1   *Borrelia burgdorferi,* **the causative agent of Lyme disease, forms**

2   **drug-tolerant persister cells.**

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8   **Running title: Persisters of *Borrelia burgdorferi***

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20

21 **ABSTRACT**

22   *Borrelia burgdorferi* is the causative agent of Lyme disease, which affects an

23 estimated 300,000 people annually in the US. When treated early, the disease usually

24 resolves, but left untreated, can result in symptoms such as arthritis and

25 encephalopathy. Treatment of the late stage disease may require multiple courses of

26 antibiotic therapy. Given that antibiotic resistance has not been observed for *B.*

27 *burgdorferi*, the reason for the recalcitrance of late stage disease to antibiotics is

28 unclear. In other chronic infections, the presence of drug-tolerant persisters has been

29 linked to recalcitrance of the disease. In this study, we examined the ability of *B.*

30 *burgdorferi* to form persisters. Killing of growing cultures of *B. burgdorferi* with antibiotics

31 used to treat the disease was distinctly biphasic, with a small subpopulation of surviving

32 cells. Upon regrowth, these cells formed a new subpopulation of antibiotic-tolerant cells,

33 indicating that these are persisters rather than resistant mutants. The level of persisters

34 increased sharply as the culture transitioned from exponential to stationary phase.

35 Combinations of antibiotics did not improve killing. Daptomycin, a membrane-active

36 bactericidal antibiotic, killed stationary phase cells, but not persisters. Mitomycin C, an

37 anti-cancer agent that forms adducts with DNA, killed persisters and eradicated both

38 growing and stationary cultures of *B. burgdorferi*. Finally, we examined the ability of

39 pulse-dosing an antibiotic to eliminate persisters. After addition of ceftriaxone, the

40 antibiotic was washed away, surviving persisters were allowed to resuscitate, and

41 antibiotic was added again. Four pulse-doses of ceftriaxone killed persisters, eradicating

42 all live bacteria in the culture.

43

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

67   the latent form of the disease. *Mycobacterium tuberculosis* hides from the immune

68   system in macrophages or in granulomas [9].

69   *B. burgdorferi* causes Lyme disease with 300,000 estimated cases annually in

70   the United States alone [10]. When treated early with antibiotics, the disease usually

71   resolves [11, 12]. If treatment is delayed, the pathogen spreads throughout the body

72   and can cause meningitis, arthritis, and carditis. Meningitis and carditis are mostly self-

73   limited, but Lyme arthritis can persist for years [13, 14]. A substantial proportion of

74   patients receiving their first course of antibiotics for Lyme arthritis do not respond fully to

75   a 28 day course of treatment. In such cases, retreatment with additional courses of

76   antibiotics is recommended [13, 15, 16]. *B. burgdorferi* avoids immune attack by

77   antigenic variation of surface components and by decreasing exposure of antigens [17-

78   19]. In this regard, Lyme disease resembles other chronic infections where the

79   pathogen is protected from the immune system, and persister cells may enable it to

80   survive treatment with antibiotics. In *Escherichia coli*, the model organism for the study

81   of persisters, dormant cells are formed primarily through expression of toxin/antitoxin

82   (TA) modules. Toxins confer dormancy by either inhibiting protein synthesis or by

83   decreasing the energy level of cells [20-22]. TA modules are widely spread among

84   bacteria, and are copiously present in some pathogens. *E. coli* has more than 30 TA

85   modules and *M. tuberculosis* over 75 [23, 24]. Interestingly, there are apparently no TA

86   modules in the genome of *B. burgdorferi* [25]. Virtually nothing is known about

87   persisters in this species. In this study, we report formation of drug-tolerant persisters in

88   *B. burgdorferi* and describe possible approaches to their elimination.

89

90   **Materials and methods**

91   **Bacterial strains and growth conditions:** *Borrelia burgdorferi* B31 5A19 that had

92   been passaged five times *in vitro* was kindly provided by Dr. Monica Embers [26]. *B.*

93   *burgdorferi* was grown in BSK-II liquid media in a microaerophilic chamber (34°C, 3%

94   O_2, 5% CO_2). Cultures were started by thawing -80°C glycerol stocks of *B. burgdorferi*

95   (titer approximately 10^7 cfu/mL) and diluting 1:20 into fresh BSK-II media.

