The infectivity gene bbk13 is important for multiple phases of the *Borrelia burgdorferi* enzootic cycle.

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Running title: *bbk13* is critical for the *B. burgdorferi* enzootic cycle

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**Abstract**

Lyme disease is a multi-stage inflammatory disease caused by the spirochete *Borrelia burgdorferi* transmitted through the bite of an infected *Ixodes scapularis* tick. We previously discovered a *B. burgdorferi* infectivity gene, *bbk13*, that facilitates mammalian infection by promoting spirochete population expansion in the skin inoculation site. Initial characterization of *bbk13* was carried out using an intradermal needle inoculation model of mouse infection, which does not capture the complex interplay of the pathogen-vector-host triad of natural transmission. Herein, we aimed to understand the role of *bbk13* in the enzootic cycle of *B. burgdorferi*. *B. burgdorferi* lacking *bbk13* were unable to be acquired by naive larvae fed on needle inoculated mice. Using a capsule-feeding approach to restrict tick feeding activity to a defined skin site, we determined that delivery by tick bite alleviated the population expansion defect in the skin observed after needle inoculation of Δ*bbk13* *B. burgdorferi*. Despite overcoming the early barrier in the skin, Δ*bbk13* *B. burgdorferi* remained attenuated for distal tissue colonization after tick transmission. Disseminated infection of Δ*bbk13* *B. burgdorferi* was improved in needle inoculated immunocompromised mice. Together, we established that *bbk13* is crucial to the maintenance of *B. burgdorferi* in the enzootic cycle and that *bbk13* is necessary beyond early infection in the skin, likely contributing to host immune evasion. Moreover, our data highlight the critical interplay between the pathogen, vector, and host as well as the distinct molecular genetic requirements for *B. burgdorferi* to survive at the pathogen-vector-host interface and to achieve productive disseminated infection.
Introduction

Lyme disease remains the predominant vector-borne disease in the United States (1). *Borrelia burgdorferi*, the causative agent of Lyme disease (2, 3), is transmitted to humans via the bite of an infected *Ixodes scapularis* tick (4). The acute manifestations of Lyme disease involve fever, nausea, and in some cases, a characteristic bull’s-eye rash (erythema migrans) at the tick bite site. If undiagnosed and untreated, Lyme disease progresses to a late stage as *B. burgdorferi* disseminates and colonizes various distal tissues such as the joints (Lyme arthritis), the heart (Lyme carditis), and the nervous system (neuroborreliosis) (5).

In nature, the *B. burgdorferi* reservoir is maintained by the enzootic cycle of transmission and acquisition of *B. burgdorferi* from various vertebrate hosts as the *Ixodes scapularis* tick feeds and progresses through its life cycle (6). After hatching, an unfed *Ixodes* larva will feed on blood from small vertebrate hosts such as rodents, triggering it to molt to the nymph stage. If the host is infected, the larva can acquire *B. burgdorferi* with the blood meal. *B. burgdorferi* will then proliferate in the fed larva and persist through the molting phase, which then gives rise to infected unfed nymphs. It is primarily the nymph stage of *Ixodes* that transmits *B. burgdorferi* to humans. The risk of contracting Lyme disease rises in areas with high numbers of *B. burgdorferi* infected nymphal ticks (7). Nymphs then molt into sexually mature adults, which mate, and lay eggs to give rise to the next generation of uninfected *Ixodes* ticks.

The molecular mechanisms that promote *B. burgdorferi* infection of its mammalian host are not completely understood and this has slowed the development of new strategies to curb the rising prevalence of Lyme disease. Genetic manipulation of
B. burgdorferi coupled with a mouse model of infection has been central to the discovery of novel B. burgdorferi infectivity genes. Needle inoculation of mouse models of infection allow for precise control of both inoculum dose and route of inoculation, enabling detailed characterization of infection kinetics. However, needle inoculation is an artificial route of infection that excludes the contribution of the tick vector to B. burgdorferi transmission—both its influence on B. burgdorferi itself and on the host microenvironment at the site of feeding. B. burgdorferi adapts to the conditions present in the unfed tick and undergoes significant transcriptional changes during tick feeding, potentiating its infectivity (8, 9). Moreover, the feeding activity of the ticks themselves, particularly the influence of the tick saliva on the skin feeding site affects B. burgdorferi infection (10-12). The tick feeding site is unique in that it is the combined interaction of the pathogen, the vector, and the mammalian host, which is likely critical to the adaptation and mechanisms of pathogenesis of B. burgdorferi, as well as other tick-borne pathogens.

We recently characterized the role of the novel B. burgdorferi infectivity gene bbk13 in the early phase of B. burgdorferi mammalian infection. Using an intradermal needle inoculation model of infection, we showed that bbk13 is important for spirochete population expansion in the skin, which then drives the downstream steps of disseminated infection, culminating in B. burgdorferi colonization of distal tissues (13). Consequently, loss of bbk13 leads to reduced colonization of distal tissue sites such as the skin, heart, and joints (13). Furthermore, we have established that BBK13 forms large oligomers in the spirochete membrane and is highly immunogenic (13, 14). In this work, we used Ixodes ticks and mice to study the role of bbk13 in the natural enzootic
cycle of *B. burgdorferi*. We demonstrate that *bbk13* is similarly required for productive disseminated infection when delivered by tick bite transmission. Interestingly, *B. burgdorferi* inoculation by tick feeding bypassed the early requirement for *bbk13* to promote *B. burgdorferi* population expansion in the skin. Despite this, *bbk13* remained critical for efficient colonization of distal tissues. In addition, we show that distal tissue colonization of Δ*bbk13* *B. burgdorferi* is restored in an immunocompromised mouse host. This work points toward a role of *bbk13* in host immune evasion and uncovers a more complex model of *bbk13* function in *B. burgdorferi* mammalian infection, emphasizing the impact of the natural tick vector on establishment of *B. burgdorferi* infection as well as the distinct molecular genetic requirements for *B. burgdorferi* survival during early localized infection at the pathogen-vector-host interface and establishment of productive disseminated infection of distal host tissues.
Results

Naive larvae fail to acquire Δbbk13 B. burgdorferi from infected mice.

