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Utilizing Two *Borrelia bavariensis* Isolates Naturally Lacking the PFam54 Gene Array To Elucidate the Roles of PFam54-Encoded Proteins

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ABSTRACT Lyme borreliosis is the most common vector-borne disease in the Northern Hemisphere, caused by spirochetes belonging to the *Borrelia burgdorferi sensu lato* species complex, which are transmitted by ixodid ticks. *B. burgdorferi sensu lato* species produce a family of proteins on the linear plasmid 54 (PFam54), some of which confer the functions of cell adhesion and inactivation of complement, the first line of host defense. However, the impact of PFam54 in promoting *B. burgdorferi sensu lato* pathogenesis remains unclear because of the hurdles to simultaneously knock out all PFam54 proteins in a spirochete. Here, we describe two *Borrelia bavariensis* strains, PBN and PNi, isolated from patients naturally lacking PFam54 but maintaining the rest of the genome with greater than 95% identity to the reference *B. bavariensis* strain, PBi. We found that PBN and PNi less efficiently survive in human serum than PBi. Such defects were restored by introducing two *B. bavariensis* PFam54 recombinant proteins, BGA66 and BGA71, confirming the role of these proteins in providing complement evasion of *B. bavariensis*. Further, we found that all three strains remain detectable in various murine tissues 21 days post-subcutaneous infection, supporting the nonessential role of *B. bavariensis* PFam54 in promoting spirochete persistence. This study identified and utilized isolates deficient in PFam54 to associate the defects with the absence of these proteins, building the foundation to further study the role of each PFam54 protein in contributing to *B. burgdorferi sensu lato* pathogenesis.

IMPORTANCE To establish infections, Lyme borreliae utilize various means to overcome the host's immune system. Proteins encoded by the PFam54 gene array play a role in spirochete survival *in vitro* and *in vivo*. Moreover, this gene array has been described in all currently available Lyme borreliae genomes. By investigating the first two *Borrelia bavariensis* isolates naturally lacking the entire PFam54 gene array, we showed that both patient isolates display an increased susceptibility to human serum, which can be rescued in the presence of two PFam54 recombinant proteins. However, both isolates remain infectious to mice after intradermal inoculation, suggesting the nonessential role of PFam54 during the long-term, but may differ slightly in the colonization of specific tissues. Furthermore, these isolates show high genomic similarity to type strain PBi (>95%) and could be used in future studies investigating the role of each PFam54 protein in Lyme borreliosis pathogenesis.

KEYWORDS *Borrelia bavariensis*, Lyme disease, spirochetes, PFam54 gene array, complement evasion, innate immunity, host-pathogen interaction

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Lyme borreliosis (LB, also termed Lyme disease in North America) is the most common vector-borne disease in the Northern Hemisphere (1, 2) with estimated annual cases between 65,000 and 200,000 in Europe (3, 4) and between 30,000 and 476,000 in the United States (5–7). This disease is caused by spirochetes belonging to the *Borrelia burgdorferi sensu lato* species complex (1, 8–10), which are maintained in an enzootic transmission cycle between ixodid ticks, normally of the genus *Ixodes*, and various vertebrate reservoir host species (8, 9, 11–13). Most LB cases in North America are caused by the genospecies *B. burgdorferi sensu stricto*, while additional genospecies, such as *B. afzelii*, *B. garinii*, and *B. bavariensis*, are causative agents of LB across Eurasia (1, 2, 8, 9). *B. bavariensis* is of particular interest in Europe, as it has a high propensity to infect humans and is associated with severe LB manifestations (i.e., neuroborreliosis) (1, 14) while additionally being rarely recovered from *Ixodes* ticks collected in the field (15–17). *B. bavariensis* was proposed as a genospecies in 2009 and validated in 2013 (18, 19), prior to which, it was considered to be a subtype (OspA serotype 4 or NT29-like) of its sister species *B. garinii* (19). *B. bavariensis* appears to primarily be a rodent-adapted genospecies (18, 20, 21), which is distributed across Eurasia (19), where it utilizes either the tick species *Ixodes ricinus* (Europe) or *Ixodes persulcatus* (Asia) as a vector (20). This genospecies exists in two distinct populations with a high-diversity, ancestral population in Asia (20, 22–24) and a genetically homogenous, almost clonal population in Europe (20, 22–24).

To establish an infection, *B. burgdorferi sensu lato* must evade complement, an important pillar of innate immunity, either indirectly through the acquisition of complement regulators or directly through interactions with complement proteins (25–29). The complement system consists of three distinct pathways (classical, lectin, and alternative), all leading to the cleavage of C3 to form activated C3b (30). This initiates the activation of other complement components, ending in the assembly of the membrane attack complex (MAC) through recruitment of late-stage complement proteins (C6, C7, C8, and C9) and ultimately leading to bacterial cell lysis (30). Additionally, cleavage of C3 and C5 by the C3 and C5 convertases, respectively, leads to the release of anaphylatoxins C3a and C5a, which can recruit additional immune cells and, therefore, are integral in mounting further host immune responses to infection (30). Host cells control complement damage by utilizing membrane-bound or fluid-phase regulatory proteins (30). All complement regulators can terminate the complement cascade at specific activation levels to protect self-cells from complement-mediated damage (30).

Lyme borreliae produce diverse outer surface proteins that bind distinct host complement components resulting in complement inactivation (27, 28, 31–34). In fact, several *Borrelia* proteins named CRASPs (complement regulator acquiring surface proteins) are capable of binding complement regulators belonging to the factor H protein family and thereby allow spirochetes to overcome the host's innate immune system (27, 34, 35). One well-studied factor H binding protein, CspA, belongs to the large paralogous protein family, PFam54, with members capable of binding complement regulatory proteins or even of direct interactions with complement components (25, 36–39). Members of the PFam54 are encoded by genes predominantly arranged in a multigene array located at the terminal end of the linear plasmid (lp) 54 in all *B. burgdorferi sensu lato* genomes studied so far (40–42). The PFam54 gene array can be separated into five major lineages, where lineages I, II, III, and V share one to one orthology among genospecies (40). Lineage IV, however, contains a variable number of paralogs, and many genospecies display unique PFam54 paralogs not found in other genospecies (40). The *B. bavariensis* type strain, PBi, contains PFam54 paralogs belonging to all of these lineages, although most do not have a described function (40). Seven of these PBi PFam54 paralogs belong to lineage IV, including *bga66* and *bga71* (40), which produce proteins that bind late-stage complement proteins (C7, C8, and C9) and thereby inhibit the assembly of a functional MAC, conferring resistance to human complement (36). In other genospecies, paralogs belonging to lineage IV have been found to facilitate human and nonhuman factor H binding (34, 39, 43, 44). Specific non-lineage IV paralogs have been studied in *B. burgdorferi sensu stricto*, where some even have

TABLE 1 Characteristics of *B. bavariensis* isolates utilized in this study

Isolate	Genospecies	Yr of culturing	Country	Biological origin	Disease manifestation	lp54 length (kb)
PBi	*Borrelia bavariensis*	<1993	Germany	Human	Neuroborreliosis	60.4
PBN	*Borrelia bavariensis*	1999	Germany	Human	Neuroborreliosis	46.6
PNi	*Borrelia bavariensis*	2000	Germany	Human	Lymphoma	46.6

proposed functions. For example, both *bba64* (PBi paralog, *bga63*) and *bba66* (PBi paralog, *bga65*) are required for tick-to-host transmission (45, 46). Even so, there are still many open questions regarding what roles the proteins encoded by the Pfam54 gene array play during the enzootic cycle of *B. burgdorferi sensu lato* spirochetes, especially in genospecies besides *B. burgdorferi sensu stricto*.