96   BSK-II liquid medium was prepared according to protocol received from Monica

97   Embers' lab by adding the following ingredients to 400 ml of deionized water and mixing

98   thoroughly: 20 g bovine serum albumin (Sigma), 2 g neopeptone (Fluka), 0.8 g

99   yeastolate (BD), 4 g HEPES sodium salt (Sigma), 2.4 g 10X CMRL (US Biologicals),

100   0.28 g sodium citrate (Fisher), 0.32 g sodium pyruvate (Sigma), 2 g glucose (Fisher),

101   0.16 g N-acetyl-glucoasmine (Sigma), 0.88 g sodium pyruvate (Sigma). The pH of the

102   medium was adjusted to 7.6 and 24 ml of rabbit serum (Sigma) was added to the

103   media. The medium was then filtered through a 0.22 μm filter.

104   Semi-solid plating was used to obtain cfu counts [27]. First, BSK 1.5X medium for

105   semi-solid plating was prepared as in Samuels 1995 [27]. The following ingredients

106   were added to 1 L of deionized water (LabChem, Inc) and mixed thoroughly: 8.33 g

107   neopeptone (Fluka), 4.22 g yeastolate (BD), 9.99 g HEPES acid (Fisher), 8.33 g

108   glucose (Fisher), 1.22 g sodium citrate (Fisher), 1.33 g sodium pyruvate (Sigma), 0.670

109   g N-acetyl-glucosamine (Sigma), 7.66 g sodium bicarbonate (Sigma). The pH of the

110   media was adjusted to 7.5 and then 83.25 g of bovine serum albumin (Sigma) was

111   added. The medium was stirred for one hour then filtered using a 0.22 μm filter. 1.5X

112   BSK-II was stored at 4°C and used within 7 days of preparation. On the day of plating,

6

113   125 ml of 1.5X BSK was mixed with 6 ml rabbit serum and 19 ml 1X CMRL (97.89

114   mg/mL) and equilibrated to 55°C. 1.7% agarose (Lonza) was melted and equilibrated to

115   55°C. When all ingredients had equilibrated to 55°C, 1.7% agarose was added to 1.5X

116   BSK at a ratio of 2:1 (BSK:agarose) to create BSK agarose. 8 ml of BSK agarose was

117   dispensed into 60mm Petri dishes as bottom agar and allowed to solidify. For top agar,

118   100 µl of the given dilution of *B. burgdorferi* was mixed with 5 ml of 55°C BSK agarose

119   and poured onto the bottom agar plates and allowed to solidify. The plates were

120   incubated in zip lock bags in microaerophilic chamber (34°C, 3% O_2, 5% CO_2) for at

121   least 21 days to obtain visible colonies.

122

123   **Antimicrobial agents:** Amoxicillin (Sigma), doxycycline hydrochloride (MP

124   Biomedicals), ceftriaxone disodium salt hemi (heptahydrate) (Sigma), and vancomycin

125   hydrochloride (Sigma) were dissolved in water. Mitomycin C (Sigma), gemifloxacin

126   mesylate (Tecoland Corporation), and spectinomycin dihydrochloride pentahydrate

127   (RPI) were dissolved in DMSO. Daptomycin cyclic lipopeptide (Sigma) was dissolved in

128   a 5 µg/ml solution of calcium chloride. Stock solutions of antibiotics were aliquotted and

129   stored at -20°C until use. Antibiotics did not undergo freeze-thaw cycles.

130

131   **Killing experiments:** *B. burgdorferi* was cultured in liquid BSK-II media for 3 days to

132   late-exponential growth phase or for 5 days to stationary phase. Antibiotics were then

133   added to the culture. The cultures were incubated in the microaerophilic chamber (34°C,

134   3% O_2, 5% CO_2). At a given time point, an aliquot of the culture was washed twice by

135   centrifuging the culture at 13.2k rpm for 5 minutes and resuspending the pellet in an

136   equal volume of fresh BSK-II medium. The cultures were then serially diluted in fresh

137   BSK-II media. 100 µl of the appropriate dilution was mixed with 5 ml of BSK agarose

138   and poured as top agar. Plates were incubated in the microaerophilic chamber until

139   visible colonies appeared (at least 21 days).

140

141   **Growth-persister experiments:** Cultures of *B. burgdorferi* were started as described

142   above. At each time point, an aliquot of a growing culture was removed, diluted, and

143   plated for cfu counts to generate the growth curve. A second aliquot (1 mL or 3 mL) was

144   removed at the same time and challenged for five days with the indicated antibiotic.