B. burgdorferi’s (Bb) enzootic cycle depends not only on successful tick transmission but also on its acquisition from the animal host by naïve larvae. Needle inoculation of Δbbk13 Bb into mice leads to reduced, but not absent, colonization of distal tissues including the skin (13). This allowed us to ask whether Δbbk13 Bb can be acquired from infected mice by feeding Ixodes larvae. We needle-inoculated cohorts of C3H/HeN mice with $10^4$ wild type, Δbbk13, or Δbbk13/Δbbk13+ Bb intradermally to generate infected mice for naïve tick feeding. Consistent with what we have shown previously (13), Δbbk13 Bb were found at reduced levels during blood dissemination (Fig. 1A) and in distal tissues (Fig. 1B). More importantly, Δbbk13 Bb was still present in the skin, as indicated by reisolation from the ear (Fig. 1B, top row).

At three weeks post-inoculation, naïve Ixodes larvae were fed to repletion on the three groups of Bb-infected mice. Individual fed larvae were assessed for Bb acquisition via homogenization and plating in solid BSK-agarose medium to quantify the number of Bb per tick. The acquisition rate of wild type Bb after larval tick feeding was 96% (48/50 larvae) with an average of ~5x$10^3$ Bb per tick (Fig. 1C). In stark contrast, only 4% of the Ixodes larvae (4/100 larvae) that fed on Δbbk13 Bb-infected mice acquired spirochetes and with Bb loads at dramatically reduced levels (Fig. 1C). The acquisition rate of Δbbk13/Δbbk13+ Bb from infected mice was 86% (43/50 larvae) with the average number of Bb per tick comparable to that of the wild type Bb group (Fig. 1C). These data indicated that there is a significant deficiency in the ability of Ixodes larvae to acquire spirochetes from Δbbk13-Bb infected mice.
Increasing the inoculum dose does not rescue low Δbbk13 B. burgdorferi numbers in distal tissues.

For *Ixodes* ticks to acquire *Bb* through feeding, the spirochetes must be present in sufficient numbers at the skin feeding site. Although both wild type and Δbbk13 Bb were present at a skin site (Fig. 1B, ear), Δbbk13 Bb were found at reduced numbers compared to wild type *Bb* during late infection (13). Reduced Δbbk13 Bb numbers in the skin likely negatively influences acquisition via tick feeding, raising the question of whether low Δbbk13 Bb acquisition was due to reduced spirochete numbers and/or a molecular function of BBK13. To address this, we attempted to increase Δbbk13 Bb load in the skin by increasing the inoculum dose. We needle-inoculated 10^5, 10^6, or 10^7 Δbbk13 Bb intradermally into C3H/HeN mice. Three weeks post-inoculation, distal tissues were collected and assessed for *Bb* colonization via semi-quantitative tissue reisolation assay (13). After 5 days of incubation, nearly all of the reisolation cultures from mice inoculated with 10^4 wild type *Bb* demonstrated a score of ‘3’ (23/24 cultures) (Fig. 2A). At the same timepoint, reisolation cultures from mice inoculated with increasing doses of Δbbk13 Bb were mostly negative for spirochetes (10^5 dose: 9/24 positive; 10^6 dose: 6/24 positive; 10^7 dose: 8/24 positive), and the positive cultures demonstrated varying spirochete densities (scores ranging from ‘1’-‘3’) (Fig. 2A). Thus, no observable increase in distal tissue colonization was detected upon increasing the inoculum dose of Δbbk13 Bb. These trends were maintained at the 2-week endpoint of the reisolation assay (Fig. 2A). Direct measurement of *Bb* loads in the distal tissues by quantitative PCR supported the findings in the tissue reisolation assay. Despite the increased inoculum doses, the mean Δbbk13 Bb loads in the ears and joints remained
consistently lower than that of wild type *Bb* at a lower inoculum (Fig. 2B, C). Moreover, 

Δ*bbk13* *Bb* loads were only above the level of detection in 2 or 3 tissues out of 6 mice 
per inoculum group, further emphasizing the reduced efficiency of distal tissue 
colonization of Δ*bbk13* *Bb*.

**bbk13 is dispensable for *B. burgdorferi* maintenance in *Ixodes* ticks.**

To further understand the role of *bbk13* in the enzootic cycle of *Bb*, we 
investigated the survival of Δ*bbk13* *Bb* in ticks after the blood meal and through the 
molt. Given the inefficiency of Δ*bbk13* *Bb* to be acquired by feeding ticks, we artificially 
infected unfed *Ixodes* larvae with wild type, Δ*bbk13*, or Δ*bbk13/bbk13+* *Bb* by 
immersion (15). After recovery from immersion, positive infection was confirmed and 
quantified by plating homogenate from groups of 10 larvae per infecting *Bb* clone. No 
significant difference was detected between the number of *Bb* per 10 larvae across the 
three *Bb* clones (Fig. 3A). The infected larvae were fed to repletion on naïve C3H/HeN 
mice. Approximately seven days post-feeding, 20 fed larvae per infecting *Bb* clone were 
individually homogenized and plated in solid BSK-agarose medium to measure 
spirochete loads. Robust multi-log expansion was detected for all *Bb* clones (Fig. 3B, 
left), with no statistical difference between the average number of Δ*bbk13* *Bb* or 
Δ*bbk13/bbk13+* *Bb* from that of wild type *Bb*. (Fig. 3B, left). A portion of the fed larvae 
were allowed to molt to unfed nymphs, from which 20 nymphs per *Bb* clone were then 
individually assayed for *Bb* load by homogenization and plating for colony forming units. 
*Bb* loads were comparable across all three groups and displayed the expected decline 
in number following the molt (Fig. 3B, middle) (16). The nymphs were then allowed to 
feed to repletion on mice and the number of *Bb* per individual fed nymph was
determined. No bbk13-dependent decrease in Bb load per fed nymph was detected, rather, the number of Δbbk13 Bb per fed nymph was found to be significantly increased compared to that of wild type Bb (Figure 3B, right). Although not statistically significant, the average number of Δbbk13/bbk13+ Bb in all tick stages except for fed nymphs tended to be lower than that of wild type and Δbbk13 Bb (Fig. 3), perhaps due to dysregulated expression of bbk13 from the shuttle vector (13). In all, loss of bbk13 did not result in a defect in the replication and survival of Bb in ticks.

**bbk13 is critical for *B. burgdorferi* infection of mice by *Ixodes* nymph**

Our previous study used needle inoculation to demonstrate the importance of bbk13 during mouse infection (13). In nature, Bb is transmitted via the feeding activity of *Ixodes* ticks. Moreover, tick feeding influences both Bb and the host to promote infection (6, 17). Unfed nymphs carrying equivalent loads of wild type, Δbbk13, or Δbbk13/bbk13+ Bb (Fig. 3B, middle) were used to test the requirement for bbk13 during natural Bb infection of mice by tick feeding. Three weeks after free feeding of infected nymphs on mice, distal tissues were collected and tested for Bb colonization via semi-quantitative tissue reisolation assay (see Materials and Methods). Briefly, reisolation cultures were inspected daily for the presence of spirochetes by darkfield microscopy. A numerical score was assigned to each culture corresponding to the observed density of spirochetes (‘0’ for no spirochetes, ‘1’-‘3’ depending on the number of spirochetes).