We recently characterized the genomes of 33 Eurasian *B. bavariensis* isolates, of which 2 European isolates, PBN and PNi, were found to contain a shorter lp54 than that of the type strain, PBi (22). In this study, we show that both PBN and PNi are naturally lacking the entire PFam54 gene array. Such findings raise the possibility of using these strains to study the roles of these genes. Therefore, we compared the complement evasion activity and infectivity conferred by a *B. bavariensis* strain that has intact PFam54 genes (PBi) and the strains deficient in those genes (PBN and PNi). We provide new insights into the potential contribution of PFam54 proteins to facilitate spirochete survival *in vitro* and *in vivo*.

RESULTS

PBN and PNi naturally lack the entire PFam54 gene array. In both long- (PacBio) and short-read (MiSeq) assemblies, PBN and PNi had a shorter lp54, of approximately 46.6 kbp, than that of type strain PBi, with a lp54 of approximately 60.4 kbp (Table 1). BLAST searches conducted for PBN and PNi returned no hits for the PBi PFam54 paralogs either on the reconstructed lp54 or when searched against all assembled contigs, whereas all paralogs (n = 11) were found in our PBi assembly (Fig. 1). These findings were further confirmed by using a paralog-specific PCR approach, which showed no amplicon for all PFam54 paralogs in PBN and PNi except for the primer pair targeting *bga68* (see Fig. S1 in the supplemental material). When using genomic DNA of PBN and PNi as a template and primers specific to amplification of *bga68*, we observed an unexpected PCR product of approximately 1,800 bp (much larger than the expected product of 1,369 bp). Following sequencing of these amplicons, sequences corresponded to a region on the *B. bavariensis* chromosome but not to any of the PFam54 paralogs. When DNA from PBi was used as a positive control, bands of expected size were observed for all PFam54 paralogs (Fig. S1) and were confirmed through Sanger sequencing. All primer pairs were designed for PBi, as the PFam54 paralogs are present in this isolate. As European *B. bavariensis* is characterized by a low-diversity, almost clonal distribution (20, 22, 23), primers designed for PBi should amplify the gene if present in PBN and PNi. Therefore, the lack of observed product is not a result of potential sequence polymorphisms in primer binding sites.

PBN and PNi display an increased serum susceptibility to human serum. Having demonstrated the absence of the entire PFam54 gene array in PBN and PNi, including *bga66* and *bga71*, serum bactericidal assays were conducted *in vitro* to explore the strains' capability to overcome complement-mediated killing. PBi and *B. garinii* strain G1 were included as a serum-resistant and serum-sensitive control, respectively. Both PBN and PNi had a significantly higher susceptibility to nonimmune human serum (NHS) than PBi, with approximately 20% of PBN or PNi cells surviving after 6 h compared to around 60% of PBi cells surviving (Fig. 2A). Additionally, PBN and PNi did not significantly differ from G1, where under the same conditions around 90% of cells were killed after 6 h (Fig. 2A). Interestingly, PBN and PNi cells which survived NHS retained a serum-sensitive phenotype after reculturing and a repeated incubation with 50% NHS (Fig. S2). When NHS was preincubated with purified proteins of PBi PFam54 paralogs

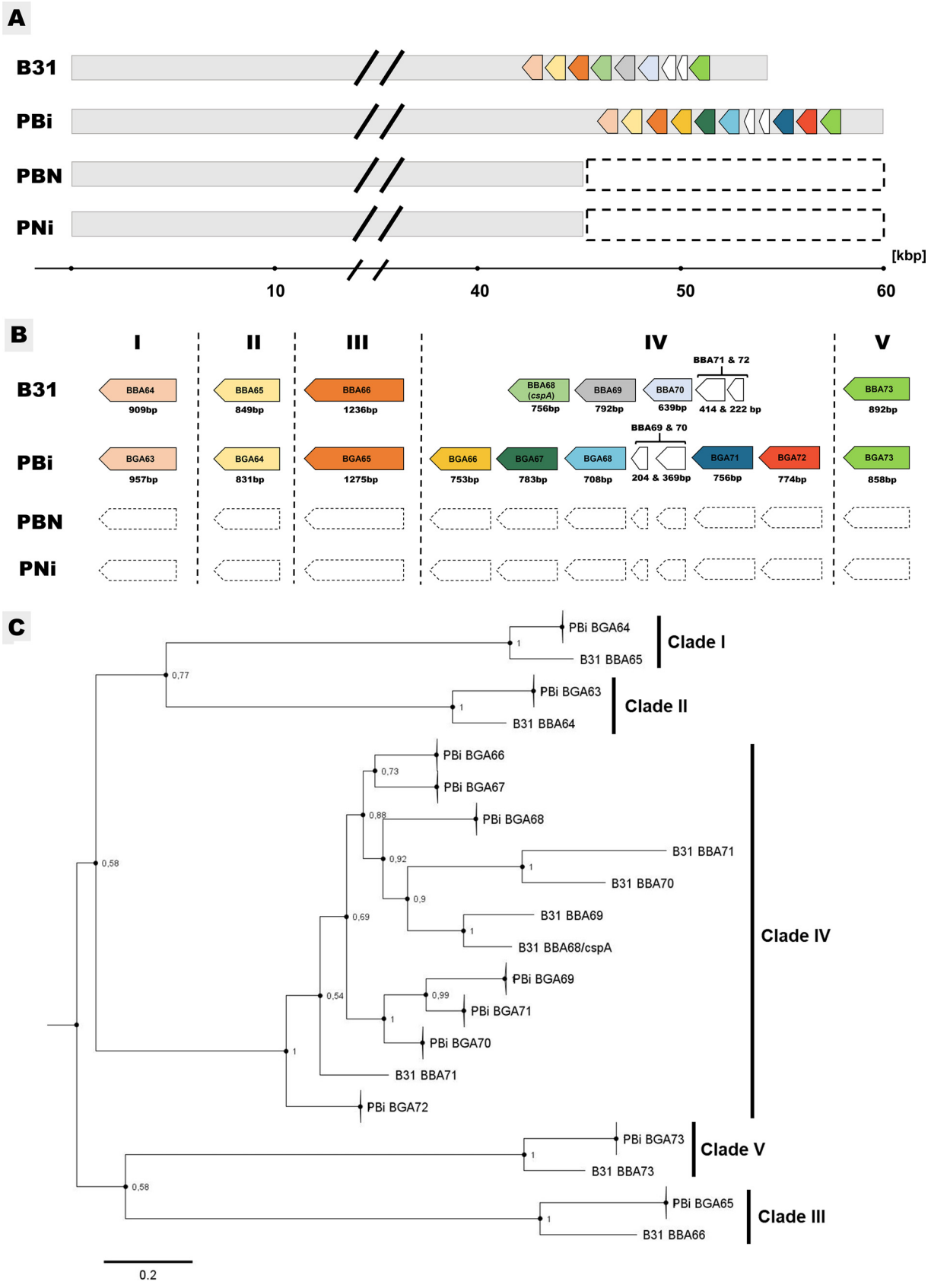

FIG 1 (A) Schematic overview of the aligned lp54 sequences for the *B. bavariensis* isolates PBi, PBN, and PNi and *B. burgdorferi sensu stricto* strain B31. Both PBi and B31 have longer lp54 sequences and contain the PFam54 gene array at the 3′ end, while PBN and PNi have a