145   After five days, an aliquot of challenged culture was removed, washed twice, diluted,

146   and plated for cfu counts to generate the persister curve.

147

148   **Minimum Inhibitory Concentration (MIC) testing:** A slightly modified version of the

149   broth microdilution [28] was used. *B. burgdorferi* was grown in liquid culture for three

150   days to reach exponential phase and then back diluted 1:10 into fresh BSK-II media to

151   make the inoculum solution. All antibiotics were prepared as stock solutions in solvent

152   (water or DMSO) based on the concentration to be tested and diluted in two fold

153   increments in a 96 well stock plate. 2 µl per well of the antibiotic stock solution was

154   transferred to the 96 well MIC plate to which 198 µl of the *B. burgdorferi* inoculum

155   solution was added (final inoculum of approximately 10^6 cells/well). Media, growth, and

156   vehicle controls were included on each plate. The MIC plate was covered with

157   Breatheasy Film (Diversified Biotech) and incubated in the microaerophilic chamber

158   (34°C, 3% O_2, 5% CO_2) for 72 hours. The lowest concentration of antibiotics that

159   showed inhibition of growth was interpreted as the MIC. All MIC assays were repeated

160   at least twice in triplicate.

161

162   **Results**

163   **Characterization of *B. burgdorferi* Persisters**

164   The presence of persisters is indicated by a biphasic killing pattern in a time-

165   dependent killing experiment. The bulk of the population is rapidly killed, followed by a

166   slower rate of death in a subpopulation of tolerant cells [29, 30]. In order to determine

167   whether *B. burgdorferi* forms persisters, time-dependent killing experiments were

168   performed with antibiotics commonly prescribed to patients with Lyme disease.

169   Doxycycline is a bacteriostatic protein synthesis inhibitor; amoxicillin and ceftriaxone

170   inhibit bacterial cell wall synthesis and are bactericidal for many bacteria. Minimum

171   inhibitory concentrations (MICs) of doxycycline, amoxicillin, and ceftriaxone were

172   determined (Table 1). Levels of antibiotics close to what is achievable with standard

173   clinically prescribed treatment dosing were chosen to evaluate persister formation in *B.*

174   *burgdorferi*, and we used colony forming unit (cfu) count to determine viability.

175   Amoxicillin (6 μg/ml, 100X MIC) and ceftriaxone (3 μg/ml, 300X MIC) at clinically

176   achievable levels killed the majority of cells in the first day, after which a slow phase of

177   death followed for the next 6 days (Figure 1a). This characteristic biphasic pattern of

178   killing is consistent with the presence of drug-tolerant persister cells.

179   Previous studies have shown that the persister fraction in other bacteria remains

180   relatively unchanged even as the antibiotic level increases. We sought to determine if *B.*

181   *burgdorferi* persisters behaved similarly in a dose-dependent killing experiment. As the

182   concentration of amoxicillin and ceftriaxone increased, the fraction of surviving cells

183   remained largely unchanged (Figures 1b-c). Doxycycline is a bacteriostatic antibiotic,

184   but at higher concentrations appeared to effectively kill *B. burgdorferi* (Figure 1d).

185   Again, the fraction of surviving cells did not change significantly with increasing levels of

186   the compound. Thus, *B. burgdorferi* forms persisters capable of surviving very high

187   concentrations of antibiotics, which exceed what is clinically achievable.

188   Density-dependent formation is a common feature of persisters reported for all

189   pathogens examined so far, including *E. coli, Staphylococcus aureus, P. aeruginosa,*

190   and *M. tuberculosis* [6, 31-33]. In order to test this property in *B. burgdorferi*, samples

191   from a growing culture were removed over time, exposed to a lethal dose of antibiotic

192   for 5 days, and then plated for cfu. There was a characteristic dip in persister levels in

193   the early log phase, which is probably due to the resuscitation of dormant cells carried

194   over from the inoculum (Figure 2). At mid-log phase, there is a sharp increase in

195   persister levels, which continues as the density of the culture rises. In *E. coli*, once the

196   culture reaches stationary state, complete tolerance is achieved for β-lactams that only

197   kill growing cells [34]. In *B. burgdorferi*, we observe a very different picture – both

198   amoxicillin and ceftriaxone kill stationary cells fairly well, yet the fraction of persisters

199   continues to increase. One possibility is that this "stationary" culture actually represents

200   a steady state where some cells die and others grow.