After 5 days of incubation, a majority (17/20) of the cultures containing tissues from mice fed on by wild type *Bb*-infected nymphs had a reisolation score of ‘3’ (Fig. 4A, 5-day culture). In contrast, nearly all (19/20) reisolation cultures from mice fed on by...
Δbbk13 Bb-infected nymphs had no spirochetes upon microscopic inspection and were scored '0' (Fig. 4A, 5-day culture). Moreover, at the 2-week endpoint, only four out of twenty of the Δbbk13 Bb reisolation cultures were positive for spirochetes, with three of the five mice having no detectable spirochetes in any distal tissue examined (Fig. 4A, endpoint). In contrast, all reisolation cultures from the wild type and Δbbk13/bbk13+ Bb infected mouse tissues were positive for spirochetes at the 2-week endpoint (Fig. 4A, endpoint). Consistent with the observed deficit in tissue reisolation, Δbbk13 Bb was below the level of detection by qPCR in the hearts (0/5 mice) and joints (0/5 mice) (Fig. 4B) of mice after tick feeding. Spirochete numbers were measurable in two out of five ear tissues from Δbbk13 Bb infected mice but were reduced compared to that of wild type and Δbbk13/bbk13+ Bb infected mice (Fig. 4B). Moreover, in agreement with reduced disseminating spirochetes and reduced overall loads in distal tissues, mice fed on by Δbbk13 Bb infected ticks exhibited a serological response with a reduced signal intensity, yet similar banding pattern to that wild type Bb or Δbbk13/bbk13+ Bb infected mice (Fig. 4C), as demonstrated previously for mice infected with spirochetes lacking bbk13 (13). The attenuated infection phenotype of Δbbk13 Bb by nymph transmission was recapitulated by free feeding artificially infected larvae (Fig. 4D), together supporting the importance of bbk13 for Bb infection by tick bite transmission.

B. burgdorferi population expansion occurs at the tick feeding site.

We previously showed that bbk13 is important early in Bb mammalian infection to promote spirochete expansion in the skin within days after intradermal needle inoculation (13). The deficiency of Δbbk13 Bb in distal tissue colonization was observed by both needle inoculation (Fig. 1A, B) (13) and natural tick transmission (Fig. 4). We
were interested in determining whether wild type *Bb* undergoes a similar population expansion in the skin after tick bite transmission and if *bbk13* contributes to this process. To achieve this, we confined wild type *Bb*-infected nymphs to capsules affixed to the dorsal skin of mice and determined the kinetics of the *Bb* population in the skin feeding site. The feeding site was harvested daily from cohorts of mice starting at day 3 out to day 12 post-nymph application. Spirochete load in the skin was assessed using the semi-quantitative reisolation assay as well as quantitative PCR. Feeding sites collected at days 3 to 6 post-nymph application showed inconsistent spirochete reisolation, with one or two of the three mice in the cohort showing absence of spirochetes and peak scores achieved only at the end of the scoring period in some cultures (Fig. 5A). However, from days 7-10 after nymph application, spirochetes were detected in all cultures as soon as day 1 of incubation and reached a peak score of ‘3’ as early as day 2 of incubation (Fig. 5A). Reisolation from skin at days 11-12 post-nymph application resembled the early timepoints, with spirochetes detected later in the scoring period (Fig. 5A). Quantitation of *Bb* loads from the skin feeding site by quantitative PCR corroborated the findings from the reisolation assay. There were little or no measurable *Bb* in the skin feeding site from days 3-7 post-nymph application, followed by a dramatic increase in *Bb* load during days 8-10 post-nymph application (Fig. 5B). A precipitous drop in *Bb* loads was detected at days 11-12 (Fig. 5B). Blood was also collected from mice throughout the duration of the nymph capsule feeding experiment and assessed for the presence of disseminating spirochetes in circulation. Plating of whole blood in solid BSK-agarose medium and colony-forming unit enumeration revealed peak numbers of *Bb* in the blood on days 8-10 post-nymph
application (Fig. 5C). Taken together, these results show that *Bb* undergoes population expansion in the skin after tick transmission and the peak of this expansion occurs at days 8-10 post-nymph application.

By tick bite transmission, *bbk13* is dispensable for *B. burgdorferi* early population expansion but critical for disseminated infection.

Having established the kinetics of wild type *Bb* population expansion in the skin following tick transmission, we used the nymph capsule feeding model to assess the contribution of *bbk13* to this process. Unfed nymphs infected with comparable numbers of either wild type *Bb* or Δ*bbk13* *Bb* (Fig. 6A) were placed in feeding capsules on the dorsal skin of C3H/HeN mice to feed to repletion. The skin feeding site was collected on days 9 and 10 post-nymph application, which corresponded to the peak of spirochete expansion of tick-transmitted wild type *Bb* (Fig. 5B). Reisolation assay of the skin site fed on by wild type *Bb*-infected nymphs collected on days 9 and 10 both demonstrated detection of spirochetes within 1 day of culture incubation (Fig. 6B). Cultures containing the skin site fed on by Δ*bbk13* *Bb*-infected nymphs showed little reisolation delay compared to wild type *Bb*, with spirochetes detected on day 2 of incubation (Fig. 6B). Surprisingly, no difference in *Bb* loads measured by quantitative PCR was detected between wild type- and Δ*bbk13* *Bb*-infected skin sites collected on either day (Fig. 6C).

These results contrasted with previous findings that, when delivered by intradermal needle inoculation, Δ*bbk13* *Bb* failed to undergo efficient population expansion in the skin inoculation site compared to wild type *Bb*, thus negatively impacting distal tissue colonization at later time points of infection (13). Yet, our data indicated that Δ*bbk13* *Bb* is highly attenuated for disseminated mouse infection by free feeding tick transmission.
(Fig. 4). Therefore, we examined the disseminated infection outcomes of $\Delta bbk13 Bb$ delivered by nymph capsule feeding. At 3 weeks after nymph application in feeding capsules, the feeding site and multiple distal tissues were dissected and assessed for spirochete colonization. Interestingly, despite the lack of $Bb$ load difference between wild type and $\Delta bbk13 Bb$ at the feeding site early in infection, $\Delta bbk13 Bb$ demonstrated reduced numbers in all tissues examined at this later time point. Nearly all reisolation cultures containing tissues from mice fed on by wild type $Bb$-infected nymphs were positive for spirochetes, while only five out of twenty cultures were positive for spirochetes from mice fed on by $\Delta bbk13 Bb$-infected nymphs (Fig. 6D). Further, quantitative PCR analysis of spirochete loads consistently showed reduced or undetectable $\Delta bbk13 Bb$ in tissues (Fig. 6E), indicating the critical requirement for $bbk13$ for productive disseminated infection.

