Cellular and immunological mechanisms influence host-adapted phenotypes in a vector-borne microparasite

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ABSTRACT

Predicting pathogen emergence and spillover to hosts relies on understanding the determinants of the pathogens' host range and the traits involved in host competence. While host competence is often considered a fixed species-specific trait, it may be variable if pathogens diversify across hosts. Balancing selection can lead to maintenance of pathogen polymorphisms (multiple niche polymorphism, MNP). The causative agent of Lyme disease, *Borrelia burgdorferi* (*Bb*), provides a model to study the evolution of host adaptation, as some *Bb* strains defined by their outer surface protein C (*ospC*) genotype are widespread in white-footed mice, and others are associated with non-rodent vertebrates (e.g. birds). To identify the mechanisms underlying potential strain x host adaptation, we infected American robins and white-footed mice, with three *Bb* strains of different *ospC* genotypes. *Bb* burdens in tissues varied by strain in a host-dependent fashion, and strain persistence largely corresponded to *Bb* survival at early infection stages and with transmission to feeding larvae('fitness'). Those early survival phenotypes are associated with cell adhesion, complement evasion, and/or inflammatory and antibody mediated induction of *Bb,* suggesting directional selective pressure for host adaptation and the potential role of MNP in maintaining OspC diversity. Such information will guide future investigations to inform eco-evolutionary models of host adaptation for microparasites.

INTRODUCTION

Predicting the potential for pathogen spillover events to human and non-human hosts has become crucial as the frequency and magnitude of emergence events continue to increase[1]. Critical traits involved in the potential for pathogen emergence are the range of hosts that a pathogen can infect (host range) and the competence of potential host species[2,3]. Host competence traits include the capacity to support initial infection establishment, development of infection over time, and eventual pathogen transmission, while the pathogen's host range trait is defined as the breadth of species for which a pathogen can achieve these three criteria[4]. Given the importance to human health, recent reviews and meta-analyses have sought to identify host- and pathogen-derived traits contributing to potential zoonotic emergence. Host-associated traits included life-history traits[5-7], relationships among hosts[8], species interaction dynamics (e.g., domestication)[9-11], and phylogenetic relationships[12,13]. Analyses of host range, adaptive potential, and mechanisms of infection support these traits as key predictors of zoonoses[2,14,15]. However, with a focus on observed patterns of host-pathogen association, these studies offer limited insights into the underlying molecular mechanisms that determine host competence and potentially shape pathogen adaptation.

A challenge in characterizing host competence is that it is a composite trait resulting from multiple processes encompassing host-pathogen interactions, any of which exhibits a broad range of variation within species[1,16,17]. Intraspecific variability in host competence has often been linked to host-specific traits such as local adaptation[16], phenotypic plasticity[1], development[18], sex[19], or environmentally-dependent physiological condition (e.g., resource availability, stressors)[20,21]. However, there is limited understanding of how competence varies depending on the host genotype-pathogen genotype interaction[16]. In fact, pathogen

74 polymorphisms within host populations (or 'niches'), have been proposed to maintain multiple
75 phenotypes within a pathogen and drive variable outcomes in host competence through a balancing
76 selection mechanism, known as "multiple niche polymorphism (MNP)"[22,23]. MNP is predicted
77 when the environment (i.e., host) is heterogeneous, and no single pathogen genotype reaches the
78 highest fitness in all hosts[24,25]. Thus, MNP assumes selective advantages for pathogen strains
79 adapting to certain hosts, i.e., to reduce their host range compared to more generalist strains [26].
80 These selective advantages may be manifested as increased probability of initial establishment,
81 faster development of infection (i.e., growth rates), or increased transmissibility[3,27].

82 *Borrelia burgdorferi* sensu stricto (*Bb*), the Lyme disease (LD) agent in North America, is an
83 ideal system to study the evolution of host-pathogen interactions because it is one of the few
84 zoonotic pathogens for which molecular mechanisms for host adaptation have been
85 proposed[22,28,29]. *Bb* is transmitted by a generalist tick vector, *Ixodes* spp., driving unique
86 selective pressures across the many hosts species, exhibiting strong variability in overall
87 competence[28,30]. MNP is proposed as a mechanism maintaining extensive *Bb* strain diversity,
88 particularly driving the polymorphism in a *Bb* gene, *ospC*[22]; Evidence for this hypothesis is
89 based on laboratory studies documenting fitness variation among *Bb* strains across hosts, as well
90 as observations of the associations between hosts and genetic markers in nature[22,31-36].
91 Determining the extent of intraspecific variation in pathogen fitness and evaluating molecular
92 mechanisms is pivotal for characterizing the potential selective pressures mediating host-pathogen
93 interactions and influencing human disease risk.

94 To understand the role of intraspecific *Bb* variation in host competence and assess the
95 plausibility of MNP as a driver of *Bb* diversification, we experimentally evaluated the interaction
96 between two reservoir vertebrate hosts and three genotypically-distinct *Bb* strains. Hosts were

rodents (white-footed mice, *Peromyscus leucopus*) and birds (American robins, *Turdus migratorius*) that are competent for *Bb* and frequently infected in nature[5]. We aimed to address the following questions: (1) Do *Bb* strains with distinct *ospC* genotypes exhibit variation in fitness (transmission to ticks) across two representative host species? (2) Are strains with higher fitness in one host (i.e., 'host adapted'), less fit in the other host compared to strains with more similar fitness across hosts (i.e., 'generalists')? Are there tradeoffs involved in host adaptation? (3) Which cellular and immunological mechanisms are involved in initial strain survival and persistence, consistent with differences in fitness, resulting in divergent strain × host phenotypes?