201   Next, we tested whether the *B. burgdorferi* cells surviving antibiotic treatment are

202   drug-tolerant persisters or resistant mutants. For this, colonies produced by the

203   surviving cells were regrown and tested for MIC. The amoxicillin and ceftriaxone MIC

204   remained unchanged, showing that surviving cells had not acquired or developed a

10

205   genetic mechanism for antibiotic resistance. The population grown from the surviving

206   cells produced the same level of persisters as the original population (Figure 3). These

207   experiments show that *B. burgdorferi* forms typical persister cells.

208

209   **Eradication of *B. burgdorferi* Persisters**

210   Drug combinations. Some antibiotics act synergistically, such as sulfonamide and

211   trimethoprim, polymixin and gentamicin, aminoglycosides and β-lactams [35], and we

212   wanted to see if a combination of compounds known to be active against *B. burgdorferi*

213   will increase efficiency of killing both regular and persister cells.

214   All possible two-drug combinations of amoxicillin, ceftriaxone, and doxycycline

215   were tested with a late-exponential phase culture in a time-dependent killing experiment

216   and found to be no more effective than the drugs used individually in killing of *B.*

217   *burgdorferi* (Figure 4a). Doxycycline actually inhibited the action of amoxicillin. We have

218   shown previously that fluoroquinolones and aminoglycosides can kill non-growing cells

219   [36, 37], and we next tested these compounds against *B. burgdorferi*. The pathogen is

220   generally poorly susceptible to compounds from these classes. However, the *B.*

221   *burgdorferi* MICs for gemifloxacin (fluoroquinolone) and spectinomycin (aminoglycoside)

222   are within achievable human dosing levels so we chose to test them [38-41] (Table 1).

223   Both gemifloxacin and spectinomycin were ineffective in killing *B. burgdorferi* at tested

224   concentrations (Figure 4b). Combining these compounds also did not improve killing

225   (Figure 4b).

226

11

227   Experimental compounds. Having shown that combinations of clinically prescribed

228   antibiotics for Lyme disease are unable to effectively kill persister bacteria, we sought to

229   examine some novel potential antimicrobial agents. We recently showed that

230   acyldepsipeptide (ADEP4), an activator of the ClpP protease, effectively kills persisters

231   in *S. aureus* [42]. In the presence of ADEP4, the protease cleaves mature proteins,

232   forcing the cell to self-digest. However, ADEP4 did not have significant activity against

233   *B. burgdorferi* (not shown), which may be due to poor penetration.

234   We then considered whether knowledge of *B. burgdorferi* biology might be

235   exploited to predict vulnerability to existing approved compounds. *B. burgdorferi* lives

236   under microaerophilic conditions, where the capacity for energy generation is limited by

237   comparison to aerobic organisms. Daptomycin is the only approved membrane-acting

238   antibiotic that disrupts the proton motive force. The *B. burgdorferi* MIC to daptomycin

239   was fairly high, 12-25 µg/ml (Table 1), in accordance with published data [43].

240   Daptomycin was highly bactericidal against *B. burgdorferi*, but a remaining

241   subpopulation of persisters survived (Figure 5), suggesting that *B. burgdorferi* persisters

242   can tolerate a drop in the energy level. Next, we tested vancomycin. This large

243   glycopeptide antibiotic binds to lipid II, precursor of peptidoglycan, on the outside of the

244   cytoplasmic membrane. Vancomycin is highly effective against Gram-positive bacteria,

245   but does not penetrate across the outer membrane of Gram-negative species.

246   Surprisingly, the vancomycin MIC with *B. burgdorferi* is low, 0.25 µg/ml, similarly to

247   Gram-positive species. *B. burgdorferi* has an outer membrane; the basis for this

248   anomaly is unclear. Vancomycin effectively killed growing cells of *B. burgdorferi*, but not

249   persisters, and was comparable to ceftriaxone (not shown). We also tested teixobactin,

250 a compound we recently discovered, which also binds lipid II [44]. At 1.2 kDa,

251 teixobactin is considerably smaller than vancomycin (1.8 kDa), but it did not exhibit

252 good activity in killing *B. burgdorferi* (not shown).

253

254 <u>Prodrugs</u>. Growth under microaerophilic conditions suggests vulnerability to compounds

255 whose action depends specifically on a low oxygen environment. Nitroaromatic

256 compounds such as metronidazole are prodrugs that are converted into reactive drugs

257 by bacterial nitroreductases. These enzymes are expressed under anaerobic or

258 microaerophilic conditions, and target pathogens living in these environments (i.e.