$\Delta bbk13 B. burgdorferi$ infectivity is restored in immunocompromised mice

The above results show that delivery by tick bite locally rescued the defect of $\Delta bbk13 B$. $B. burgdorferi$ in spirochete expansion in the skin during early infection. It is known that the feeding activity of $Ixodes$ ticks leads to immunomodulation at the bite site, promoting $B. burgdorferi$ survival (9, 17), therefore, prompting the question as to whether $bbk13$ is involved in immune evasion. To test this, we determined whether $\Delta bbk13 Bb$ mammalian infectivity was restored if the host immune response was reduced overall. We delivered $10^4$ wild type, $\Delta bbk13$, or $\Delta bbk13/\Delta bbk13$+ $Bb$ by intradermal needle inoculation into highly immunocompromised Nod- scid IL2R$\gamma^{null}$ (NSG) mice and the congenic control strain, Nod/ShiLtJ. At day 6 post-inoculation, blood was collected from mice and plated in solid BSK-agarose medium to enumerate circulating $Bb$. No $Bb$...
clone-specific differences were observed between the number of circulating *Bb* in immunocompromised NSG mice and in the control mouse strain (Fig. 7A). At three weeks post-inoculation, infection of the inoculation site and distal tissues was assessed by semi-quantitative reisolation assay. As expected, ∆*bbk13 Bb* were highly attenuated for infection of the control mice with only six out of thirty cultures scoring ‘1’ or ‘2’ following five days of incubation. In contrast, twenty-nine out of thirty and twenty-four out of thirty reisolation cultures from wild type *Bb* and ∆*bbk13/bbk13+ Bb* infected NOD mice, respectively, received a score of ‘1’–‘3’ at the same time point (Fig. 7B, top). Strikingly, in immunocompromised NSG mice, ∆*bbk13 Bb* demonstrated robust infection. Twenty-one out of thirty reisolation cultures from ∆*bbk13 Bb* inoculated NSG mice received a score of ‘3’ following five days of incubation, congruent with that of wild type *Bb* (25/30 cultures) and ∆*bbk13/bbk13+ Bb* (22/30 cultures) (Fig. 7B, bottom). These data indicated that, without host immune pressure, the infection defect of ∆*bbk13 Bb* was overcome resulting in productive disseminated infection of distal tissues and suggestive of a role for BBK13 in immune evasion.
Discussion

The maintenance of *B. burgdorferi* in the natural reservoir depends on its ability to colonize a vertebrate host, to position itself to be acquired during tick feeding, and to subsequently be transmitted to a new vertebrate host. Though *B. burgdorferi* colonizes several mammalian tissues that contribute to the pathogenesis of Lyme disease in humans, colonization of the skin of reservoir hosts is most pertinent to the upkeep of the enzootic cycle. Intravital imaging in skin of infected mice revealed that *B. burgdorferi* undergoes directed migration towards a feeding tick (18). Furthermore, *B. burgdorferi* has shown positive chemotactic responses to components of the tick salivary gland and that these factors are important during spirochete acquisition by feeding ticks (19, 20). This evidence supports the ability of *B. burgdorferi* to sense the presence of the feeding tick and to position itself to best be taken up by a feeding tick. However, these cues are likely restricted to the immediate vicinity of an active tick feeding site. Therefore, efficient *B. burgdorferi* acquisition likely depends on achieving the appropriate density of *B. burgdorferi* in the skin such that enough *B. burgdorferi* will encounter the cues from a feeding tick. *B. burgdorferi* must then be present in the feeding site at a sufficient number for successful tick acquisition and continuation of the enzootic cycle.

We and others have previously described the population expansion of *B. burgdorferi* at the site of needle inoculation (13, 21-24). We further showed that this population expansion is promoted by gene *bbk13* and the inability to expand in numbers at the skin inoculation site leads to attenuated dissemination and distal tissue colonization (13). We now show that naïve larvae fail to acquire Δ*bbk13* *B. burgdorferi* from needle inoculated mice. Our data suggest that the number of Δ*bbk13* *B. burgdorferi*
*B. burgdorferi* colonizing the skin is likely below the threshold required for successful tick acquisition, but do not rule out the possibility of a specific function of *bbk13* as a driver of the acquisition defect. Matching the wild type and Δ*bbk13* *B. burgdorferi* numbers in the skin would allow for any *bbk13*-specific requirement to be tested during tick acquisition. However, increasing the needle inoculum dose of the mutant did not result in increased spirochete loads in the skin.

Using the semi-quantitative tissue reisolation assay as the measure of tissue burden and highly immunocompromised NSG mice, we discovered that when host immune pressures are relieved, Δ*bbk13* *B. burgdorferi* is able to colonize distal tissues similar to that of wild type *B. burgdorferi*. Thus, it appears that *bbk13* is important for circumventing the host immune response, either directly or indirectly, to promote *B. burgdorferi* survival in the mammalian host. The restoration of Δ*bbk13* *B. burgdorferi* loads in the tissues of NSG mice to apparent wild type levels suggests that this model could be used to test for a *bbk13*-specific requirement for tick acquisition. However, there are a number of limitations to this model in its ability to address this question. The semi-quantitative nature of the tissue reisolation assay does not provide sufficient resolution at the score of a ‘3’ (>15 spirochetes in the field of view) to precisely distinguish quantitative differences in tissues loads between the wild type and Δ*bbk13* *B. burgdorferi* infections. Furthermore, assessment of spirochete loads in distal tissues of NSG mice using quantitative PCR enumeration of *B. burgdorferi* genome copies was unreliable. Tissues that had no reisolation of live spirochetes recorded high spirochete loads by qPCR (data not shown). This observation indicates that *B. burgdorferi* DNA is retained in the tissues despite the absence of viable spirochetes. The NSG mice and
the congenic control strain used in our studies are of the Nod/ShiLtJ background,
commonly called NOD (Non-obese diabetic). NOD mice have been shown to have poor
clearance of apoptotic cells and the generation of autoantibodies against DNA (25),
which indicates poor clearance of DNA. Moreover, hyperglycemic mice are suspected to
suffer from deficiencies in clearing *B. burgdorferi* debris and DNA from tissues (26). This
is an important consideration for mouse infection studies since quantitative PCR
measurement of *B. burgdorferi* genome copies in distal tissues, the current gold
standard for assessing *B. burgdorferi* tissue loads, assumes a correlation between the
amounts of *B. burgdorferi* genomic DNA and viable spirochetes. The caveats of precise
quantitation of *B. burgdorferi* in the tissues of NSG mice have the potential to confound
the interpretation of tick acquisition, or lack thereof, of Δbbk13 *B. burgdorferi*. Therefore,
NSG mice were deemed not to be the appropriate model to test this question. In
addition, while removal of immune pressures clearly allowed Δbbk13 *B. burgdorferi* to
achieve disseminated infection, given the technical limitations of these mice, we cannot
rule out the potential that BBK13 also contributes to a broader mechanism of
dissemination not overcome by the immunodeficiency of the NSG background.
Nonetheless, the broadly immunocompromised background of NSG mice is a useful
tool to eliminate a large swath of the host immune response across both innate and
adaptive arms. Future studies will focus on identification of the specific aspect(s) of the
host immune response that BBK13 contributes to providing protection against.