RESULTS

1. *Bb* strains vary in their fitness across hosts. We compared fitness between *Bb* genotypes commonly found in wild-caught rodents (*ospC* type A, K)[14,27,28] or rarely found in rodents but infectious in birds (*ospC* type E)[29]. These strains included tick-isolated B31-5A4 (*ospC* type A) and cN40 (*ospC* type E), or human patient-isolated 297 (*ospC* type K) (Table S1) and displayed similar growth rates in culture *in vitro* (Fig.S1). We generated *I. scapularis* nymphs carrying each of these strains and permitted these nymphs to feed on robins and white-footed mice. We found indistinguishable bacterial burdens between the three strains in flat and engorged nymphs (Fig.S2, Table S2). We then compared the ability of B31-5A4, 297, and cN40 to transmit from these *Bb*-infected robins and mice to naïve ticks by determining the *Bb* burdens in xenodiagnostic *I. scapularis* larvae at various time points. We found significantly higher proportions of robin-fed cN40-positive larvae than 297-positive larvae, when all time points were combined (Table S3-S4). Additionally, the proportion of cN40-positive larvae was significantly lower at 14 days post feeding (dpf), but significantly higher at 28dpf, than B31-5A4-positive larvae (Table S3-S4). We

also found significantly greater *Bb* loads in the larvae that fed on cN40-infected robins relative to 297-infected robins throughout the experiments. The bacterial burdens in larvae from cN40-infected robins were significantly lower at 14dpf but significantly higher at 28 and 56dpf than larvae from B31-5A4-infected robins (Fig.1A-1D, Table S2). Although the percent positivity of larvae varied over time and among different strains (Fig. 1E), the percentage of positive larvae at 56dpf (45, 27, 25% for larvae that fed on cN40-, B31-5A4-, 297-infected robins, respectively) suggest that cN40 is more fit in robins than B31-5A4 and 297.

In white-footed mice, the proportion of cN40-positive larvae was significantly lower than B31-5A4- or 297-positive larvae when all time points were combined (Table S3-S4). At 28 and 35dpf, there were significantly lower bacterial burdens in 297-positive than B31-5A4-positive larvae (Fig. 1F-1I, Table S2). We also found *Bb* burdens in cN40-positive larvae were significantly lower than those from B31-5A4- or 297-positive larvae throughout the experiments (Fig.1F-1I, Table S2-S4). While the percent positivity differed in the larvae from B31-5A4-, 297-, or cN40-infected mice at different time points and among different strains (Fig. 1J), the percentage of positive larvae at 56dpf (7, 69, 65% for larvae that fed cN40-, B31-5A4-, 297-infected mice, respectively) suggest cN40 is less fit in mice than B31-5A4 or 297.

2. The fitness of *Bb* strains corresponds with their ability of initial survival and persistence. Because tick-transmitted bacterial loads can vary[37], we confirmed the above data and also evaluated early injection site survival by intradermally injecting robins and white-footed mice with each strain (10^4 spirochetes per individual). At one day post injection (dpi), B31-5A4- or cN40-infected robins showed significantly greater burdens at the injection sites than uninfected robins, whereas B31-5A4- or 297-infected mice had significantly higher burdens at these sites than

143 uninfected mice (Fig. 2A-2B, Table S2). These findings indicate that B31-5A4, 297, and cN40
144 differ in their initial survival in robins and white-footed mice, corresponding with their fitness.
145 Because of the correspondence of data trends during both needle and tick infections, we utilized
146 a more physiologically relevant route of tick infections to evaluate the ability of these strains to
147 survive in the bloodstream of American robins and white-footed mice. At 7dpf, B31-5A4- and
148 cN40-, but not 297-infected, robins developed significantly greater levels of bacteremia than
149 uninfected robins, whereas robins infected with all strain displayed significantly greater
150 bacteremia than uninfected robins at 14dpf (Fig.2C-2D, Table S2). At 21dpf, cN40-infected robins
151 yielded significantly greater levels of bacteremia than uninfected robins, and bacteremia in cN40-
152 infected robins was significantly greater than B31-5A4-infected robins (Fig.2E, Table S2). At
153 28dpf, robins infected with all strains displayed levels of bacteremia indistinguishable from
154 uninfected robins (Fig.2F, Table S2). Thus, cN40 induces longer-lasting bacteremia in robins. In
155 contrast, the bacteremia levels from B31-5A4- or 297-, but not cN40-, infected white-footed mice
156 were significantly greater than uninfected mice at 7 and 14dpf, and B31-5A4- or 297-infected mice
157 had significantly higher bacteremia levels than cN40-infected mice at 14dpf (Fig.2G-2H, Table
158 S2). None of these strains were detected in mouse blood at 21 and 28dpf (Fig.2I-2J). Thus, cN40
159 is less capable than B31-5A4 and 297 at surviving in the white-footed mouse bloodstream.
160 We also evaluated the ability of B31-5A4, 297, and cN40 to colonize robin and white-footed
161 mouse tissues at 64dpf. Robins infected with all strains had significantly greater spirochete burdens
162 in skin than uninfected robins (Fig.2K, Table S2). Additionally, *Bb* burdens were significantly
163 greater in the heart and brain of cN40-infected, and the heart of B31-5A4-infected, than uninfected
164 robins (Fig.2L-2M, Table S2). The burdens in livers from robins infected with each strain were
165 indistinguishable from uninfected robins (Fig.2N, Table S2). These data showed persistent robin