259 *Helicobacter pylori, Clostridium difficile, E. coli*). We found that some nitroaromatic

260 compounds like nitrofurantoin are effective in killing *E. coli* persisters [45]. However, we

261 did not detect homologs of nitroreductases in the genome of *B. burgdorferi*. The MIC for

262 nitroaromatic compounds (nitrofurantoin, nitrofurazone, and metronidazole) was too

263 high to make them useful agents for killing *B. burgdorferi* persisters (data not shown).

264   Another compound that depends on a reductive environment for action is the

265 prodrug mitomycin C. Upon entering the cell, mitomycin C is reduced into an active drug

266 which then forms covalent adducts with DNA [46]. Originally discovered in a screen for

267 antibiotics, mitomycin C is now used as an anticancer agent. Cancers often create a

268 microaerophilic environment, which, together with rapid cell division, accounts for the

269 relatively selective action of mitomycin C against them. Functional RecBC and RecFOR

270 pathways are required to repair DNA damaged by mitomycin C in *E. coli* [46].

271 Interestingly, according to genomic data, *B. burgdorferi* lacks the genes of the RecFOR

272 pathway [47], further suggesting vulnerability to this compound.

13

273   Mitomycin C eradicated a late exponential culture of *B. burgdorferi* within 24

274   hours, with no detectable persisters remaining (Figure 6a). This was observed with a

275   low, clinically achievable dose of the compound - 1.6 μg/ml, or 8X MIC. In a dose-

276   dependent experiment, eradication of a late exponential culture was achieved within 5

277   days with a 0.8 μg/ml (4X MIC) dose of the compounds (Figure 6b). Finally, mitomycin

278   C was tested against a stationary culture of *B. burgdorferi*. Surprisingly, eradication was

279   achieved with a low dose of 4X MIC within 24 hours (Figure 6c). It appears that a

280   stationary population is more susceptible to this compound than an exponentially

281   growing one.

282

283   Pulse-dosing. Apart from identifying compounds capable of killing persisters, it may also

284   be possible to eliminate them with conventional bactericidal antibiotics using pulse-

285   dosing. Based on our results, the level of persisters is lowest during early exponential

286   growth (Figure 2). We reasoned that allowing growth to resume and then re-treating

287   them as they enter exponential phase could kill persisters surviving an antibiotic

288   challenge. Eradication of the culture could then be achieved after several rounds of

289   killing and regrowth. To test this, a culture of *B. burgdorferi* was exposed to amoxicillin

290   or ceftriaxone. The surviving persisters were allowed to resuscitate for a short period of

291   time in fresh media, and then exposed to antibiotic again for a second round of killing.

292   Persisters were substantially diminished after four rounds of killing with amoxicillin, and

293   were eradicated below the limit of detection after four rounds of killing with ceftriaxone

294   (Figure 7). Additionally, we found that a ceftriaxone solution stored under experimental

295   conditions (in BSK-II media at 34°C, 3% O_2, 5% CO_2) does not lose activity, as

14

296   measured by MIC against *B. burgdorferi,* for up to 20 days. The activity of amoxicillin

297   measured similarly, however, dropped 20-fold over 20 days which suggests degradation

298   over time. The resulting MIC was still lower than the concentration used in killing

299   experiments. This pulse-dosing experiment shows that a population of the pathogen can

300   be eradicated with conventional antibiotics commonly used to treat the disease.

301

302   **Discussion**

303   The presence of drug-tolerant persisters can explain the recalcitrance of chronic

304   infections to antimicrobial therapy, especially in cases when the disease is caused by a

305   susceptible pathogen. While some chronic infections are ancient – leprosy, syphilis,

306   tuberculosis – many cases in developed countries are consequences of otherwise

307   successful medical intervention. Various indwelling devices (catheters, prostheses,

308   heart valves) provide a substratum for biofilms that protect persisters from the immune

309   system [2]. Even in bacterial infections that are routinely successfully treated with

310   antibiotics, there is dependence upon the host immune system to control persisting

311   bacteria that are not eradicated by antibiotics. The role of the immune system becomes

312   evident when these same infections involve immunocompromised hosts and antibiotic

313   eradication of the infection becomes much more difficult.