Consistent with previous findings for *B. burgdorferi* lacking the entire linear
plasmid (lp) 36 (27), on which the *bbk13* gene resides (28), no *bbk13*-dependent defect
in *B. burgdorferi* replication or survival was detected across the tick life stages. Tick bite
transmission of *B. burgdorferi* induces significant changes in both the spirochete and the mammalian host to promote infection (8, 9), which are not recapitulated by *in vitro* grown *B. burgdorferi* delivered by needle inoculation. Therefore, we sought to assess the contribution of the *bbk13* gene to mouse infection by tick bite transmission. *B. burgdorferi* lacking *bbk13* were highly attenuated for disseminated infection three weeks following free feeding of infected ticks on mice. Our previous findings indicated a role for *bbk13* in *B. burgdorferi* population expansion in the skin site of infection and the importance of this population expansion for establishment of productive disseminated infection (13). To investigate the contribution of *bbk13* to this model of infection in the context of tick bite transmission, we first established the early kinetics of wild type *B. burgdorferi* in the skin site of tick feeding using capsule-confined nymphs. Similar to the spirochete numbers in the skin delivered by needle inoculation, tick-delivered *B. burgdorferi* underwent population expansion in the feeding site, with peak numbers of spirochetes detected on days 8-10 post-nymph application. Given that *B. burgdorferi* transmission can occur 24-48 hours after the start of tick feeding and that tick feeding is not synchronized, the observed kinetics of spirochete population expansion in the skin was comparable to that determined by needle inoculation (13). Using the nymph capsule feeding approach, analysis of the numbers of Δ*bbk13* *B. burgdorferi* in the tick feeding site on days 9 and 10 post-nymph application revealed the striking result that loss of *bbk13* had no impact on spirochete population expansion in the skin. It is important to note that peak numbers of wild type *B. burgdorferi* in the blood were also detected on days 8-10 post-nymph application. As the skin is a highly vascularized tissue and the mice were not perfused prior to harvest of the tick feeding site, it remains
a possibility that circulating Bb in the blood may have contributed to the numbers of wild

type and Δbbk13 B. burgdorferi detected in the skin at these time points. Despite the
amelioration of the early infection defect of Δbbk13 B. burgdorferi when delivered by tick
feeding, assessment of mice fed on by capsule-confined nymphs three weeks post-
nymph application revealed that the bbk13 gene remained critical for disseminated
infection. Together, these data suggest that one or more of the biological changes that
the spirochetes and/or the host undergo during tick feeding allowed Δbbk13 B.

burgdorferi to overcome the barrier to population expansion in the skin. However, the
relief of this early phenotype was not sufficient to overcome the need for bbk13 during
disseminated infection. These findings emphasize that B. burgdorferi population
expansion in the skin and dissemination to distal tissues are distinct events of
mammalian infection that are uniquely influenced by the feeding tick and likely require
different sets of B. burgdorferi genes. Furthermore, this work underscores the
significance of the vector as a driver of vector-borne pathogen adaptation and
pathogenesis.

Our studies have provided greater insight into the role of bbk13 in the biology of
B. burgdorferi, yet the molecular function of BBK13 remains unknown. Recently, we
established that BBK13 forms large oligomeric complexes in the spirochete membrane
(14). Current work is focused on investigating how BBK13 oligomerization contributes to
the mechanisms by which B. burgdorferi surmounts immune barriers in the mammalian
host in order to establish a disseminated infection. In sum, this work cements the
importance of bbk13 in promoting B. burgdorferi mammalian infectivity and highlights
the influence of the tick vector on the pattern of infection, particularly at the skin—the interface between host, vector, and pathogen.
**Materials and methods**

**B. burgdorferi clones and growth conditions.** The *Borrelia* (*Borreliella*) *burgdorferi* clones used in this study were derived from the low-passage infectious clone B31 A3-68 Δbbe02, herein referred to as wild type, which lacks plasmids cp9 and lp56, as well as the bbe02 gene on lp25 (29). The Δbbk13 mutant clone and the Δbbk13/bbk13+ complement have been described previously (13). *B. burgdorferi* cultures were grown at 35°C in liquid Barbour-Stoenner-Kelly II (BSKII) medium containing gelatin and 6% rabbit serum. Alternatively, *B. burgdorferi* were plated in solid BSK-agarose medium and incubated at 35°C under 2.5% CO₂. The following antibiotics were used, as needed: kanamycin (200 μg/ml), streptomycin (50 μg/ml), and gentamicin (40 μg/ml).

**Ethics statement.** The University of Central Florida is accredited by the International Association for Assessment and Accreditation of Laboratory Animal Care. Protocols for all animal experiments were prepared according to the guidelines of the National Institutes of Health and were reviewed and approved by the University of Central Florida Institutional Animal Care and Use Committee.