166 skin colonization of all tested strains, but the ability of each strain to colonize other tissues varied.
167 In mice, the bacterial burdens in the outer ears (skin), joints, heart, and bladder from B31-5A4 or
168 297 infections were significantly greater than uninfected or cN40-infected mice (Fig.2O-2R, Table
169 S2). Thus, B31-5A4 and 297, but not cN40, persistently colonized these mice. Taken together,
170 these results showed a positive correspondence of these *Bb* strains' ability initial survival and
171 persistence in robins and white-footed mice.
172
173 **3. Multiple cellular and immunological phenotypes correspond with *Bb* strain-specific**
174 **fitness, providing mechanistic insights for *Bb* survival and persistence within a host.**
175
176 3.1. Cell adhesion: *Bb* strains differed in adhesion to robin and white-footed mouse fibroblasts.
177 We incubated B31-5A4, 297, and cN40 with robin or white-footed mice fibroblasts and compared
178 the levels of bacterial attachment microscopically with a non-adherent *Bb* strain, B314[38](Fig.S3).
179 Compared to B314, we detected significantly greater attachment of B31-5A4 and cN40(Fig.3A,
180 Table S2). We also observed significantly greater numbers of B31-5A4 and 297 attaching to white-
181 footed mouse fibroblasts relative to B314, and 297 had greater attachment than cN40(Fig.3B,
182 Table S2). These results showed differential cell adhesion of these *Bb* strains as a cellular
183 mechanism that positively corresponds with these strains' fitness in robins and white-footed mice.
184
185 3.2. Complement evasion: Robin, but not white-footed mouse complement, differentiated between
186 the *Bb* strains serum survivability. We first defined the capability of B31-5A4, 297, and cN40 to
187 evade complement-mediated killing by evaluating the spirochete survival after incubating these
188 strains with naïve robin or white-footed mouse sera. More than 88.2% of the spirochetes for these

189 strains survived in heat inactivated robin sera (rendered complement non-functional)[38], as did
190 the high passage, non-infectious, and serum sensitive control *Bb* strain, B313 (Fig.4A). When
191 incubated in normal robin sera, 8.6% of B313 survived, verifying the bactericidal activity of robin
192 sera (Fig.4A). 95.5% of B31-5A4 and 94.1% of cN40, but 43.6% of 297, survived normal robin
193 sera (Fig.4A). All strains survived indistinguishably (at least 80.6% survival) in robin sera treated
194 with *Ornithodoros moubata* complement inhibitor (OmCI), which inactivates avian complement
195 (Fig. 4B) [37], indicating that 297 is more vulnerable to robin complement-mediated killing than
196 B31-5A4 and cN40. Similarly, at least 97.1% of all strains survived in heat-inactivated, white-
197 footed mouse sera (Fig.4C). Whereas only 29.1% of B313 survived normal white-footed mouse
198 sera, at least 97% of all other strains survived in this sera (Fig.4C). Additionally, at least 89.1% of
199 all strains survived in white-footed mouse sera treated with cobra venom factor (CVF), which
200 inactivates mammal complement (Fig.4D)[37]. These results indicate that these strains evade
201 killing by white-footed mouse complement at indistinguishable levels.
202
203 3.3. Cytokine induction: *Bb* induced strain-specific varying levels of robin and white-footed mouse
204 pro-inflammatory cytokines during infection. As an indicator of the overall immune response [39,
205 40], we measured the ex-vivo expression levels of the genes encoding pro-inflammatory cytokines,
206 IFNγ, TNF, or TNF-induced protein, after incubating robin or white-footed mouse fibroblasts with
207 each *Bb* strain for 24h. The expression levels of housekeeping genes (18S rRNA and β-actin) were
208 included as controls. We found the expression levels for these genes in cells treated with all *Bb*
209 strains were indistinguishable from mock-treated cells at 1:10 (fibroblasts: spirochetes) (Fig. S4,
210 Table S2). At a ratio of 1:100, 297-incubated robin cells had significantly greater expression of
211 IFNγ and TNF-induced protein than mock-treated robin cells (Fig.5A-C, Table S2). Conversely,

212 cN40-treated mouse cells showed significantly greater expression of IFNγ and TNF than mock-
213 treated cells (Fig.5D-F).

214 We also assessed the levels of the pro-inflammatory cytokine markers IFNγ and TNFα *in vivo*
215 in robin and white-footed mouse sera at various times after tick infection. For 14 and 21dpf, we
216 found comparable levels of these cytokines in robins and mice, regardless of *Bb* strain (Fig. S5,
217 Table S2). At 7dpf, 297-infected robins had significantly greater levels of IFNγ and TNFα than
218 uninfected robins and greater levels of TNFα than cN40-infected robins, whereas cytokine levels
219 in cN40 or B31-5A4-infected robins were indistinguishable from uninfected robins (Fig.5G-5H,
220 Table S2). Conversely, cN40-infected mice displayed significantly greater levels of IFNγ and
221 TNFα than uninfected mice and TNFα than 297-infected mice, while 297-infected mice showed
222 indistinguishable cytokine levels from uninfected mice (Fig.5I-5J, Table S2). B31-5A4-infected
223 mice had significantly greater levels of IFNγ at 7dpf than uninfected mice (Fig.5J, Table S2).
224 These data indicate differences of pro-inflammatory cytokine induction *ex vivo* and *in vivo* by
225 these *Bb* strains, consistent with the fitness of these strains in robins and white-footed mice.
226
227 3.4. Antibodies: *Bb* strains differed in their ability to induce antibodies in a host- and infection
228 stage-dependent manner. We quantified the levels of antibodies induced by infection of each *Bb*
229 strain. To avoid strain-specific antibody recognition[41,42], we quantified the IgG titers in sera
230 from infected robins and mice that recognized an equal ratio of mixed lysates of B31-5A4, 297,
231 and cN40 (Fig. 6A-J). At 7dpf, 297-infected robins generated higher anti-*Bb* IgG titers than
232 uninfected or other strain-infected robins (Fig.6B, Table S2). At 14 and 21dpf, robins infected with
233 any strain developed significantly greater IgG titers than uninfected robins (Fig.6C-D, Table S2).
234 By 28dpf, cN40-infected robins generated significantly greater IgG titers than uninfected or other