314   *B. burgdorferi* is a pathogen that can affect immunocompetent hosts. It

315   establishes long term infections of years to lifelong in both its natural (i.e. mice) and

316   incidental (i.e. humans) hosts in the absence of antibiotic therapy [14, 48]. Treatment in

317   the early stages of disease results in good outcomes. Delays in diagnosis and treatment

318   lead to sequelae that may require additional treatment. For example, patients who

15

319   develop arthritis, which typically begins after 1 month of untreated infection, often do not

320   respond fully to a first course of 28 days of antibiotics [49]. The majority of these

321   patients have evidence of *B. burgdorferi* DNA in their synovial fluid and will respond to

322   additional one or two month courses of antibiotics [13, 16]. A smaller minority of patients

323   referred to as "antibiotic resistant Lyme arthritis" will continue to have arthritis with

324   synovial fluid that is PCR negative for *B. burgdorferi* DNA. These patients typically

325   respond to anti-inflammatory agents such as methotrexate or TNF-inhibitors. Both these

326   groups of patients should be distinguished from the highly controversial group of

327   patients with "chronic Lyme disease" that exhibit fatigue, myalgias and arthralgias

328   without objective evidence of disease. For the first group of Lyme arthritis patients

329   responsive to antibiotics, given that there is no reported resistance to clinically used

330   tetracyclines, β-lactams, and cephalosporins in the pathogen, the need for lengthy

331   courses of therapy is unclear. The presence of persister cells is one possible

332   explanation and this is a pattern that is seen in other infections where persister cells are

333   thought to be relevant for disease *in vivo.*

334   We found that similar to other pathogens, the pattern of killing of *B. burgdorferi*

335   by bactericidal antibiotics is biphasic, with a small subpopulation of surviving persisters.

336   These surviving clones are not resistant mutants; upon regrowth they form a new

337   persister subpopulation. Also similar to *E. coli, S. aureus,* and other pathogens, the

338   density of persisters increases as the culture deviates from strictly exponential growth,

339   reaching a maximum at stationary state. This is probably due to a deterioration of

340   growth conditions resulting in increasing numbers of dormant cells. However, of note,

341   the stationary state in *B. burgdorferi* is atypical, as amoxicillin and ceftriaxone continue

342   to kill the majority of cells despite an increase in the level of persisters in the population.

343   Cell-wall acting antibiotics do not normally kill non-growing cells; one possibility is that

344   stationary state *B. burgdorferi* cultures represent a steady state of growing and dying

345   cells. The ability of β lactams to kill non-growing cells has also been observed in *M.*

346   *tuberculosis* where a combination of meropenem and a β lactamase inhibitor was able

347   to kill viable but non-replicative cells [50]. The authors speculate that peptidoglycan

348   remodeling continues in these non-replicating cells allowing for the activity of the β

349   lactam. This is another possible explanation of the killing we observe of stationary

350   phase *B. burgdorferi* with amoxicillin and ceftriaxone.

351   In a recently published study, Iyer, et al. [51] treated two different strains of *B.*

352   *burgdorferi* with ceftriaxone and were unable to detect live *B. burgdorferi* by subculture

353   in liquid medium. However, the cell density in that study was 10^7 cells/ml, and according

354   to our data, persister levels in this early exponential culture are low. In some of the

355   biological replicates treated with ceftriaxone, we have not been able to recover live

356   cells. At higher cell densities, the presence of persisters is unambiguous.

357   One common strategy for improving elimination of infective agents is to combine

358   existing compounds. For example, β lactams and aminoglycosides are known to

359   synergize with each other to achieve effective killing of *Enterococci* [52]. We tested

360   combinations of standard antibiotics used in treatment of Lyme disease as well as a

361   combination of a fluoroquinolone and an aminoglycoside, compounds that often

362   synergize and are capable of killing non-growing cells. However, there was no synergy

363   in killing *B. burgdorferi* with any of the tested combinations.

364   We recently described efficient killing of persisters in *S. aureus* [42] and in *E. coli*

365   [45], and tested these compounds against *B. burgdorferi*. ADEP4, an activator of the

366   Clp protease, causes massive protein degradation in *S. aureus*, killing regular cells and

367   persisters. However, ADEP4 was not active against *B. burgdorferi*. We also reported

368   that nitrofuran prodrugs are effective in killing *E. coli* persisters. Nitrofurans are reduced

369   by bacterial nitroreductases into generally reactive compounds, explaining their activity

370   against persisters. Nitroreductases are expressed under anaerobic or microaerophilic

371   conditions. *B. burgdorferi* is a microaerophilic organism, but does not have obvious

372   homologs of a nitroreductase, and nitrofurans we tested were fairly inactive.