**Needle inoculation of mice.** *B. burgdorferi* cultures were grown from frozen glycerol stocks in BSKII medium with the appropriate antibiotics to stationary phase (1x10⁹/ml). *B. burgdorferi* cultures were kept at stationary phase for ~24 hours prior to mouse inoculation. Culture density was determined using a Petroff-Hauser chamber under dark-field microscopy. *B. burgdorferi* cultures were diluted in BSKII to the desired inoculum dose. Groups of C3H/HeN, Nod-scid IL2Rgamma<sup>null</sup> (NSG) or NOD/ShiLtJ (NOD) mice were inoculated intradermally into the shaved dorsal skin with wild type, Δbbk13, or Δbbk13/bbk13+ *B. burgdorferi* at a dose of 10⁴, 10⁵, 10⁶ or 10⁷ spirochetes.
All inoculum cultures were analyzed for plasmid content by PCR and plated in solid BSK-agarose medium to verify the presence of virulence plasmids lp25, lp28-1, and lp36 in individual colonies (27). All inoculum cultures had the expected plasmid profile, and 80%-100% of individual clones examined contain all three virulence plasmids.

Seven days or 3-4 weeks post-tick feeding mice were assayed for *B. burgdorferi* infection by semi-quantitative tissue reisolation assay (as described below) (13), quantitative PCR loads in tissues (13, 27) and/or serology (30).

**B. burgdorferi acquisition by naïve *Ixodes* larvae.** Three weeks post-inoculation with wild type, Δbbk13 or Δbbk13/Δbk13+ *B. burgdorferi*, mice were fed upon by groups of ~150 naïve *Ixodes scapularis* larval ticks (CDC, BEI resources or Oklahoma State University). Replete larvae were collected and individually assessed for *B. burgdorferi* infection, as described below.

**Artificial infection of *Ixodes* larvae and free feeding tick transmission.**

Approximately 4-month-old naïve *Ixodes scapularis* larval ticks (CDC, BEI resources or Oklahoma State University) were dehydrated by exposure to saturated ammonium sulfate for 24 hr. Log phase grown *B. burgdorferi* clones were diluted to 2x10^7 cells/ml in BSKII. 500 µl of spirochetes were incubated with dehydrated ticks at 35°C for ~1.5 hrs and washed twice with PBS (15). The inoculum cultures were verified to contain the expected endogenous plasmids (27, 31). Infectious plasmids lp25, lp28-1 and lp36 were present in 80-100% of individuals of each inoculum culture. Three days post artificial infection cohorts of ~150 larvae were fed to repletion on groups of 5 naïve C3H/HeN mice (Envigo) per *B. burgdorferi* clone. A subset of fed larvae was allowed to molt into nymphs and subsequently cohorts of 25 nymphs fed on groups of 5 naïve C3H/HeN
mice (Envigo) per *B. burgdorferi* clone. Three weeks post tick feeding mice were assayed for *B. burgdorferi* infection by semi-quantitative tissue reisolation assay (as described below) (13), quantitative PCR loads in tissues (13, 27) and/or serology (30).

**Capsule feeding tick transmission.** The top portion of a 2 ml screw-cap tube with perforated cap (capsules), was glued onto the shaved dorsal skin of groups of 2-6 C3H/HeN mice using veterinary tag cement (Nasco) and allowed to completely adhere overnight. Groups of 15 *B. burgdorferi*-infected unfed nymphs were added to each capsule and allowed to feed. Seven days post-nymph application, replete nymphs were collected. Seven days or 3 weeks post-nymph application mice were euthanized and the skin feeding site and distal tissues were collected for analysis. Infection was determined by semi-quantitative tissue reisolation assay (as described below) (13), quantitative PCR loads in tissues (13, 27) and/or serology (30).

**Semi-quantitative tissue reisolation scoring method.** Semi-quantitative tissue reisolation scoring was performed as described previously (13). Briefly, tissues were dissected at designated timepoints after inoculation and placed in BSKII containing an antibiotic cocktail of rifampin (50 μg/ml), amphotericin B (2.5 μg/ml), and phosphomycin (20 μg/ml) (RPA cocktail). *B. burgdorferi* is naturally resistant to these antibiotics. Reisolation cultures were incubated at 35°C. Cultures were visually inspected daily for the presence of spirochetes using dark-field microscopy. While viewing at 200x magnification, a numerical score is assigned, as follows: 0, absence of spirochetes; 1, low spirochete density (<5 spirochetes in the field of view); 2, medium spirochete density (5 to 15 spirochetes in the field of view); and 3, high spirochete density (>15 spirochetes in the field of view). Lastly, presence or absence of spirochetes (denoted by
‘y’ or ‘n’, respectively) was scored at the endpoint of the assay, after 14 days of incubation.

**Quantification of B. burgdorferi loads in Ixodes ticks.** The *B. burgdorferi* densities in ticks were assessed before and 7-14 days after feeding to repletion on mice. Ticks were surface sterilized using sequential washes with 3% hydrogen peroxide, 70% ethanol, and sterile water. Groups of 10 unfed larvae, individual fed larvae, or individual nymphs (fed or unfed) were crushed and homogenized in BSKII using a plastic pestle in microcentrifuge tubes, first by hand then by motorized adapter. Homogenates were serially diluted and plated in solid BSK-agarose medium supplemented with RPA cocktail. Colony-forming units were enumerated and *B. burgdorferi* load per tick calculated (30, 32). Alternatively, total DNA was isolated from groups of 40 unfed larvae using a NucleoSpin tissue kit (Clontech Laboratories) according to the manufacturer’s specifications and qPCR performed as previously described (30).

**Quantification of B. burgdorferi density in the blood.** Peripheral blood was collected from mice on day 6 post-needle inoculation with *B. burgdorferi* or day 3-12 post-application of *B. burgdorferi* infected ticks. Whole blood was serially diluted in BSKII then plated in solid BSK-agarose medium plus RPA cocktail and incubated at 35°C under 2.5% CO₂. Colony-forming units were enumerated and spirochete density in circulating blood was calculated (13).

**Graphs, figures, and statistical tests.** Graphpad Prism v.9 was used to generate graphs and perform statistical tests. Adobe illustrator was used for figure assembly minor editing of graphic elements.
Acknowledgements

We would like to thank the three expert reviewers for their thoughtful comments. Thank you to Travis Jewett and members of the Jewett labs for insightful discussions. Thank you to the UCF NAF animal care staff. The following reagent was provided by Centers for Disease Control and Prevention for distribution by BEI Resources, NIAID, NIH: *Ixodes scapularis* Larvae (Live), NR-44115.

FOOTNOTES

Conflicts of interest statement: None declared.

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Figure legends

Figure 1. Naïve larval ticks fail to acquire Δbbk13 B. burgdorferi during feeding on infected mice.