(Continued on next page)

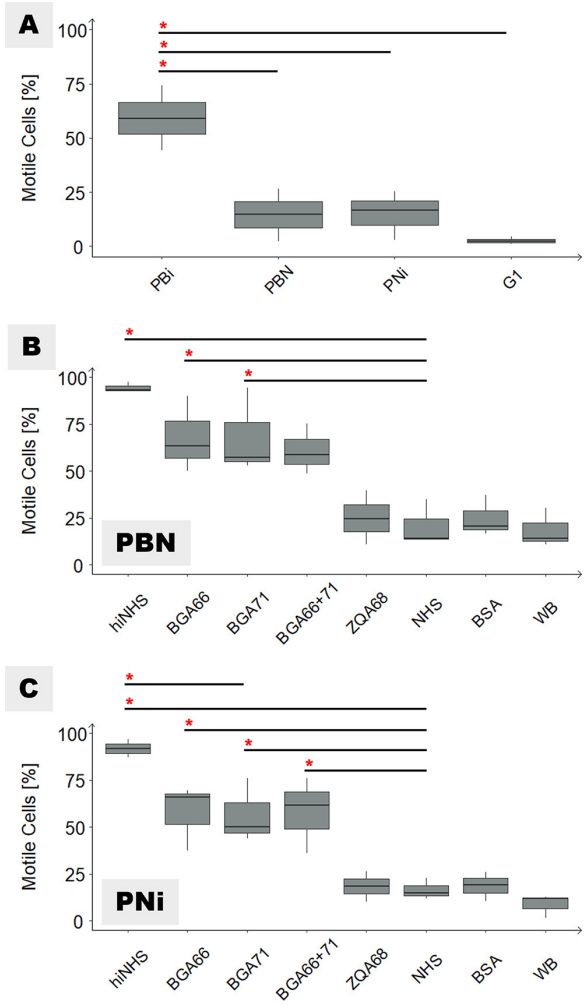

FIG 2 Serum susceptibility of *B. bavariensis* strains PBN and PNi lacking the PFam54 gene array after 6 h of incubation at 33°C. *B. bavariensis* strain PBi and *B. garinii* strain G1 were included as serum-resistant and -susceptible controls, respectively. (A) Percentage of motile PBN, PNi, PBi, and G1 after 6 h of incubation in 50% NHS. (B) Percentage of motile PBN cells after 6 h of incubation with NHS which was preincubated with 10 μM purified BGA66, BGA71, or a combination of BGA66 and BGA71. (C) Percentage of motile PNi cells after 6 h of incubation with NHS which was preincubated with 10 μM purified BGA66, BGA71, or a combination of BGA66 and BGA71. For both cases PBN (B) and PNi (C) controls were included where cells were incubated with NHS, heat-inactivated NHS (hiNHS), wash buffer (WB), or bovine serum albumin (BSA). Additionally, the PFam54 orthologous protein ZQA68 from *B. garinii* was included as a negative control. All tests were done in triplicate. Significance was tested using pairwise *t* tests with a Bonferroni multiple-testing correction, and significant differences are marked with a red asterisk. For all time points (0, 1, 2, 4, and 6 h) see Fig. S3.

BGA66 and BGA71 (36), both PBN (Fig. 2B) and PNi (Fig. 2C) were rescued to serum resistance levels comparable to those of PBi after 6 h of incubation (Fig. 2B and C). Moreover, each recombinant protein conferred serum resistance, while a combination of both did not increase the resistance level compared to the individual molecule (Fig. 2B and C). Additionally, incubation with a different PFam54 protein, ZQA68 from *B. gar-*

FIG 1 Legend (Continued)
shorter lp54 and lack ~14 kb from the 3′ end of the aligned sequences, where the PFam54 gene array is located. Individual genes are shown as arrows, and colors denote orthologous genes between isolates. Arrow direction denotes on which DNA strand the gene is found. (B) Synteny map of the PFam54 gene array. Genes are colored according to orthology and broken down into the five clades originally described in reference 40. These colors correspond to panel A. PBN and PNi are missing all orthologs belonging to all five clades (shown as dashed outlines in reference to the PBi paralogs). (C) Phylogeny to check the orthology of PBi and B31 PFam54 paralogs run in MrBayes (see Supplemental File 1 for further details). References for PBi and B31 were downloaded from GenBank for all PFam54 paralogs present in Wywial et al. (40) (B31, accession number AE000790.2; PBi, accession number CP000015.1). PBi clades include the orthologs found in the PacBio assembly from Margos et al. (54). All PBi sequences were the same and are displayed as a single node.