373   We also tested daptomycin, a lipopeptide that acts by increasing K^+ permeability

374   of the membrane. Being in a low-energy (microaerophilic) environment, the pathogen

375   may be vulnerable to membrane-acting compounds. Daptomycin killed the majority of

376   cells in a stationary culture, but the level of surviving persisters was comparable to that

377   of a stationary culture treated with ceftriaxone. In a recent publication daptomycin was

378   reported to kill *B. burgdorferi* persisters more effectively than regular cells [43]. This

379   conclusion was based on equating stationary cells with persisters. As follows from our

380   experiments, a stationary culture harbors a small subpopulation of persisters. The

381   actual level of stationary cells apparently surviving treatment by daptomycin in that

382   study was very high, 28%, as determined by live/dead staining. Under similar

383   conditions, we detect about 10^3 (0.002%) surviving persisters by cfu count. It appears

384   that live/dead staining may be over reporting the level of live *B. burgdorferi* cells.

385   Another weakness of the pathogen is its apparently limited ability for DNA repair.

386   Based on the genome, *B. burgdorferi* lacks *recFOR*. In *E. coli,* both RecBC and

18

387   RecFOR are required for repair of DNA damage caused by mitomycin C, an anticancer

388   drug. Mitomycin C at a low, clinically achievable dose (8X MIC), eradicated *B.*

389   *burgdorferi* persisters in both exponential and stationary cultures within 24 hours. A

390   highly reduced environment activates mitomycin C, and this contributes to its selective

391   action in microaerophilic tumors. While the killing of persisters by mitomycin C is

392   impressive, given the toxicity of this drug, this is more of a proof-of-principle for a

393   compound exploiting the weaknesses of this pathogen rather than a clinically useful

394   agent. Treatment with mitomycin C can result in serious negative side effects and it

395   should not be used for treatment of Lyme Disease. This agent will be useful to examine

396   the possible contribution of persisters to the disease in an animal model of infection.

397   Another peculiar feature of *B. burgdorferi* and a weakness of the pathogen is the

398   lack of development of resistance to any antibiotic used to treat Lyme disease. Even

399   attempts to raise mutants resistant to amoxicillin and ceftriaxone *in vitro* have been

400   unsuccessful. Joseph Bigger proposed an interesting strategy for elimination of

401   persisters in 1944, in the first publication describing these cells [53]. The rationale is to

402   add antibiotic to kill off regular cells; wash it away; allow the culture to start regrowing, at

403   which point persisters will resuscitate. Reintroducing antibiotics will kill the regrowing

404   bacteria. The argument against pulse dosing is that this protocol invites resistance

405   development. Given that this is not a concern for *B. burgdorferi*, pulse dosing may be an

406   effective strategy and we performed pulse dosing with amoxicillin and ceftriaxone.

407   Persisters were eradicated with ceftriaxone in four pulses. These experiments form the

408   basis for testing pulse dosing in an animal model, and if successful, in humans.

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409   While we have identified the presence of *B. burgdorferi* persisters in cultures of

410   the organism, the mechanisms by which they are able to survive remain unknown.

411   There are multiple pathways of persister formation in other bacteria. The study of

412   persisters so far identified redundant TA modules as a main component responsible for

413   persister formation in *E. coli* and *S. typhimurium* [8, 20, 22]. TA modules are widely

414   spread among bacteria, but are surprisingly absent from the genome of *B. burgdorferi*.

415   Other components leading to persister formation in *E. coli* have been detected as well -

416   the stringent response [54], various metabolic processes [55, 56], global regulators, and

417   protein stabilizing chaperones [56] . Future work will determine if these or other

418   processes are involved in persister formation in *B. burgdorferi* and if persisters play a

419   role in the pathogenesis of Lyme disease in humans.

420

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603

604   **Figure Legends**

605   **FIG 1**

606   Killing of *B. burgdorferi* by antibiotics*.* (a) Time-dependent killing. Antibiotics were

607   added to an exponentially growing culture, samples were taken over time, washed,

608   diluted, and plated in semi-solid BSK-II media for cfu counts. The culture was treated

609   with amoxicillin (Amox) (6 μg/ml) or ceftriaxone (Cef) (3 μg/ml) (n=9). (b-d) Dose-

610   dependent killing. A late-exponential culture of *B. burgdorferi* culture was exposed to

611   antibiotics for 5 days, and surviving cells were determined by cfu count. The culture was

612   treated with amoxicillin (b), ceftriaxone (c), or doxycycline (Dox) (d) (n=6). Error bars

613   represent standard error.