Groups of six C3H/HeN mice were needle-inoculated intradermally with 10^4 WT, Δbbk13, or Δbbk13/Δbbk13+ B. burgdorferi. (A) At six days post-inoculation, disseminating B. burgdorferi were enumerated by plating blood in solid BSK-agarose medium and enumerating colony-forming units. Each dot represents an individual mouse. The mean value is indicated by a black horizontal line. The Kruskal-Wallis test with Dunn’s multiple comparisons was used to determine statistical significance (**, p≤0.01). (B) Four weeks post-inoculation, the indicated tissues were assessed for B. burgdorferi by reisolation assay. Semi-quantitative scoring of reisolation cultures was performed (‘0’ indicates no spirochetes, ‘1’-‘3’ indicates increasing spirochete density). (C) Naïve tick larvae fed to repletion on C3H/HeN mice three weeks after needle inoculation with 10^4 WT, Δbbk13, or Δbbk13/Δbbk13+ B. burgdorferi. Individual fed larva were homogenized and plated in solid BSK-agarose medium and B. burgdorferi load determined by enumerating colony-forming units. Each data point represents an individual tick. The tick infection rate for each B. burgdorferi clone is indicated above the corresponding scatterplot. The mean is indicated by a horizontal black line. The Kruskal-Wallis test with Dunn’s multiple comparisons was used to determine statistical significance (****, p<0.0001; n.s., not significant).

Figure 2. Increasing the inoculum dose does not alleviate the infectivity defect of Δbbk13 B. burgdorferi.
Groups of six C3H/HeN mice were intradermally inoculated with $10^4$ WT *B. burgdorferi* or increasing doses ($10^5$, $10^6$, $10^7$) of Δbbk13 *B. burgdorferi*. (A) At three weeks post-inoculation, tissues were assessed for *B. burgdorferi* by reisolation assay. Semi-quantitative scoring of reisolation cultures was performed following 5 days (‘0’ indicates no spirochetes, ‘1’-‘3’ indicates increasing spirochete density) and 14 days (endpoint, scored for presence, ‘y’, or absence, ‘n’, of spirochetes) of culture incubation. (B, C) Total DNA was extracted from the ears (B) and joints (C). *B. burgdorferi* load was measured by quantifying *B. burgdorferi* flaB copies normalized to $10^6$ mouse nid copies using quantitative PCR. In cases where not all samples per group had detectable *B. burgdorferi* DNA, the number of positive samples out of the total mice per group are indicated in the scatterplot. Data points below the level of detection are not shown. The mean is indicated by a horizontal black line. The Kruskal-Wallis test with Dunn’s multiple comparisons was used to determine statistical significance. (*, p<0.05; **, p<0.01)

**Figure 3. bbk13 is dispensable for replication and survival in ticks.**

Naïve larvae were artificially infected by immersion with wild type (WT), Δbbk13, or Δbbk13/bbk13+ *B. burgdorferi*. *B. burgdorferi* loads in ticks at various stages of development were measured by plating tick homogenate in solid BSK-agarose medium and enumerating colony-forming units. (A) Unfed larvae were assessed for *B. burgdorferi* load following artificial infection. Each data point represents a group of 10 larvae. (B) *B. burgdorferi* loads in fed larvae, unfed nymphs and fed nymphs were determined. Each data point represents an individual tick. The tick infection rate for each *B. burgdorferi* clone is indicated above the corresponding scatterplot. The mean is indicated by a horizontal black line. The Kruskal-Wallis test with Dunn’s multiple comparisons was used to determine statistical significance. (*, p<0.05; **, p<0.01)
comparisons was used to determine statistical significance (***, p<0.001; n.s., not significant).

**Figure 4.** *bbk13* is critical for *B. burgdorferi* infection of mice by tick transmission.

Unfed nymphs infected with wild type (WT), Δ*bbk13*, or Δ*bbk13*/*bbk13* + *B. burgdorferi* were fed to repletion on groups of five C3H/HeN mice. Mice were assessed for *B. burgdorferi* infection three weeks post-tick feeding by reisolation assay, quantitative PCR, and serology. (A) Distal tissues were collected for spirochete reisolation. Semi-quantitative scoring of reisolation cultures was performed following 5 days (‘0’ indicates no spirochetes, ‘1’-’3’ indicates increasing spirochete density) and 14 days (endpoint, scored for presence, ‘y’, or absence, ‘n’, of spirochetes) of culture incubation. (B) Total DNA was extracted from ear, heart, and joint tissues. *B. burgdorferi* loads in each tissue were measured by quantifying *B. burgdorferi flaB* copies normalized to 10^6 mouse *nid* copies using quantitative PCR. The mean for each group is represented by a horizontal line. In cases where not all samples per group had detectable levels of *B. burgdorferi* DNA, the number of positive samples out of 5 mice per group are indicated. The Kruskal-Wallis test with Dunn’s multiple comparisons was used to determine statistical significance (n.s., not significant). (C) Pre-immune and post-immune sera were assessed by immunoblot for the presence of anti-*B. burgdorferi* antibodies. Post-immune serum from individual mice was used to blot total *B. burgdorferi* lysate. Pre-immune sera were pooled across each of the three groups. Molecular weight standards are shown in kilodaltons (kDa). (D) Unfed *Ixodes* larvae were artificially infected with wild type (WT) or Δ*bbk13* *B. burgdorferi* by immersion. Artificially infected larvae were allowed to feed freely to repletion on groups of five C3H/HeN mice. Three weeks after
larva application, tissues were assessed for *B. burgdorferi* by reisolation assay. Semi-quantitative scoring of reisolation cultures was performed following 5 days (‘0’ indicates no spirochetes, ‘1’-‘3’ indicates increasing spirochete density) and 14 days (endpoint, scored for presence, ‘y’, or absence, ‘n’, of spirochetes) of culture incubation.

**Figure 5.** *B. burgdorferi* delivered by tick bite undergo population expansion in mouse skin.

Unfed nymphs infected with wild type (WT) *B. burgdorferi* were placed in capsules adhered to the dorsal skin of naïve C3H/HeN mice. Cohorts of three mice each were assessed for *B. burgdorferi* infection at the indicated days after nymph application. (A) The tick feeding site was assessed for *B. burgdorferi* by tissue reisolation assay. Semi-quantitative scoring of reisolation cultures was performed daily over 5 days of culture incubation (‘0’ indicates no spirochetes, ‘1’-‘3’ indicates increasing spirochete density). (B) Total DNA was extracted from the skin feeding site and *B. burgdorferi* loads were measured by quantifying *B. burgdorferi flaB* copies normalized to $10^6$ mouse *nid* copies using quantitative PCR. The mean *B. burgdorferi* load (black dot) for each cohort was plotted over time. Error bars represent standard deviation. (C) Peripheral blood from each mouse was plated in solid BSK-agarose medium and circulating *B. burgdorferi* was enumerated by counting colony-forming units. Gray dots represent individual mice. Error bars represent standard deviation.