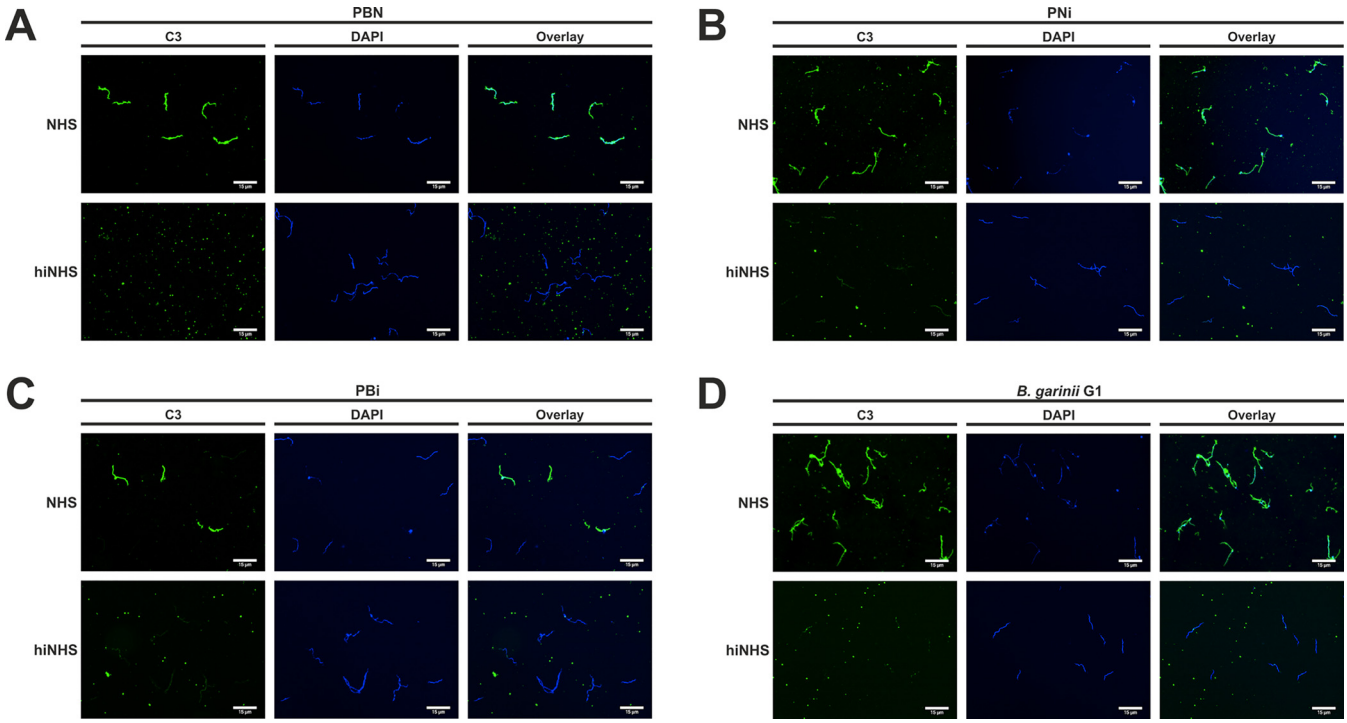

FIG 3 (A to D) Deposition of complement component C3 after challenging viable spirochetes from PBN (A), PNi (B), PBi (C), and serum-sensitive *B. garinii* G1 (D) with NHS (top row) and heat-inactivated NHS (hiNHS, bottom row). After fixation, deposition of C3 (green) was visualized with a polyclonal anti-C3 antibody (1:1,000), and spirochetal DNA (blue) was stained by using DAPI. Shown are representative data from two independent experiments. All scale bars are equal to 15 μm. The spirochetes were observed at a magnification of $\times$1,000. The data were recorded with an Axio Imager M2 fluorescence microscope (Zeiss) equipped with a Spot RT3 camera (Visitron Systems).

inii ZQ1, which does not confer resistance to human NHS, did not rescue PBN and PNi to resistance levels comparable to PBi (Fig. 2B and C). Refer to Fig. S3 for all serum assay time points. To further support PBN and PNi's increased susceptibility to complement, deposition of activated complement components of C3 (i.e., C3b) and the MAC was investigated by employing immunofluorescence microscopy. These complement deposition assays revealed that the majority of PBN and PNi cells showed deposition of C3 including activated C3b on the spirochetal surface (Fig. 3A to C). Concerning PBi, deposition of C3 components was also detected to some extent. Moreover, the staining pattern for PBN and PNi was similar to that of serum-sensitive *B. garinii* G1 (Fig. 3D). The same staining pattern was observed in relation to the pore-forming MAC when cells were stained for late-stage complement proteins (C5b to C9) (Fig. S4).

Both PBN and PNi were isolated from patients and are hypothesized to be clonal populations representing a single isolate. To determine if the serum susceptibility pattern of PBN and PNi could result from these isolates representing mixed cultures of two unique isolates (one serum-sensitive and one serum-resistant), the remaining spirochetes which survived NHS treatment after 6 h were transferred to fresh BSK-H (named for Barbour, Stoenner, and Kelly) medium and grown until they reached the exponential phase. After DNA isolation, PBN-ST and PNi-ST (where ST indicates serum treated) were sequenced using the Illumina MiSeq platform (see Materials and Methods). The genomes of PBN-ST and PNi-ST were identical to those of the original PBN and PNi genomes, including all plasmids and the shortened lp54 (22). Only very few variants were observed in repeat regions or regions which are known to be challenging to assemble using Illumina data (22, 47). The only notable exception was a single nonsynonymous mutation in a hypothetical protein found on lp28-9 of PBN-ST only. An HHPred structure-based search (48, 49) suggested similarities with dynamin-like proteins from *Bacillus* species. No BLAST hits for PFam54 paralogs were found in PBN-ST and PNi-ST, and they did not produce amplicons in the PFam54 paralog-specific PCRs (Fig. 1; Fig. S2).

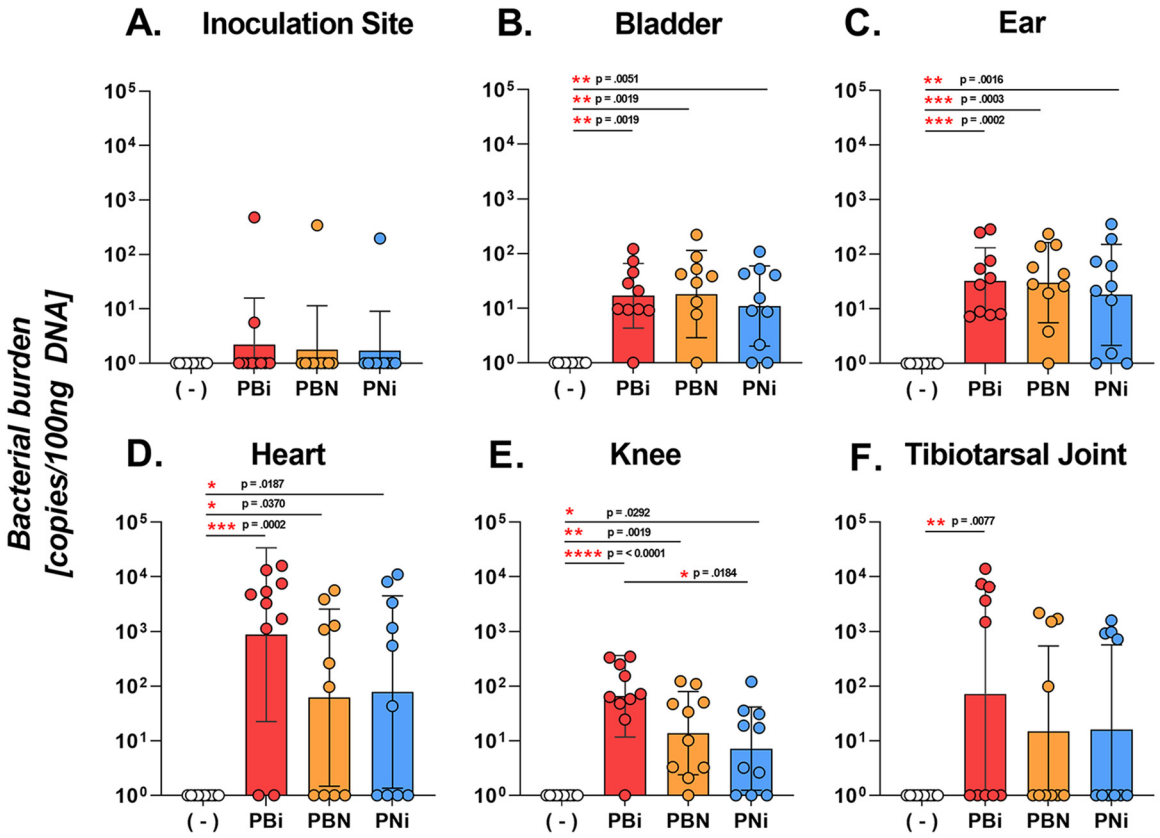

FIG 4 (A to F) Bacterial burden of mice at the inoculation site (A), bladder (B), ear (C), heart (D), knee (E), and tibiotarsal joint (F) 21 days post-needle inoculation with 1×10^5 bacteria of PBN, PNi, PBi, or BSK-II medium not supplemented with serum as a negative control. After 21 days, mice were sacrificed, and the bacterial burden was determined using a qPCR targeting the 16S rRNA gene of *Borrelia* which was normalized to the total amount of DNA. Differences between bacterial burdens were tested for significance using the Kruskal-Wallis test with the two-stage step-up method of Benjamini, Krieger, and Yekutieli. Significant differences are marked with an asterisk. Error bars show the standard deviation.