614

615   **FIG 2** Growth dependent persister formation in *B. burgdorferi.* Growth in BSK-II medium

616   was determined by cfu count. Persister levels were determined by taking samples from

617   the growing culture, exposing to antibiotic for 5 days, and counting cfu. (a), amoxicillin

618   (Amox) (6 μg/ml); (b), ceftriaxone (Cef) (3 μg/ml). N=6. Error bars represent standard

619   error.

620

621   **FIG 3** Persister formation is not heritable. Colonies recovered from a persister

622   experiment before and after antibiotic treatment were used to inoculate fresh BSK-II

623   media. The colonies were allowed to grow for 3 days and treated with the same

624   antibiotic used in the original persister experiment for 5 days. Persister levels of the

625   colonies recovered after antibiotic treatment (Persisters) were not significantly different

626   than the colonies recovered before antibiotic treatment (Control). N=5. Error bars

627   represent standard error. Amox = amoxicillin, Cef = ceftriaxone.

628

629   **FIG 4** Killing of *B. burgdorferi* with drug combinations. (a) Time-dependent killing of late

630   exponential *B. burgdorferi* cultures exposed to the indicated antibiotics in combination.

631   Amoxicillin (Amox) (6 µg/ml), ceftriaxone (Cef) (3 µg/ml), and doxycycline (Dox) (2.5

632   µg/ml) (n=6). (b) Killing of late exponential *B. burgdorferi* exposed to gemifloxacin

633   (Gemi) (1.5 µg/ml) and/or spectinomycin (Spec) (160 µg/ml) singly or in combination

634   (n=6). An aliquot was taken at indicated time points, washed, diluted, and plated on

635   semi-solid BSK-II media for cfu counts. Error bars represent standard error.

636

637   **FIG 5** Killing of *B. burgdorferi* by daptomycin. Time dependent killing of stationary phase

638   *B. burgdorferi* exposed to daptomycin (81 µg/mL) (n=3). Error bars represent standard

639   error.

640

641   **FIG 6** Killing of *B. burgdorferi* by mitomycin C (MMC). (a, c) Time-dependent killing of *B.*

642   *burgdorferi*. Three independent cultures of *B. burgdorferi* either at late exponential

643   phase (a) or stationary phase (c) of growth were treated with MMC: 0.8 µg/ml (4X MIC)

644   or 1.6 µg/ml (8X MIC). (b) Dose-dependent killing of late exponential cultures of *B.*

645   *burgdorferi* culture after 5-day exposure to increasing concentrations of MMC. An

646   aliquot was taken at indicated time points, washed, diluted, and plated on semi-solid

647   BSK-II media for cfu counts. N=6. The x-axis is the limit of detection. An asterisk

648   represents eradication to the limit of detection.

649

650   **FIG 7** Pulse dosing results in effective killing of *B. burgdorferi* persisters. Late

651   exponential cultures of *B. burgdorferi* were treated with (a) ceftriaxone (Cef) (3 µg/ml) or

652   (b) amoxicillin (Amox) (6 µg/ml) for 5 days. This represents the first round of killing. The

653   cultures were then washed and allowed to recover in fresh BSK-II media for 24 hours.

654   They were then treated again with amoxicillin (6 µg/ml) or ceftriaxone (3 µg/ml) for a

655   further 5 days to give the second round of killing. This was repeated for a total of four

656   rounds of killing with a 24 hours period of growth in fresh media between each round.

657   Bars represent the average of at least three independent cultures (n=3-6) and error bars

658   represent standard error. The x-axis is the limit of detection. An asterisk represents

659   eradication to the limit of detection.

660 **Table 1:** Selected antibiotics tested against *B. burgdorferi.*

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

The MIC was determined by broth microdilution method.

a

b

a

b

a

b

a

Cef Pulse Dosing

Log (CFU/ml)

9
7
5
3
1

Round 1 Round 2 Round 3 Round 4

*

■ Day 0
■ Day 5

b

Amox Pulse Dosing

Log (CFU/ml)

9
7
5
3
1

Round 1 Round 2 Round 3 Round 4

■ Day 0
■ Day 5