Genomic and phenotypic characterization of Borrelia afzelii BO23 and Borrelia garinii CIP 103362

Sébastien Bontemps-Gallo, Kevin A. Lawrence, Crystal L. Richards, Frank C. Gherardini*

Laboratory of Bacteriology, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, Montana, United States of America

* frank.gherardini@nih.gov

Abstract

In recent years, the number of Lyme disease or borreliosis cases in Eurasia has been dramatically increasing. This tick-borne disease is caused by Borrelia burgdorferi sensu lato, which includes B. burgdorferi sensu stricto, the main species found in North America, and B. afzelii and B. garinii, which are primarily responsible for the disease in Eurasia. Currently, research on Lyme disease has focused mainly on B. burgdorferi while B. afzelii and B. garinii, which cause disease with distinctly different symptoms, are less studied. The purpose of this study is to evaluate B. afzelii BO23 and B. garinii CIP 103362 as model organisms to study Eurasian Lyme disease. To begin our analyses, we sequenced, annotated the chromosomes of both species and compared them to B. burgdorferi strain B31. We also assayed shuttle vector, pBSV2, for transformation efficacy and demonstrated that these strains can be cultured on solid media. In addition, we characterized how physicochemical parameters (e.g., oxygen, osmolarity, oxidative stress) affect both growth and motility of the bacteria. Finally, we describe each strain’s antibiotic susceptibility and accessed their ability to infect mice. In conclusion, B. afzelii BO23 was more practical for in vitro and in vivo studies than B. garinii CIP 103362.

Introduction

Lyme disease or borreliosis, caused by Borrelia (Borrelia) burgdorferi sensu lato (s.l.) [1], is transmitted to mammalian hosts through a bite of hard Ixodes ticks [2–5]. Lyme disease is the most common tick-borne disease with 300,000 estimated new cases in the United States of America [6, 7] and 85,000 detected new cases in Europe per year [8]. Climate change has expanded the geographic distribution of the vector leading to an increase of the number of cases [8, 9].

Thirty-five years ago, Dr. W. Burgdorfer and colleagues at the Rocky Mountain Laboratories (USA) identified the spirochete B. burgdorferi as the etiologic agent of Lyme disease [5] and were able to grow Borrelia spp. in a complex, chemical undefined rich medium (Barbour-Stoenner-Kelly, BSK) [10, 11]. Ten years later, Dr. G. Baranton and colleagues from the
Institute Pasteur (France) demonstrated the genetic diversity in the Lyme disease-Borrelia related species [12]. Over the last 20 years, scientists and physicians have characterized distinct species belonging to the Lyme disease Borrelia and described related symptoms in more detail. In North America, only two species (B. burgdorferi sensu stricto (s.s.) and B. mayonii) have been described to cause disease in humans [3, 13]. In Eurasia, two species (B. garinii and B. afzelii) are the main cause of Lyme disease in humans [14, 15] while other species (B. burgdorferi sensu stricto, B. spielmanii, B. bisetii, B. lusitaniae and B. valaisiana) are occasionally found in patients suffering from borreliosis in Eurasia [2].

Symptoms can vary significantly from one species to another [16]. After transmission by infected ticks, an erythema migrans appears in 70–80% of the people infected by B. burgdorferi s.s. In contrast, only 20–30% of the people infected by B. afzelii or B. garinii develop this characteristic rash. All patients will develop flu-like symptoms, regardless of the causative species. During the early stage of disease, bacteria primarily localize at the bite site. As the disease progresses, bacteria disseminate to the skin and other tissues, including heart and central nervous system. About 15% of untreated people develop acute Lyme disease or a neuroborreliosis. Finally, in the late disseminated infection, bacterial spread throughout the whole body can lead to an Acrodermatitis Chronica Atrophins (ACA, B. afzelii infection), treatment resistant arthritis (B. burgdorferi s.s. infection), muscle weakness, cardiomypathy or gastritis [2, 15, 16].

The genomes of Borrelia species are intriguing as they consist of a linear chromosome (about 910 kb) as well as of a set of circular and linear plasmids (10 circulars and 12 linears in B. burgdorferi B31 strain) [17, 18]. The number of plasmids varies depending on the species and seems to be affected by frequent reorganization [18–22]. The linear chromosome harbors the majority of essential genes involved in metabolism or regulation. Only a small set of genes encoding proteins that are required for growth localize to plasmids [18, 23]. Furthermore, surface proteins involved in virulence are mainly expressed from plasmids [4, 16, 24, 25]. Genetic tools have been developed to study gene function and regulation, making Borrelia species suitable for genetic studies to better understand biochemistry, physiology and virulence [26].