PBN and PNi remain infectious to BALB/c mice but show a trend for less efficient joint tropism after intradermal inoculation. We further determined the infectivity of PBi, PBN, and PNi *in vivo* by intradermally inoculating each of these isolates into BALB/c mice and a mock-infected group with culture medium as a control. At 21 days postinoculation, we found that the spirochete burdens at the inoculation site of skin from the mice infected with PBi, PBN, or PNi were indistinguishable from those in the mock-infected group (Fig. 4A). Conversely, the spirochete burdens in heart, bladder, and ear tissue derived from each of these three isolates did not statistically differ from each other but were significantly higher than those from mock-infected mice (Fig. 4B to D). All three isolates further showed significantly higher bacterial burdens at knee joints in comparison to the negative control, but PNi showed significantly lower burdens in comparison to PBi (Fig. 4E). Similarly, at tibiotarsal joints, the spirochete burdens from PBi-infected mice but not PBN- or PNi-infected mice were significantly greater than those burdens from mock-infected mice, although the three isolates did not significantly differ among themselves (Fig. 4F). These results demonstrate the ability of PBi, PBN, and PNi to yield productive infections in mice, suggesting that PFam54 is not essential for spirochete persistence in mice after intradermal infection, but lack of PFam54 could lead to slight strain-to-strain differences in the efficiency of colonizing specific murine tissues.

DISCUSSION

Lyme borreliae utilize a number of sophisticated strategies to successfully infect and colonize a host, including evading diverse host immune responses to survive in

the host's bloodstream, tissues, and organs (27, 28, 34, 35). Proteins encoded by the PFam54 gene array play an important role in some of these processes (34, 37–39, 50, 51), and these genes have been found in all sequenced *B. burgdorferi sensu lato* genomes to date (41, 42, 52). The functional characterization of these proteins has been challenging, as they are sequentially similar at different extents, reflecting their functional redundancy (36, 51, 53). However, simultaneously knocking out PFam54 proteins from a spirochete to study the functions of these proteins is not feasible because each spirochete carries more than five PFam54 proteins in any given Lyme borreliae species or strain (40). In a recent study using Illumina MiSeq assemblies of *B. bavariensis* genomes (22), we found that strains PBN and PNi contain a shorter lp54 plasmid of 46.6 kb compared to lp54 of type strain PBi (60.4 kb) (22, 54). For confirmatory purposes, long-read sequencing (PacBio) was conducted, as a combination of long and short sequencing technologies enables proper plasmid reconstruction (47). Comparative analyses of those genomes clearly indicated that the entire PFam54 gene array was missing in PBN and PNi. Such a novel identification of two strains deficient in the entire PFam54 provides a tool to study the functions of PFam54 *in vitro* and *in vivo* by comparing the phenotypes of these strains with the strains that carry the entire PFam54 (e.g., PBi).

We hypothesized that the absence of these genes in PBN and PNi could lead to an increased susceptibility to human complement, which was supported by our analyses. Compared to PBi, we found that PBN and PNi displayed decreased survival in human serum and increased deposition of the major complement component, C3. This result is consistent with the fact that PFam54 from PBi encodes BGA66 and BGA71, two proteins that bind to C7, C8, and C9 to inactivate terminal complement pathway in promoting the serum survivability in a gain-of-function spirochete background in *B. garinii* (36). In fact, we found that the presence of BGA66 and BGA71 rescues the serum sensitivity of PBN and PNi to levels indistinguishable from those from PBi. In contrast to CspA, which inhibits complement activation at the level of C3 activation, BGA66 and BGA71 terminate the complement cascade at the formation of the lytic MAC. Thus, it would be expected that serum-resistant PBi did show some deposition of complement components, in particular, C3 (36). It is also well known that complement activation occurs predominately on the surface of dead cells that are present even in the minority in the whole cell population. Thus, staining of serum-resistant cells is most likely due to dying or dead cells. Overall, these findings support the role of PFam54 in promoting evasion of human complement in *B. bavariensis* (34, 36, 39, 51, 55).

Both PBN and PNi, were isolated from patients with confirmed LB (Table 1) indicating that both isolates were able to establish human infection. Unfortunately, the time point at which PBN and PNi lost this region of the lp54 could not retrospectively be determined. The loss could have occurred before or during the infection process or during *in vitro* cultivation as described previously for other plasmids (47, 56, 57). It is tempting to speculate that the LB patients themselves could have had other circumstances (e.g., immunocompromised) which would have increased the likelihood of infection with PBN and PNi. Interestingly, spirochetes that survived serum treatment after 6 h of incubation (PBN-ST and PNi-ST) did not differ along their entire genome, including the shortened lp54, indicating that spirochetes that are killed and those that survived have the same genetic makeup. Furthermore, both PBN-ST and PNi-ST further retained the same serum-sensitive phenotype compared with the original isolates when challenged again with 50% NHS (Fig. S3). Serum survival of a subpopulation could be a result of a stochastic process involving transcriptional changes due to external signals which equip spirochetes with complement-interfering factors. *Borrelia* transcriptomes, including members of the PFam54 family, do change in response to stressors stemming from both reservoir hosts (i.e., temperature, pH) and tick vectors (i.e., low nutrients, oxidative stress) (9, 58, 59). Utilizing a transcriptional approach could therefore highlight which genes are transcribed by these two isolates while under serum stress to understand which mechanisms PBN and PNi, or even a subpopulation

thereof, utilize to overcome complement-mediated killing. *B. burgdorferi sensu lato* genomes are quite redundant, containing a number of genes with similar functions (41, 60, 61). Therefore, the presence and potential variation in other known anti-complement lipoproteins may contribute to such a strain-to-strain variation in complement evasion (31, 33, 34, 39). Additionally, considering that the majority of *B. burgdorferi sensu lato* plasmid-encoded proteins are either hypothetical or uncharacterized (42), it is likely that there are further gene products which confer serum resistance through yet uncharacterized immune evasion mechanisms (26–28, 34) and are contributing to the observed phenotype of PBN and PNi.