strain-infected robins (Fig.6E, Table S2). In mice, cN40-infection induced significantly greater anti-*Bb* IgG titers than uninfected or other strain-infected mice at 7dpf (Fig.6G, Table S2). At 14dpf, white-footed mice infected with any strain developed significantly greater anti-*Bb* IgG titers than uninfected mice (Fig.6H). B31-5A4- or 297-infected white-footed mice generated significantly greater IgG titers than uninfected or cN40-infected mice at 21 and 28dpf (Fig.6I-6J, Table S2). Thus, whereas *Bb* strains differed in their ability to induce antibodies in a host- and infection stage-dependent manner, the levels of anti-*Bb* antibodies in robins and white-footed mice at 7dpf negatively corresponded to spirochete fitness in each of these hosts.

In addition to whole cell lysates, we also compared IgG titers recognizing purified major *Bb* antigens in robins and white-footed mice infected with each *Bb* strain at 7dpf and found similar negative correspondence between IgG titers and spirochete fitness (Fig. S6, Table S2). These findings raised the hypothesis that 7dpf antibodies are bactericidal. To avoid strain-specific bacterial killing, we incubated an equal ratio of B31-5A4, 297, and cN40 with different dilutions of sera from robins or white-footed mice infected with each strain at 7dpf. We found B31-5A4- and cN40-infected robin sera did not kill spirochetes, but 297-derived sera eliminated 50% of spirochetes at the dilution rate of 1:52 (Fig.6K). Conversely, cN40-infected white-footed mouse sera eliminated 50% of spirochetes at the dilution rate of 1:32 (Fig.6L). These results correlate both titers and bactericidal activities of anti-*Bb* antibodies with the fitness of these strains at early stages of infection. Note, the lower IgG titers at late stages of infection (28dpf) may reflect the lower fitness of the strains in their respective hosts, which warrants further investigations. However, our findings showed early stages-specific titers and bactericidal activities from the antibodies against B31-5A4-, 297-, or cN40, consistent with the fitness of these strains in robins and white-footed mice.

DISCUSSION

Our results provide clear evidence for strain-specific fitness variation across American robins and white-footed mice, two host species previously identified as highly competent for *Bb.* Based on significant differences in pathogen acquisition by xenodiagnostic larvae, strains 297 and cN40 appear to be white-footed mouse- and robin-adapted, respectively, and B31-5A4 seems to be more generalist (particularly at early stages). While we observed fitness disadvantages for host-adapted strains in their non-adapted hosts, our results suggest that *Bb* strains do not occupy strictly specialist or generalist phenotypes, as both strains 297 and cN40 achieved low levels of transmission in their non-adapted hosts.

Competing hypotheses exist for the fundamental drivers of *Bb* diversity, including MNP, suggesting that polymorphisms are maintained through differential fitness experienced by strains across different host environments. An alternative hypothesis, supported by parsimonious models of genome evolution, posits negative frequency-dependent selection (NFDS) to maintain high *Bb* diversity via adaptive immune responses that continually select against common genotypes in a host-independent manner[43]. Although the evolution and maintenance of *Bb* diversity has been the subject of much theoretical discussion[29,44-46], empirical and mechanistic approaches addressing this are uncommon. Previous evidence for host-adapted phenotypes among different *ospC* genotypes revealed weak associations from natural populations[22,47] and fitness variation for multiple *Bb* strains in a single species[29,41,48]. By demonstrating the presence of divergent host-adaptation phenotypes among *Bb* strains across different host species representative of the Lyme disease ecology system in nature, as well as the cellular and immunological mechanisms that influence host-pathogen interactions, this study provides novel evidence for the potential role

281 of MNP in driving patterns of *Bb* diversity. This does not preclude the potential for NFDS to
282 additionally structure *Bb* strain communities, and indeed synergistic effects of multiple
283 evolutionary drivers have been suggested[46].

284 Our results highlight that intraspecific pathogen variability drives variation in competence
285 within and between hosts. While microparasite competence is defined as the capacity for hosts to
286 permit establishment, development, and transmission of infection, "realized host competence" for
287 *Bb* has historically been measured as the percent of field-derived hosts, or individuals infected
288 with field-derived nymphs, from which xenodiagnostic larvae can successfully acquire
289 infection[30]. Our findings suggest that measures of competence may be influenced by the relative
290 frequency of different host-adapted genotypes in the environment, as local *Bb* communities
291 (measured by *ospC* types) appear to vary over time and space[33,47,49]. Thus, host overall
292 competence would be an integrated measure of host genotype-specific competence.

