 µg/mL
7 x 10^6 cells/mL	0.25 µg/mL
8 x 10^7 cells/mL	1.0 µg/mL
1.25 x 10^8 cells/mL	2.5 µg/mL

Table 2a. Probability Assay I			
Cell concentration*	**Early**	**Mid**	**Late**
Tubes with Motile Spirochetes	23	36	36
Tubes with Nonmotile Spirochetes	37	14	0
Tubes with Motile Spirochetes/ Total Tubes	23/60	36/50	36/36
Percent Tubes with Motile Spirochetes	38%	72%	100%
Statistical Significance (Chi-Squared with Yates' Continuity Correction)	Early v. Mid p = 0.000857	Mid v. Late p = 0.00154	Early v. Late p = 6.89 x 10^9

412 *diluted to: early (2 x 10^6 cells/mL), mid (2 x 10^7 cells/mL), and late (1 x 10^8 cells/mL)

Table 2b. Probability Assay II			
Cell density*	**Early**	**Mid**	**Late**
Tubes with Motile Spirochetes	0	0	12
Tubes with Nonmotile Spirochetes	22	22	31

Tubes with Motile Spirochetes/ Total Tubes	22	22	43
Percent Tubes with Motile Spirochetes	0%	0%	28%
Statistical Significance (Chi-Squared with Yates' Continuity Correction)	Early v. Mid null	Mid v. Late (approximation) p = 0.0162	Early v. Late (approximation) p = 0.0162

*grown to: early (2-3 x 10^6 cells/mL), mid (2-3 x 10^7 cells/mL), and late (1 x 10^8 cells/mL)

Table 3. Comparison of Post-treatment Growth Between Persister Isolate and Untreated Isolate

	Early Log B31 5A19	**Early Log Persister Isolate**	**Mid Log B31 5A19**	**Mid Log Persister Isolate**	**Late Log B31 5A19**	**Late Log Persister Isolate**
Tubes with Motile Spirochetes	23	5	36	10	36	9
Tubes with Nonmotile Spirochetes	37	26	14	20	0	17
Tubes with Motile Spirochetes/ Total Tubes	23/60	5/31	36/50	10/30	36/36	9/26
Percent Tubes with Motile Spirochetes	38%	16%	72%	30%	100%	34%
Statistical Significance	p = 0.0355 (Fisher exact test)		p = 0.001 (Fisher exact test)		p = 4.224 x 10^{-9} (Fisher exact test)	

423 **Table 4. Persister Growth Calculations**

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Calculation of *k*				P	P_o	*t* (days)
log 4 to log 6	1.23289		Pulse I	1.00E+07	0.67	18
log 5 to log 6	0.75165		Pulse II	1.00E+06	0.94	11
log 6 to log 7	2.02125		Pulse III	4.17E+05	0.692	14
Avg. log 4-7	**1.3352633**		Pulse IV	8.00E+05	1.018	10

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Figure Legends

Figure 1. Relationship between *B. burgdorferi* population density and minimum inhibitory concentration of doxycycline. The largest cell density with a population decrease from day 0 to day 5 is plotted against the lowest concentration of doxycycline required to cause the decrease. The relationship suggests a linear correlation with R^2=0.8721. For each data point, corresponding to input cell density (log 5-log 8), *n*=4 tubes; bars are standard error of the mean.

Figure 2. Number of motile *B. burgdorferi* per concentration of doxycycline on day 5 post-treatment in the MBC assay. A growth inhibition assay was performed, whereby cultures of differing initial concentrations and seed concentrations were treated with multiple concentrations of doxycycline. The number of motile spirochetes per culture was counted 5 days after the cessation of treatment. No growth was seen in any culture at this time point with treatments of 25 and 50 µg/ml.

Figure 3. Pulse-dose assay. (A) Representative figure of the pulse-dose assay. (B) Time to regrowth for each consecutive cycle of the assay. *B. burgdorferi* cultures (n=34) were seeded initially at 7 x 10^5 cells/mL, treated with 50 µg/mL of doxycycline, then switched to doxycycline-free media. Cultures were monitored for regrowth, then those that regrew (n=5) were treated again before they reached their initial concentration. The process was repeated 2 more times for those that regrew (n=2). The time to regrowth after each treatment cycle is displayed. Error bars are SD. No significant difference was observed in the time to regrowth between cycles 2 and 3.

Figure S1. *B. burgdorferi* growth curve. Plot of growth for untreated *B. burgdorferi* and cultures treated with 0.1, 0.25 and 0.5 µg/ml doxycycline. The initial concentration was 4.0 x 10^5 spirochetes/mL (early log).