Though PBN and PNi are more susceptible to complement-mediated killing than PBi, when we compared the late-stage colonization (21 days post-intradermal infection in mice) of these strains with that of PBi, we did not notice differences between all three tested strains in heart, bladder, ear, and the initial infection site. Such heart- and bladder-specific colonization by *B. bavariensis* is consistent with the previous findings in PBi (62–65). It is important to note that laboratory mice may differ from naturally occurring rodent species which act as reservoir hosts for *B. bavariensis* in Europe, meaning that infection and tissue tropism may differ in these naturally occurring host species. Our results still suggest that PFam54 is not essential for persistent survival of spirochetes and that the persistence of PBi, PBN, and PNi is independent on the ability of these strains to evade complement. In fact, such a complement-independent persistence is consistent with several documented studies showing that the role of complement in discriminating strain-to-strain differences of infectivity is more apparent at infection onset than at the later stages of infection (66, 67). Hart et al. reported that CspA and its orthologous proteins from several isolates of *B. burgdorferi sensu stricto* or *B. afzelii* confer spirochete survival in fed ticks and thus promote tick-to-host transmission (39). Other *B. burgdorferi sensu stricto* PFam54 paralogs (i.e., *bba64* and *bba73*) are additionally upregulated during tick feeding, suggesting that PFam54 genes, and encoded products, may be important to tick-to-host transmission (58). As PBi contains paralogs of these upregulated genes which PBN and PNi are lacking, this could hint at an additional possibility that PBN and PNi may differ in their transmissibility during tick infection or the ability to successfully colonize the tick vector, requiring additional investigation.

Spirochetes of all three tested isolates did not differ from mock-infected mice at the initial infection site in the skin (Fig. 4C), unlike isolates from other Lyme borreliae genospecies (*B. burgdorferi sensu stricto*, *B. afzelii*, and *B. garinii*) (62–65). In contrast to clearance at the initial inoculation site, spirochetes of all three isolates were able to colonize and persist in ear skin (Fig. 4C). Many Lyme borreliae genes required for infection are regulated differently at different infection stages (58, 68). Thus, this finding raises the possibility of transcriptional differences of the *B. bavariensis* genes that are essential for skin colonization at early and late infection onsets, which warrants further investigations. We did find, however, that spirochete burdens at knee and tibiotarsal joints varied among the three isolates, with PBN and PNi showing potentially lower efficiency in establishing infection in these tissues but still being able to produce a viable infection. Strain-to-strain differences in tissue colonization have been observed in studies using strains of other Lyme borreliae genospecies (69–72). A possible contributing factor in the variation in tissue colonization efficiency could be the presence or absence of spirochete adhesins, including variability in the said adhesins. In fact, some PFam54 proteins, including *bga71* from *B. bavariensis*, were shown to confer spirochete attachment to mammalian cells (37, 38, 53). Additionally, some spirochete polymorphic adhesins, e.g., outer surface protein C (OspC) and decorin binding protein A (DbpA), promote differential levels of tissue colonization among *Borrelia* isolates and genospecies (35, 62, 73), although PBN, PNi, and PBi do share the same *ospC* (22) and *dbpA* sequences (our unpublished data), suggesting that these are most likely not causing the observed phenotype. Therefore, our results suggest that the PFam54 proteins may play a role in the efficiency in which spirochetes colonize specific host tissues, but this will require

future research to be done. It is important to note that our study design only measured spirochete burden using a quantitative PCR (qPCR) methodology, so we are not able to comment on the viability of spirochetes at these tissues. Even so, the method allows us to determine where spirochetes disseminate and the relative burden of spirochetes present, which further allows us to answer questions of tissue- or strain-specific infection dynamics.

The PFam54 gene array is found across the *B. burgdorferi sensu lato* species complex and therefore has been maintained throughout the evolutionary history of these spirochetes (40, 94). As genospecies besides *B. burgdorferi sensu stricto*, including *B. bavariensis*, have been challenging to genetically modify (75), these isolates with almost identical genetic make-ups to type strain PBi (>95% identity over all genomic compartments) (22) and naturally lacking the PFam54 array offer a unique opportunity to study the role of these proteins in infection and tick-to-host transmission in humans and reservoir hosts. The natural loss of the PFam54 gene array in *B. bavariensis* is associated with an increase of susceptibility to human immune serum with a corresponding increase in complement deposition as hypothesized. Even so, isolates lacking the PFam54-gene array remained infectious to mice at multiple tissues with only slight variation in tissue-dependent persistence after intradermal inoculation. These findings highlight that PFam54-related proteins are probably not the sole proteins that can lead to an infectious phenotype and the novel idea that human versus host infectivity may occur from different mechanisms. As a majority of *Borrelia* proteins remain uncharacterized without orthologs outside other *Borrelia* genospecies (22, 60, 61, 76), such findings pave the road to utilize isolates such as PBN and PNi to further study novel infection genes as well as the function of each PFam54 protein and their contribution to the Lyme borreliae infectivity.

MATERIALS AND METHODS

Ethics statement. Collection of blood samples and consent documents were approved by the ethics committee at the University Hospital of Frankfurt (control numbers 160/10 and 222/14), Goethe University of Frankfurt am Main. All healthy blood donors provided written informed consent in accordance with the Declaration of Helsinki. The mouse experiments were performed in strict accordance with all provisions of the Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals, and the PHS Policy on Humane Care and Use of Laboratory Animals. The mouse infection protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the Wadsworth Center, New York State Department of Health (protocol docket number 19-451). All efforts were made to minimize animal suffering.

Bacterial cultivation and DNA extraction. *B. bavariensis* isolates PBN, PNi, and PBi (Table 1) were provided by the German National Reference Centre for *Borrelia* from the Bavarian Food and Health Safety Authority. Bacterial isolates were maintained according to standard procedures (77, 78) in either in-house-made modified Kelly-Pettenkofer (MKP) medium (77), in-house-made BSK-H medium (78), or a commercially available BSK-H medium (Bio&Sell, Germany) supplemented with 6% rabbit serum (Sigma-Aldrich, Germany). Once cultures reached a density of 1×10^8 cells per mL, whole genomic DNA was extracted using a Maxwell 16 LED DNA kit (Promega, Germany). DNA quality (260/280) and concentration were measured using a NanoDrop 1000 photometer (Thermo Fisher Scientific, USA) and a Qubit 3.0 fluorometer (Thermo Fisher Scientific), respectively.

Next-generation sequencing and genome assembly. All isolates were sequenced using both long- and short-read sequencing methods. Short-read libraries were produced using the Nextera XT DNA library preparation kit or the Illumina (San Diego, CA, USA) DNA prep kit according to the manufacturer's protocol. Library quality was evaluated using a fragment analyzer (Agilent, Germany) or an Agilent TapeStation 2200. Sequencing was performed using a MiSeq reagent V2 kit on an Illumina MiSeq platform according to standard protocol that produced paired-end reads of 250 bp. Illumina reads were first trimmed for Illumina MiSeq adapter sequences using Trimmomatic v.0.38 (79) before being assembled using SPAdes v.3.14.1 (80), which has been shown to be the best option for *de novo* assemblies of *Borrelia* genomes (22, 47). SPAdes contigs were then mapped to references using NUCmer v.3.23 from the package MUMmer (81, 82) as described in Becker et al. (22).