293 Theory suggests that more generalist parasites should experience fitness disadvantages
294 compared to more host-adapted species[3]. However, our results did not indicate the presence of
295 a fitness tradeoff during the first two weeks of infection: B31-5A4 exhibited similar levels of *Bb*
296 positive xenodiagnostic larvae and their bacterial burdens in both hosts at 14dpf. Yet, we did
297 observe a fitness cost for B31-5A4 infecting robins during later stages of infection, where
298 acquisition by ticks was significantly reduced compared to cN40 at all time points past 14dpf. This
299 shift in the infectivity profile[45], from "persistent" in white-footed mice to "rapidly-cleared" in
300 robins, can influence *Bb* fitness depending on the timing of tick feeding in nature (phenology).
301 "Persistent" genotypes were found to be more frequent in the Northeast US than the "rapidly-
302 cleared" genotypes, presumably due to positive selection for persistent infection form the nymphal
303 abundance peak until they are acquired by the larvae that host-seek nearly two months later[50,51].

304 Future experiments using combinatorial *Bb* infections across different host species could reveal
305 mechanisms that alter the fitness or infectivity profiles and improve our understanding of the
306 evolutionary drivers of host adaptation and the elevated *Bb* strain diversity observed in nature.
307 Our results further support several cellular and immunological mechanisms mediating the
308 ability to "survive" at early stages and "persist" at late stages. These mechanisms include
309 differential cell adhesion, cytokine induction, complement evasion, and/or triggering of antibodies.
310 During early stages of infection, each strains' ability to adhere to host fibroblasts positively
311 corresponded to its overall fitness in that host, consistent with a role for *Bb* attachment to cells in
312 permitting infection establishment[52]. Additionally, the tested cytokine markers (IFN, TNF) were
313 selected as the documented strain-specific induction of those markers, representing overall
314 immune responses[40]. Although these cytokines impact LD severity in humans, we did not notice
315 any apparent similar symptoms in those reservoir animals (data not shown)[40]. Instead, we found
316 that the extent of cytokine levels *in vitro* and *in vivo* corresponded with *Bb* fitness in reservoir
317 hosts. These findings suggest cytokine marker-mediated signaling promotes *Bb* clearance, and
318 host-adapted *Bb* strains avoid triggering this signaling in their adapted-reservoir host. Although it
319 is not clear if this is a result of active immune suppression or lack of stimulation, a lack of early
320 immune responses in adapted-reservoir hosts would presumably reduce selective pressures and
321 fitness costs, increasing host permissiveness to *Bb* infection establishment, and paving the road for
322 longer-term persistence[53]. Moreover, the timing and intensity of antibody response also
323 appeared to mediate variation in strain fitness across hosts, as both 297 and cN40 triggered
324 antibody production earlier in their non-adapted hosts. Given the shift in the infection profile
325 exhibited by B31-5A4 from persistent in mice to rapidly-cleared in robins, the increased antibody
326 response at 7dpf by infected robins to *Bb* lipoproteins relative to mice is also striking. This

suggests the capacity to delay hosts' adaptive immune defenses may be a key trait in determining infection persistence[53]. Finally, an important difference we observed among hosts is the role for complement-mediated clearance during early infection, which has been identified as a crucial bottleneck of transmission for Lyme borreliae[54].While robin complement effectively reduced 297 survival, mouse complement did not have a similar effect on cN40 survival, consistent with the idea that some mechanisms limiting strain fitness are host-strain specific[54].

We acknowledge limitations of our findings, including the *in vitro* cultivated fibroblasts don't necessarily reflect *in vivo* results and the chosen *Bb* strains and reservoir hosts do not always recapitulate all the bacterial strains and hosts in nature. Thus, future studies should aim to increase both experimental hosts and strain diversity to better characterize these eco-evolutionary dynamics. Additionally, host-independent factors proposed to confer *Bb* adaptation to different hosts (e.g., host-tick species association), should also be considered[55]. Despite these limitations, our findings provide novel insight into the mechanisms structuring *Bb* diversity and provide a model for understanding host adaptation in a complex microparasite-host system. The patterns and mechanisms discussed here can guide future efforts to characterize host-pathogen interactions, reservoir competence dynamics, and the potential epidemiological impacts of intraspecific phenotypic variation.

MATERIALS AND METHODS

Intradermal and tick infection. The source of American robins and white-footed mice and the details of the animal infections are described in Supplementary information. For tick infections, unfed infected nymphs were placed in the ear canals of mice (5 nymphs per ear, 10 totals per

mouse) or allowed to feed freely on robins contained in a PVC pipe (10 nymphs per bird). Xenodiagnostic larval placement is detailed in Supplementary information (SI).

Quantitative (qPCR) and reverse transcription PCR (RT-qPCR). DNA was extracted from ticks and tissues of white-footed mice and robins. *Bb* genomic equivalents were calculated using a Real-Time PCR system, based on amplification of the *Bb 16S rRNA* gene using primers shown in Table S5, detailed in the SI.

Adhesion assays. The primary fibroblasts from robin neck skin and white-footed mouse ears were cultivated on cover slips in 24-well plates and then incubated with *Bb* strains in BSK-II medium (2×10^5 cells and 2×10^6 spirochetes per well), detailed in the SI.

Serum resistance, ELISA, and borreliacidal assays. For serum resistance assays, the sera were obtained from uninfected white-footed mice and robins. For ELISA and bactericidal assays, sera were collected from nymph infected white-footed mice and robins carrying *Bb* strains at 7, 14, 21, and/or 28dpf, as described in the SI.