**Figure 6.** When delivered by tick transmission *bbk13* is not required for *B. burgdorferi* population expansion in the skin but is critical for disseminated infection.
Unfed nymphs infected with wild type (WT) or Δbbk13 B. burgdorferi were placed in capsules adhered to the dorsal skin of groups of six naïve C3H/HeN mice to feed. The tick feeding site was collected at days 9 and 10 post-nymph application for analysis. (A) Prior to feeding, subsets of individual unfed B. burgdorferi-infected nymphs were homogenized and plated in solid BSK-agarose medium to assess spirochete load by enumerating colony-forming units. (B) The tick feeding site was assessed for B. burgdorferi by tissue reisolation assay. Semi-quantitative scoring of reisolation cultures was performed daily over 4 days of culture incubation ('0' indicates no spirochetes, '1'- '3' indicates increasing spirochete density). (C) Total DNA was extracted from the tick feeding site. B. burgdorferi load was measured by quantifying B. burgdorferi flaB copies normalized to 10^6 mouse nid copies using quantitative PCR. In cases where not all samples per group had detectable B. burgdorferi DNA, the number of positive samples out of 6 mice per group are indicated in the scatterplot. Data points below the level of detection are not shown. The mean is indicated by a horizontal black line. The Mann-Whitney test was used to determine statistical significance (n.s., not significant). (D, E) Unfed nymphs infected with WT or Δbbk13 B. burgdorferi were placed in capsules adhered to the dorsal skin of groups of 2-4 naïve C3H/HeN mice to feed. At 3 weeks post-nymph application, the tick feeding site and various distal tissues were collected for analysis. (D) Tissues were assessed for B. burgdorferi by tissue reisolation assay. Semi-quantitative scoring of reisolation cultures was performed on day 6 of culture incubation ('0' indicates no spirochetes, '1'- '3' indicates increasing spirochete density). (E) Total DNA was extracted from the indicated tissues. B. burgdorferi loads were measured by quantifying B. burgdorferi flaB copies normalized to 10^6 mouse nid copies.
using quantitative PCR. In cases where not all samples per group have detectable *B. burgdorferi* DNA, the number of positive samples out of the total mice per group are indicated in the scatterplot. Data points below the level of detection are not shown. The mean is indicated by a horizontal black line.

**Figure 7. Infectivity of Δ*bbk13* *B. burgdorferi* is restored in immunocompromised mice.**

Groups of six control NOD/ShiLtJ (NOD) or immunocompromised Nod-scid IL2Rgamma<sup>null</sup> (NSG) mice were intradermally inoculated with 10<sup>4</sup> wild type (WT), Δ*bbk13*, or Δ*bbk13/bbk13+* *B. burgdorferi*. (A) Six days post-inoculation, blood was collected and assessed for disseminating spirochetes by plating in solid BSK-agarose medium and enumerating colony-forming units. (B) Three weeks post-inoculation, tissues were assessed for *B. burgdorferi* by reisolation assay. Semi-quantitative scoring of reisolation cultures was performed on day 5 of culture incubation (‘0’ indicates no spirochetes, ‘1’-‘3’ indicates increasing spirochete density).
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#### 5-day culture

- ear
- heart
- bladder
- joint

#### Endpoint

- ear
- heart
- bladder
- joint

### Graphs

**B. Ear**

- Log plot of flaB copies / 10⁶ nld copies
- Significance: *p < 0.05, **p < 0.01
- Key:
  - 10⁴ WT
  - 10⁵ Δbbk13
  - 10⁶ Δbbk13
  - 10⁷ Δbbk13

**C. Joint**

- Log plot of flaB copies / 10⁶ nld copies
- Significance: *p < 0.05, **p < 0.01
- Key:
  - 10⁴ WT
  - 10⁵ Δbbk13
  - 10⁶ Δbbk13
  - 10⁷ Δbbk13
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B

![Graphs showing flaB copies per 10^6 nid copies](image)

C

![Image showing gel electrophoresis results](image)

D

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B. burgdorferi/mL blood

*Note: Days 1-5 reisolation culture age*
**A**

Bar graph showing the concentration of *B. burgdorferi* in mL blood for WT, Δbbk13, and Δbbk13/bbk13+ strains. The y-axis represents the concentration in a logarithmic scale, ranging from 1 to 10^4.

**B**

Table summarizing the inoculation sites and outcomes in WT, Δbbk13, and Δbbk13/bbk13+ strains. The table includes columns for inoculation site (inoc. site), ear, heart, bladder, and joint, with mouse numbers 1 to 6.

- **NOD**
  - inoc. site
    - WT: 2 2 1 2 1 1
    - Δbbk13: 0 0 0 0 0 0
    - Δbbk13/bbk13+: 0 3 1 0 0 1
  - ear
    - WT: 3 3 3 0 3 3
    - Δbbk13: 1 1 0 1 0 0
    - Δbbk13/bbk13+: 3 1 0 0 0 3
  - heart
    - WT: 1 1 1 1 2 1
    - Δbbk13: 0 0 0 0 0 0
    - Δbbk13/bbk13+: 1 2 2 1 1 2
  - bladder
    - WT: 3 3 3 3 3 3
    - Δbbk13: 0 2 0 0 1
    - Δbbk13/bbk13+: 3 3 3 3 3 3
  - joint
    - WT: 3 3 2 3 3 3
    - Δbbk13: 0 0 0 0 0 1
    - Δbbk13/bbk13+: 3 2 3 2 3 3

- **NSG**
  - inoc. site
    - WT: 3 3 3 3 1 3
    - Δbbk13: 3 0 1 3 2 0
    - Δbbk13/bbk13+: 3 1 0 2 0 0
  - ear
    - WT: 3 3 2 2 3 0
    - Δbbk13: 3 3 2 3 1 3
    - Δbbk13/bbk13+: 2 3 3 0 2 3
  - heart
    - WT: 3 3 3 3 3 3
    - Δbbk13: 3 3 3 3 3 3
    - Δbbk13/bbk13+: 3 3 3 3 3 3
  - bladder
    - WT: 3 3 3 3 3 3
    - Δbbk13: 3 3 3 3 3 3
    - Δbbk13/bbk13+: 3 3 3 3 3 3
  - joint
    - WT: 3 3 3 3 3 3
    - Δbbk13: 2 3 2 3 3 2
    - Δbbk13/bbk13+: 3 3 3 3 3 3

All results are given in terms of 3 weeks post-inoculation.