Long-read sequence data of PBN and PNi were generated using Pacific Biosciences single-molecule, real-time (SMRT) technology by the Norwegian Sequencing Center (www.sequencing.uio.no). Libraries were prepared from genomic DNA sheared to 12 kb using Pacific Biosciences' protocol for SMRTbell libraries and PacBio barcoded adapters for multiplex SMRT sequencing. Libraries were size selected using 0.45 AMPure paramagnetic beads (PBs). The library was sequenced on a Pacific Biosciences Sequel instrument using Sequel Polymerase v.3.0, SMRT cells v.3 LR, and sequencing chemistry v.3.0 (movie time, 15 h). Reads were demultiplexed using the demultiplex barcodes pipeline on SMRT Link v.8.0.0.80529 (SMRT Tools v.8.0.0.80502). A minimum barcode score of 40 was used. Reads were assembled using the microbial

TABLE 2 Overview of all primers used in this study

Gene	Reference	Primer	Sequence (5′ to 3′)	No. of amplicons (bp)	Citation
BGA63	PBi	BGA63_PBi_F1	AACTGGGCTAATTTTGCTTTC	1,161	This study
		BGA63_PBi_R1	TTACTGAATTGGGGCAAGAA		
BGA64	PBi	BGA64_PBi_F3	TAACATTGGGGATAATAACATTT	686	This study
		BGA64_PBi_R3	ATCGTATTGCAGCTCTAAGG		
BGA65	PBi	BGA65_PBi_F1	GCTCAACAGATGATCAAGCAAAGA	1,481	This study
		BGA65_PBi_R1	AAGCTGTGATTTTGATTCTCCTG		
BGA66	PBi	BGA66_F_PK	CGTTGCACTTGATATTTTTTAAGAAGAGAAGC	1,395	36
		BGA66_R_PK	GGCTATGCACTTTAAAGGTATTAATGATTTAATTTTCAAGATG		
BGA67	PBi	BGA67_PBi_F1	CATCTCATGTGCCGTTAATAAAATT	1,001	This study
		BGA67_PBi_R1	AGTTGTTCTGCACTGGTTTTAATGT		
BGA68	PBi	BGA68_F_PK	CATTAAAACCTATGTGTACGAACTAGCAGCATATGGAG	1,369	36
		BGA68_R_PK	GTTAATGTCGTTGTAAGAATATTAAGCTTAATT		
BGA71	PBi	BGA71_F_PK	GGCAGATATATGAAGTTTGTAAAGAACTTGGTACTTTTAAGCCTATTG	1,406	36
		BGA71_R_PK	GTTTAGTTTTTGGCAAAATCCTTTCCTTATAACTAATATTTTAACC		
BGA72	PBi	BGA72_F_PK	CTAAAAGCCTGTTGTGATCACATACACTACTAATTGG	1,298	36
		BGA72_R_PK	CTTTAATTATATTTAGTTTAGTTTTTGTCAAAATACTCTCC		
BGA73	PBi	BGA73_F_PK	GTTCTACAACATTGGATTGAGATTGTAGAACGCTTAC	1,779	36
		BGA73_R_PK	GTATCAATTTGATCAACAAGTGAGCATGAGATGCAC		
recG		recF890	CCCTTGTTGCCTTGCTTTC	741	74
		recR1694	GAAAGTCCAAAACGCTCAG		
16S rRNA		16SrRNAfp	GCTTCGCTTGTAGATGAGTCTGC		62, 97
		16SrRNArp	TTCCAGTGTGACCGTTCACC		

assembly application on SMRT Link (v.8.0.0.80529, SMRT Link Analysis Services and GUI v.8.0.0.80501) and additionally utilizing hybridSPAdes (83), which utilized both long- and short-read sequence data. The final plasmids arising from long-read sequencing were constructed by comparing the microbial and hybridSPades assemblies and concatenating sequences if needed. Long-read sequence data of PBi are available at NCBI (accession number CP028873.1) (54). In short, the library was generated using the Pacific Biosciences 20-kb library preparation protocol. Size selection of the final library was performed using BluePippin with a 10-kb cutoff. The library was sequenced on a Pacific Biosciences RS II instrument using P6-C4 chemistry with a 360-min movie time. PacBio reads were assembled using HGAP v.3 (Pacific Biosciences, SMRT Analysis software v.2.3.0). Overlapping contig regions due to the circularization during the PacBio library preparation were removed. To polish indels and sequencing errors that may be present in the PacBio contigs, the Illumina short reads were mapped to the PacBio contigs, and a consensus sequence was extracted using the CLC Genomic Workbench v.11.

Characterizing the PFam54 gene array in PBN, PNi, and PBi. Sequences for all PFam54 paralogs described for PBi in Wywial et al. (40) (*bga63* to *bga73*) were downloaded from GenBank (accession numbers CP000015.1) and used as queries. We used BLAST v.2.8.1 (84, 85) (algorithm, blastn) to search for the PFam54 paralogs described above in all assembled contigs. BLAST hits shorter than 500 bp and with a percentage identity lower than 80% compared to PBi were not considered paralogous to their reference. Further BLAST hits were removed if they were overlapping with regions previously assigned to a result of higher quality. The presence and absence of PFam54 orthologs were further checked through paralog-specific PCRs with primers designed for the paralogs present in PBi. All PCR products were cleaned using a DNA Clean and Concentrator-5 kit (Zymo Research, USA) and sequenced at the Sequencing Service of Ludwig-Maximilians University according to the standard protocol for Sanger sequencing (http://www.gi.bio.lmu.de/sequencing/help/protocol). All primers are listed in Table 2. For further detail on PCR analyses see Supplemental File 1. Gene orthology was confirmed through phylogenetic reconstruction performed in MrBayes (86, 87) based on all GenBank references and extracted PFam54 paralog sequences from PBi, PBN, and PNi (see Supplemental File 1).

Human serum, proteins, and antibodies. Nonimmune human serum (NHS) obtained from healthy volunteers was initially tested for anti-*Borrelia* IgM and IgG antibodies as previously described (88) and for complement activity employing the Wieslab functional complement assays (SVAR, Malmö, Sweden). Only serum samples considered to be negative for anti-*Borrelia* antibodies and displaying complement activity were used to form the serum pool. Polyclonal anti-C3 antisera were obtained from Merck Biosciences (Bad Soden, Germany), and the neoepitope-specific monoclonal anti-C5b-9 antibody was purchased from Quidel (San Diego, CA, USA). Generation and purification of His-tagged proteins have been previously described (88). Alexa Fluor 488-conjugated anti-goat IgG and Alexa Fluor 488-conjugated anti-mouse IgG were purchased from Thermo Fisher (Langenselbold, Germany).

Serum susceptibility assays. To test serum susceptibility of PBN and PNi to NHS, 1×10^7 highly viable *Borrelia* cells suspended in 50 μL BSK-H medium (Bio&Sell, Germany) were incubated with 50 μL NHS at 33°C as described previously (89). *B. bavariensis* type strain PBi was included as a serum-resistant control, and *B. garinii* strain G1, as a serum-sensitive control (36, 90). The number of motile cells was counted at different time points (0, 1, 2, 4, and 6 h) using dark-field microscopy. Prior to counting, cultures were diluted 1:100 with phosphate-buffered saline (PBS) (negative controls, 10 to 20 cells/field). To facilitate counting,

each slide was separated into nine quadrants which were further subdivided into nine boxes. One box per quadrant was randomly chosen so that, in total, nine microscopy fields were counted for each time point per strain. *Borrelia* clumps were not considered, and if they were present, a new field was chosen to facilitate counting. Each assay was conducted at least three times. Determination of the spirochete motility by dark-field microscopy has been shown to be a viable method to assess cell viability after serum treatment (39, 89, 91, 92), which correlates with other methods, such as dead/live staining of *Borrelia* cells (93).