Statistical analyses. Kruskal-Wallis tests followed by a two-stage step-up method with a Benjamini, Krieger, and Yekutieli correction was used for all comparisons[56], detailed in the SI.

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FIGURE LEGENDS

Figure 1. *Bb* displayed strain-specific, robin- or white-footed (WF) mouse-dependent spirochete transmission to larvae. Nymphs carrying *Bb* strains B31-5A4, 297, cN40, or naïve nymphs (Uninfect.) fed to repletion on robins or WF mice. (**A-D, F-I**) Data represent the geometric mean ± geometric s.d. of *Bb* burdens in xenodiagnostic larvae from indicated animals. Significance ($p \leq 0.05$) from the uninfected group (*) or between groups (#) is indicated. (**E&J**) Data represent the geometric mean ± SEM of *Bb* percent positivity in the xenodiagnostic larvae derived from each animal.

Figure 2. *Bb* strains varied in their ability for early colonization, bacteremia induction and persistence at tissues in robins and white-footed (WF) mice. (A-B) Robins (four/group) and WF mice (five/ group) were intradermally inoculated with indicated *Bb* strains or BSK-II medium (Uninfect.). The inoculation site of skin (Inoc. site) was collected at 1-day post injection (dpi) to determine bacterial burdens by qPCR. **(C-R)** The bacterial loads in blood and tissues at indicated time points post nymph feeding (dpf) determined by qPCR. Data represent geometric mean ± geometric s.d. Significance ($p \leq 0.05$) from the uninfected group (*) or between groups (#) is indicated.

Figure 3. *Bb* strains differed in the levels of cell adhesion in a robin and/or white-footed (WF) mouse-specific manner. Spirochete strains (2×10^6 spirochetes) were incubated with fibroblasts (2×10^5 cells) from robins and WF mice, stained by anti-*Bb* antibodies and DAPI, and counted microscopically from four determinations at one hour post incubation (hpi). Data represents mean

± s.d. Statistical significance ($p \leq 0.05$) from B314-treated group (*) or between groups is indicated (#).

Figure 4. *Bb* strains varied in their ability to survive in robin but not white-footed (WF) mouse sera in a complement-dependent fashion. Percent survival of indicated *Bb* strains is shown in uninfected robin sera in the **(A)** absence or **(B)** presence of OmCI, or uninfected WF mouse sera in the **(C)** absence or **(D)** presence of CVF. Heat treated sera were also included. Data represent mean ± s.d. of percent survival from three occasions. Significance ($p \leq 0.05$) between groups is indicated (#).

Figure 5. *Bb* strains triggered different levels of cytokine *ex vivo* and at early robin and white-footed (WF) mouse infection. (A-F) Fibroblasts (2×10^5 cells) from robins and WF mice were incubated with *Bb* strains (2×10^7 spirochetes) or cell medium (Mock). The expression levels of indicated genes were determined at 24-h post incubation (hpi) by qRT-PCR and normalized to expression of β-actin genes. **(G-J)** Sera were obtained 7 days post nymph feeding (dpf) to determine levels of cytokines using ELISA. Data represent the geometric mean ± geometric s.d. Significance ($p \leq 0.05$) from the uninfected group (*) or between groups (#) is indicated.

Figure 6. *Bb* strains differed in their ability to induce anti-spirochete antibodies in robin and white-footed (WF) mouse infection. (A-J) Sera were obtained at indicated days post nymph feeding (dpf). The levels of IgG against an equal ratio of B31-5A4, 297, and cN40 lysates were determined by ELISA. Data represents the geometric mean ± geometric s.d. Significance ($p \leq 0.05$) from the uninfected group (*) or between groups (#) are indicated. **(K-L)** Sera from infected robins

and WF mice at 7dpf, or naïve nymphs (Uninfect.). Data represent the mean ± SEM of the survival percentage. 50% BA, the dilution rate that killed 50% of spirochetes, was obtained from curve-fitting. N.F., not fittable.