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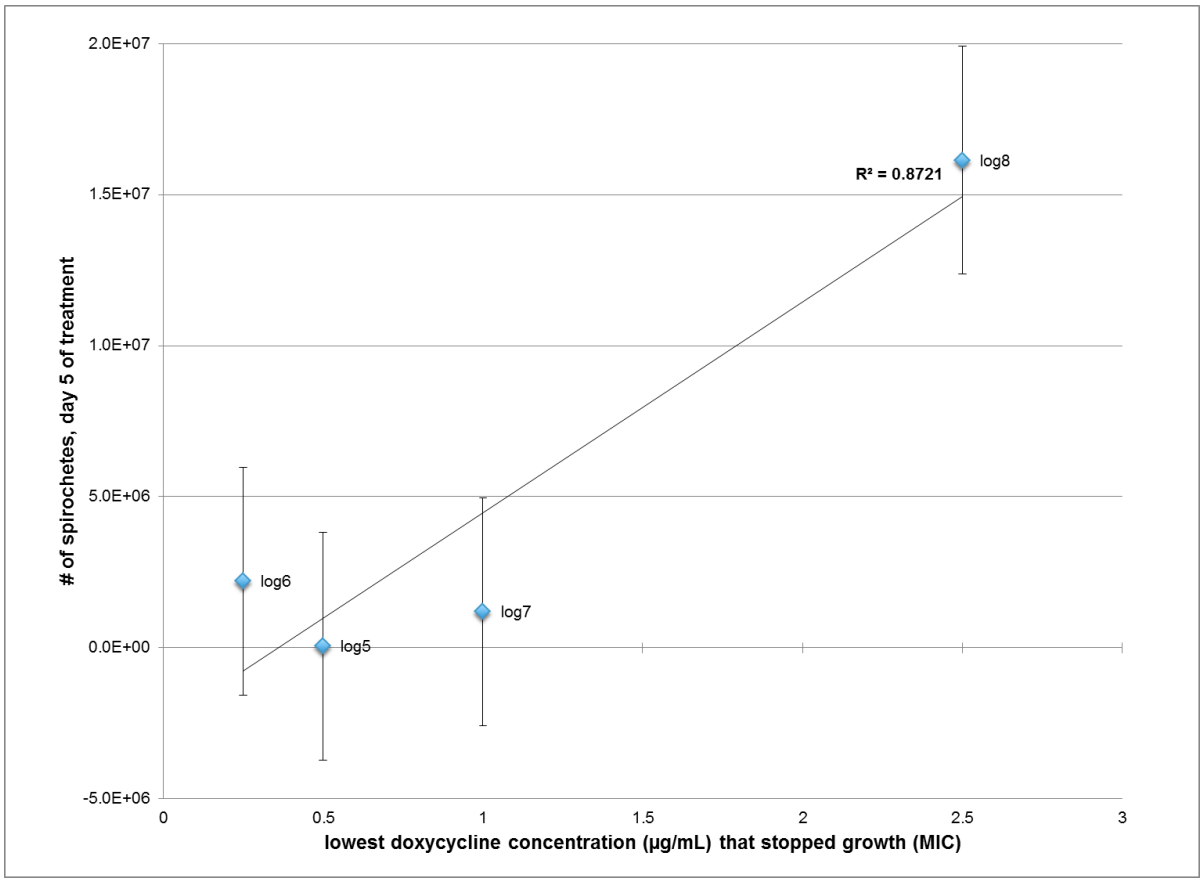