BGA66 and BGA71 are known to facilitate serum resistance of *B. bavariensis* PBi (36), and to further explore if they could rescue serum-sensitive *Borrelia* isolates from complement-mediated killing, a modified serum bactericidal assay was conducted. For this, 50 μL NHS was preincubated with either 10 μM purified BGA66, 10 μM purified BGA71, 10 μM bovine serum albumin (BSA), or a combination of BGA66 and BGA71 (5 μM each) for 15 min at 37°C. As an additional control, the PFam54 paralog ZQA68 from *B. garinii* ZQ1, known to lack anticomplement activity, was employed (39, 94). Thereafter, 1×10^7 *Borrelia* cells suspended in 50 μL BSK-H medium were added to 50 μL preincubated NHS and further incubated at 33°C. The number of motile cells was counted at different time points (0, 1, 2, 4, and 6 h) using dark-field microscopy. As further controls, reaction mixtures containing heat-inactivated NHS (hiNHS), NHS alone, and wash buffer (50 mM Tris/HCl, pH 8.0) were also included. Each assay was conducted in triplicate.

Genetic characterization of serum survivors. To further characterize PBN and PNi cells which survived treatment with 50% NHS, spirochetes (labeled as PBN-ST or PNi-ST for post-serum treatment) were recultured in BSK-H medium (Bio&Sell, Germany) until they reached a density of 1×10^8 cells per mL (77, 78). PBN-ST or PNi-ST were then retreated with 50% NHS to determine the serum-susceptible phenotype. Additionally, whole genomic DNA was extracted using a Maxwell 16 LED DNA kit (Promega, Germany). *Borrelia* DNA was then sequenced on a MiSeq platform using the protocol described above for short-read library preparation and assembly. We used BLAST v.2.8.1 (84, 85) (algorithm, blastn) to again search for the PFam54 paralogs described above and paralog-specific PCRs to support the presence or absence of PFam54 paralogs in PBN-ST and PNi-ST (see Supplemental File 1). All assembled contigs of PBN-ST or PNi-ST were then mapped back to the original PBN or PNi assembly (22) using NUCmer v.3.23 from the package MUMmer (81, 82). Single nucleotide polymorphisms (SNPs) and indels between the two assemblies were then called using the program show-snps from the package MUMmer (81, 82). The locations of SNPs were compared to a previously published annotation for PBN and PNi (22) to determine if they were located in proposed open reading frames. Gene annotations without proposed function (i.e., hypothetical proteins) were subjected to a protein structure similarity search using the online-based HHpred server (48, 49).

Immunofluorescence microscopy. To determine the deposition of activated complement components on the *Borrelia* surface, an immunofluorescence assay was conducted as previously described (95, 96). For this, 6×10^6 cells were suspended in 150 μL GVB^{++} (gelatin veronal buffer containing Ca^{2+}/Mg^{2+}), and either 50 μL NHS or 50 μL hiNHS was added. Following incubation for 1 h at 37°C, 10 μL of the suspension was transferred to a glass slide, air-dried overnight, and then fixed using a glyoxal solution (Merck, Germany). Slides were then incubated for 1 h at 37°C with either an anti-C3 (1:1,000) or a neoepitope-specific anti-C5b-9 antibody (1:70) to detect C3 and the assembled MAC, respectively. After washing with PBS, Alexa Fluor 488 conjugated antibodies (1:1,000) (Life Technologies, Eugene, OR, USA) were applied, and the slides were incubated for 1 h at 37°C in the dark. After washing, the slides were overlaid with a DAPI (4′,6-diamidino-2-phenylindole) solution (1:500) and incubated for 10 min at 4°C. Finally, the glass slides were enclosed with a coverslip using fluorescence mounting medium (Agilent Technologies Denmark ApS, Glostrup, Denmark). The evaluation was carried out with an Axio Imager M2 fluorescence microscope (Zeiss, Germany) equipped with a Spot RT3 camera (Visitron Systems, Germany).

Intradermal infection of *Mus musculus* mice and the quantification of bacterial burdens in tissues. First, 4-week-old female BALB/c mice ($n = 10$) were intradermally inoculated with 1×10^5 bacteria of *B. bavariensis* strains PBi, PBN, or PNi as described previously (39). Control mice were inoculated in parallel with BSK-H medium unmodified with serum. As a plasmid profiling procedure for these isolates is not currently available, the isolates were cultured at less than 10 passages to avoid decreased infectivity due to potential plasmid loss events during *in vitro* cultivation. At 21 days postinfection, mice were sacrificed and harvested for organs and tissues including the inoculation site of the skin, ankle joints, heart, bladder, ear tissue, and knee joints. These were collected and processed for quantitative assessment of bacterial burdens during infection. DNA was extracted from tissues using the EZ-10 genomic DNA kit (Bio Basic, Canada), and the quantity and quality of DNA were assessed using a NanoDrop 1000 UV/Vis spectrophotometer (Thermo Fisher Scientific, USA). The 280:260 ratio of these samples was between 1.75 and 1.85, indicating no contamination of RNA or proteins in those DNA samples. Quantitative PCR was performed as described previously (39). In brief, spirochete genomic equivalents were calculated using an ABI 7500 real-time PCR system (Thermo Fisher Scientific) in conjunction with PowerUp SYBR green master mix (Thermo Fisher Scientific), based on amplification of the Lyme borreliae 16S rRNA gene using primers 16SrRNAfp and 16SrRNArp (Table 2) as described previously (62, 97). Cycling parameters for SYBR green-based reactions were 50°C for 2 min, 95°C for 10 min, and 45 cycles of 95°C for 15 s, 52°C for 30 s, and 60°C for 1 min. The number of 16S rRNA copies was calculated by establishing a quantification cycle (C_q) standard curve of a known number of 16S rRNA genes extracted from each *B. bavariensis* strain PBi, PBN, or PNi and then comparing the C_q values of the experimental samples for each strain.

Statistical analysis. Differences in serum sensitivity, including trials utilizing preincubated NHS, were tested using pairwise *t* tests with a Bonferroni multiple testing correction in R v.3.6.1 (98), while for the mouse experiments, differences were tested using the Kruskal-Wallis test with the two-stage step-up

method of Benjamini, Krieger, and Yekutieli. For all tests, a P value of <0.05 (*) was considered significant.

Data availability. The sequence data for PBN and PNi can be found in GenBank under BioProject number PRJNA327303.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.6 MB.

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R.E.R., N.S.B., J.W., S.H., and P.K. conceptualized the project, which was then coordinated by P.K. and N.S.B. G.M. and V.F. provided the isolates, which were cultivated by R.E.R., F.R., and T.A.N. S.H., G.M., and V.F. organized all whole-genome sequencing of samples, which was then processed by N.S.B., R.E.R., and S.H. J.W. and R.E.R. characterized the PFam54 gene family in all samples, including developing the PCRs for confirming the absence or presence of the genes. F.R. performed the serum bactericidal assays and immunofluorescence analyses under the guidance of P.K. T.A.N. performed all mouse infection studies under the guidance of Y.-P.L. Throughout the study, expert opinions and advice were provided by P.K., Y.-P.L., N.S.B., G.M., and V.F. R.E.R. wrote the manuscript with the help of N.S.B., J.W., P.K., and Y.-P.L., and it was then approved by all coauthors.

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