Fig. 1

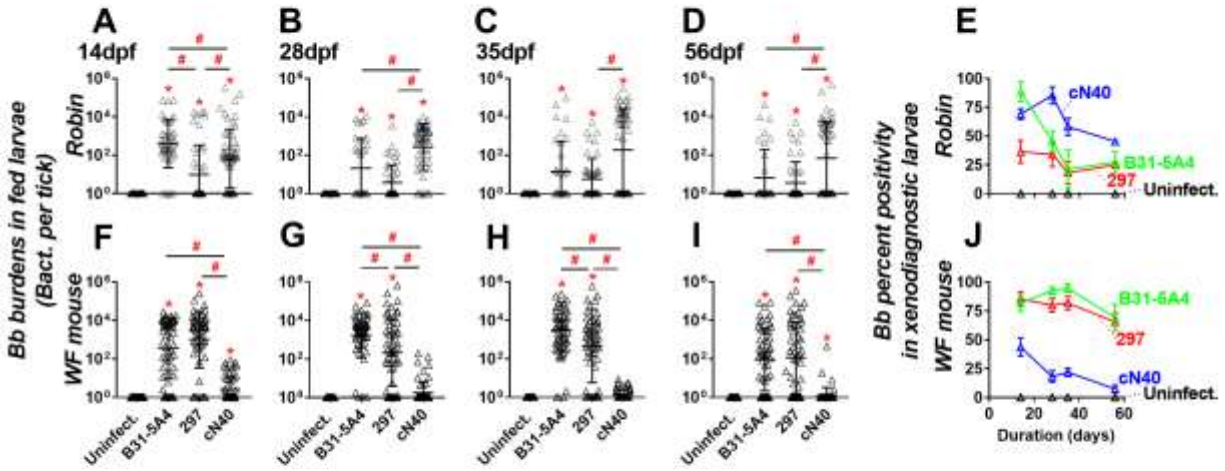

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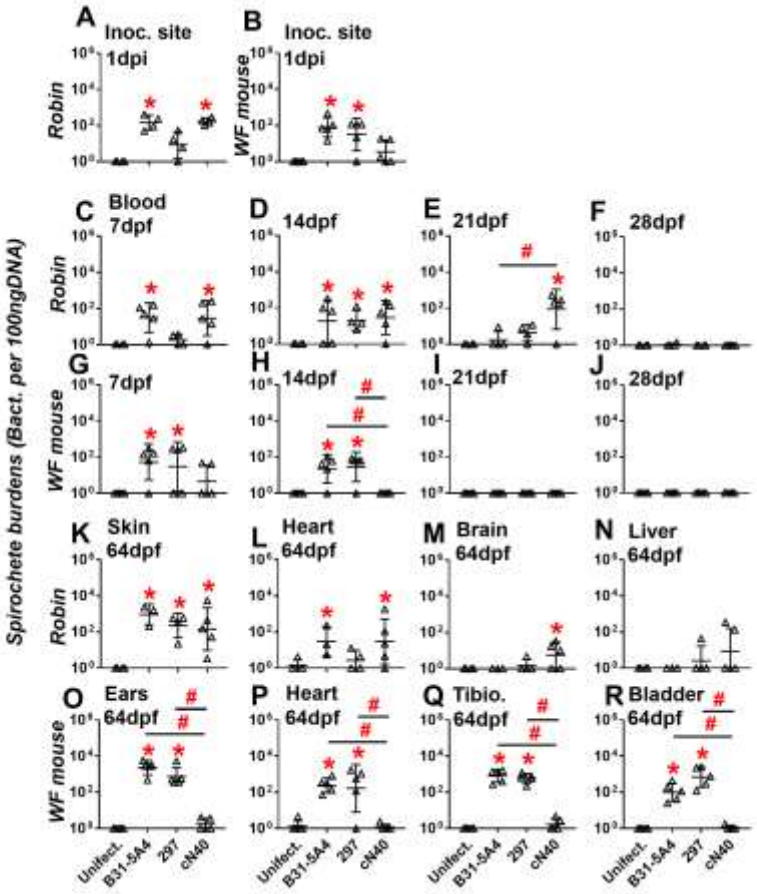
Fig. 2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B Inoc. site 1dpi
C Blood 7dpf
D 14dpf
E 21dpf
F 28dpf
G 7dpf
H 14dpf
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J 28dpf
K Skin 64dpf
L Heart 64dpf
M Brain 64dpf
N Liver 64dpf
O Ears 64dpf
P Heart 64dpf
Q Tibio. 64dpf
R Bladder 64dpf
Robin
WF mouse
Spirochete burdens (Bact. per 100ngDNA)
Unifect.
B31-5A4
297
cN40