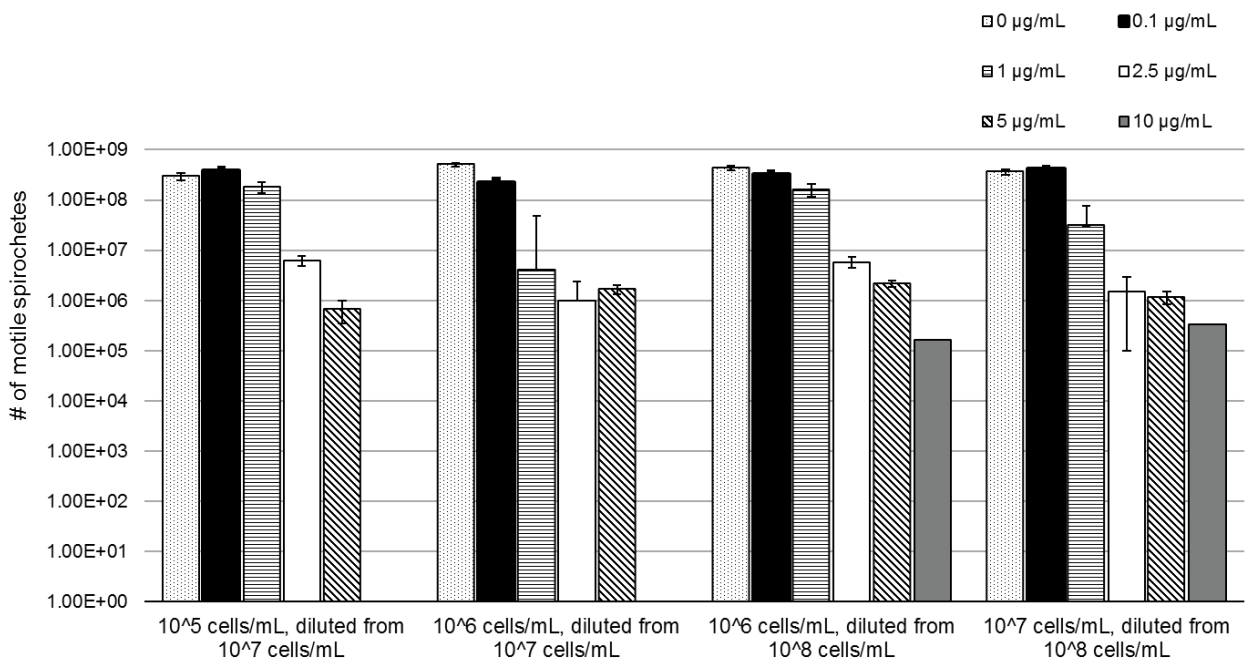

Figure 2. Number of motile *B. burgdorferi* per concentration of doxycycline on day 5 post-treatment in the MBC assay. A growth inhibition assay was performed, whereby cultures of differing initial concentrations and seed concentrations were treated with multiple concentrations of doxycycline. The number of motile spirochetes per culture was counted 5 days after the cessation of treatment.

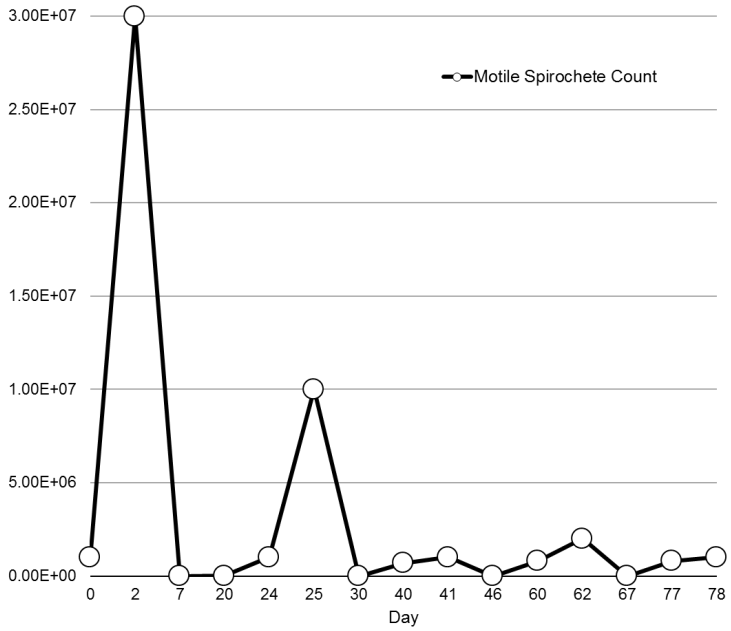

Figure 3A

Figure 3B

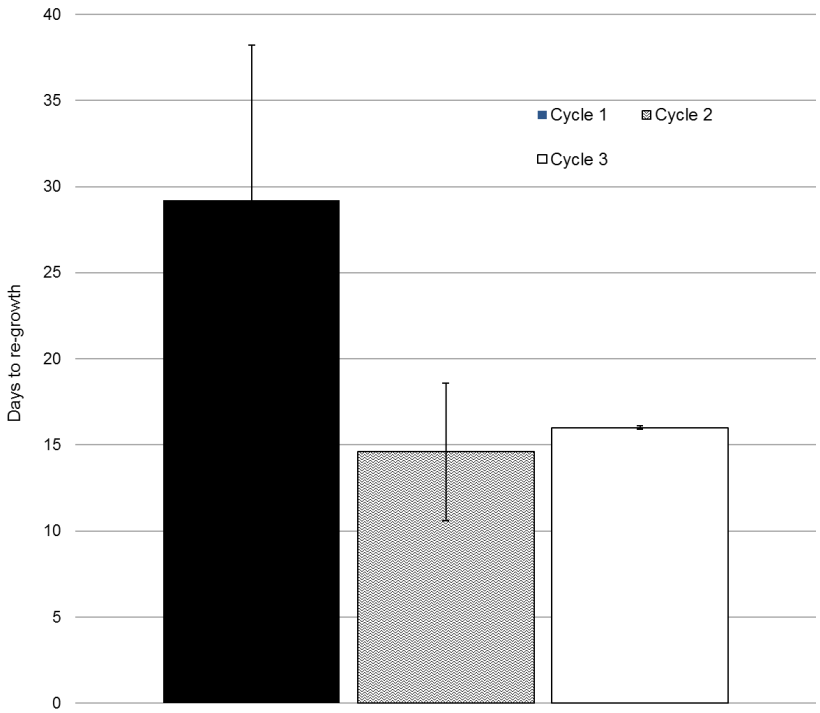

Figure 3. Pulse-dose assay. (A) Representative figure of the pulse-dose assay. (B) Time to regrowth for each consecutive cycle of the assay. B. burgdorferi cultures (n=34) were seeded initially at 7 x 105 cells/mL, treated with 50 µg/mL of doxycycline, then switched to doxycycline-free media. Cultures were monitored for regrowth, then those that regrew (n=5) were treated again before they reached their initial concentration. The process was repeated 2 more times for those that regrew (n=2). The time to regrowth after each treatment cycle is displayed. Error bars are SD. No significant difference was observed in the time to regrowth between cycles 2 and 3.