Fig. 3

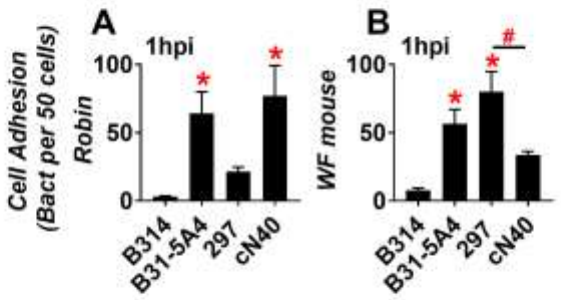

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Fig. 4

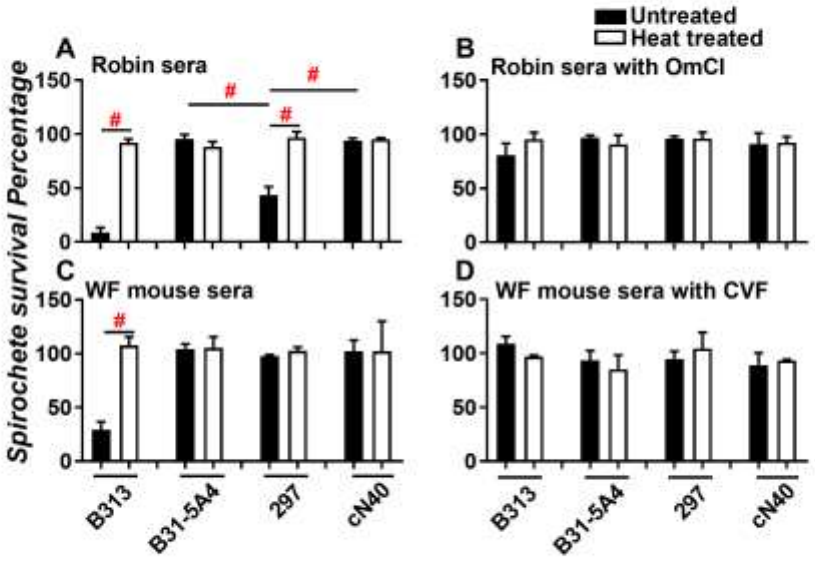

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Fig. 5

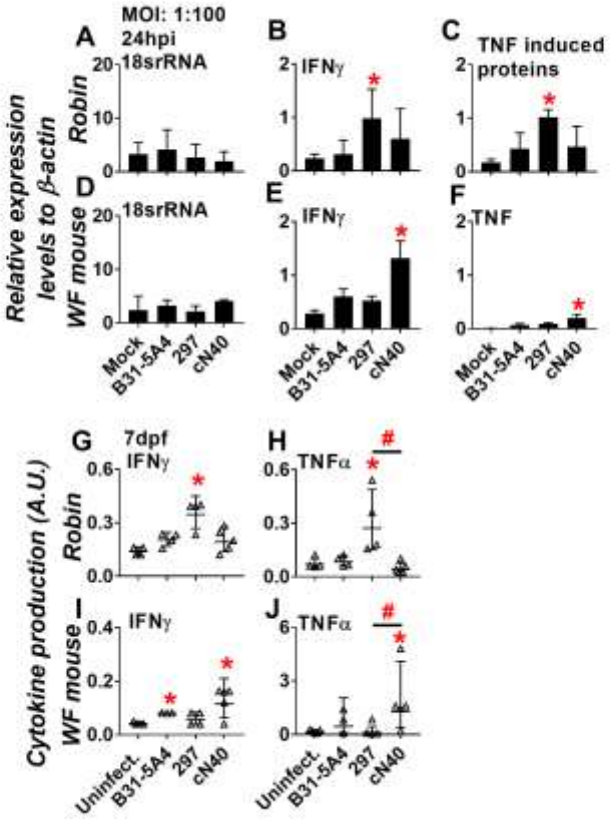

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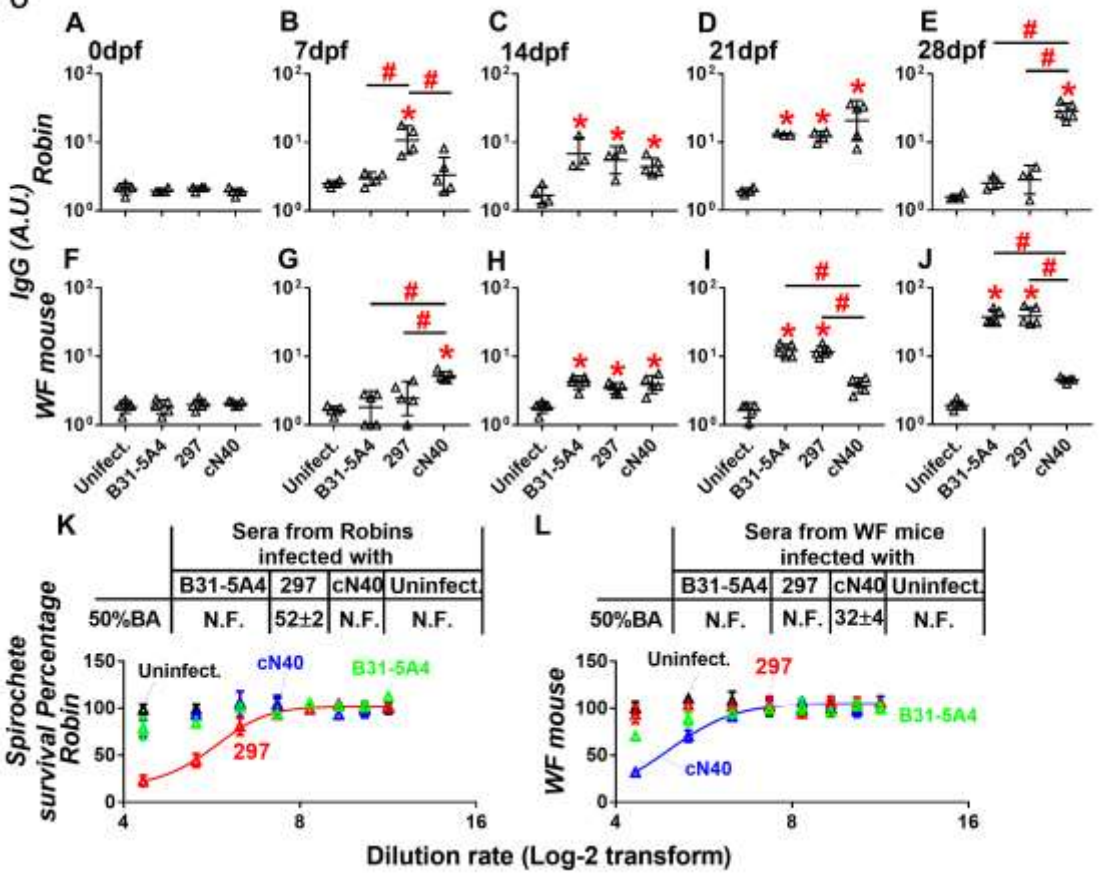
Fig. 6


A
0dpf
B
7dpf
C
14dpf
D
21dpf
E
28dpf
IgG (A.U.)
Robin
WF mouse
F
G
H
I
J
Unifect.
B31-5A4
297
cN40
K
Sera from Robins infected with
B31-5A4
297
cN40
Uninfect.
50%BA
N.F.
52±2
N.F.
N.F.
Spirochete survival Percentage
Robin
L
Sera from WF mice infected with
B31-5A4
297
cN40
Uninfect.
50%BA
N.F.
N.F.
32±4
N.F.
WF mouse
Dilution rate (Log-2 transform)

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