The increasing number of cases of Lyme disease in Eurasia has driven the scientific community to develop appropriate models to study and understand this disease. In this study, we investigated if B. afzelii strain BO23 and B. garinii strain CIP 103362 (both available from the ATCC strain collection) can be used as model organisms to study Eurasian Lyme disease. B. afzelii BO23 was isolated from human skin in Germany [27] while B. garinii CIP 103362 was isolated from an Ixodes ricinus tick in France [12]. Since genome sequence information is indispensable today, we first sequenced and annotated the genome of both strains. We also demonstrated that B. afzelii was transformable with the shuttle vector, pBSV2. We further studied the effects of physicochemical parameters encountered during the infectious cycle (oxygen, osmolarity, oxidative stress) on the growth as well as on the motility of these strains. Finally, we assayed the antibiotic susceptibility and infectivity of these two strains.

**Results**

**Comparisons of the genome of the three strains**

In modern biology, a strain has to satisfy several prerequisites to be considered a model organism. Among them, the availability of the genome sequence to study gene function and regulation is one of the most important. To establish if B. afzelii strain BO23 and B. garinii strain CIP 103362 can be used as model organisms for Eurasia Lyme disease, we first sequenced and annotated the genome of the two strains (Table 1).
The final assembled genome of *B. afzelii* consists of 32 contigs, including 1 linear chromosome (905,394 bp) and 31 contigs with lengths ranging from 1,117 to 85,180 bp. The final assembled genome of *B. garinii* consists of 12 contigs, including 1 linear chromosome (905,638 bp) and 11 contigs with lengths ranging from 1,085 to 57,532 bp.

Chromosomes of all strains were about the same size with an average 28.4% GC content (Table 1). Interestingly, less protein-encoding genes (797) are predicted for the chromosome of *B. burgdorferi* compared to *B. afzelii* (812) and *B. garinii* (808). We decided to look more closely at this difference (S1 Table). The three chromosomes shared 783 common protein-encoding genes. Eighteen genes were specific to *B. afzelii* and *B. garinii*. Furthermore, each chromosome had unique genes: 6 in *B. burgdorferi*, 7 in *B. afzelii* and 3 in *B. garinii* (S1 Table).

Based on the annotation of the respective genes, none of these differences seems to provide new metabolic activities. Interestingly, *B. garinii* CIP 103362 has lost the gene encoding for PdeA, a cyclic di-GMP phosphodiesterase while other strains of *B. garinii* (BgVIR, NMJW1, SZ or PBr) harbor the gene. In *B. burgdorferi*, PdeA and PdeB are required to degrade c-di-GMP (cyclic diguanylate) into 2 GMP (guanosine monophosphate) [28]. The c-di-GMP signaling pathway is essential to complete the infectious cycle. In *B. burgdorferi*, disruption of pdeA negatively affects both motility and the ability to establish an infection in mice. [29].

### Table 1. Comparison of the genome of the three *Borrelia* strains.

|                   | *B. burgdorferi* B31 |                   |                   | *B. afzelii* BO23 |                   |                   | *B. garinii* CIP 103362 |
|-------------------|----------------------|-------------------|-------------------|-------------------|-------------------|-----------------------|-------------------------|
|                   | Accession # | Size (bp) | Gene | Protein | Accession # | Size (bp) | Gene | Protein | Accession # | Size (bp) | Gene | Protein |
| Chromosome        | AE000783      | 910,724   | 860  | 797     | CP018262     | 905,394   | 856  | 812     | CP018744     | 905,638   | 856  | 808     |
| Plasmids          |                       |                 |       |         |                       |                 |       |         |                       |                 |       |         |
| cp26              | AE000792      | 26,498    | 26   | 24      | CP018266     | 26,523    | 26   | 25      | CP018750     | 27,038    | 26   | 25      |
| cp32-1            | AE001575      | 30,750    | 42   | 41      |                       |                 |       |         |                       |                 |       |         |
| cp32-3            | AE001576      | 30,223    | 42   | 39      |                       |                 |       |         |                       |                 |       |         |
| cp32-4            | AE001577      | 30,299    | 42   | 40      |                       |                 |       |         |                       |                 |       |         |
| cp32-6            | AE001578      | 29,838    | 41   | 40      |                       |                 |       |         |                       |                 |       |         |
| cp32-7            | AE001579      | 30,800    | 44   | 44      |                       |                 |       |         |                       |                 |       |         |
| cp32-8            | AE001580      | 30,885    | 42   | 40      |                       |                 |       |         |                       |                 |       |         |
| cp32-9            | AE001581      | 30,651    | 42   | 32      |                       |                 |       |         |                       |                 |       |         |
| cp9               | AE000791      | 9,386     | 11   | 11      | CP018274      | 8,898     | 10   | 10      |                       |                 |       |         |
| lp38              | AE000787      | 38,829    | 36   | 28      |                       |                 |       |         |                       |                 |       |         |
| lp54              | AE000790      | 53,657    | 65   | 55      | CP018745      | 57,532    | 70   | 63      |                       |                 |       |         |
| lp54-lp38 fusion  |                       |                 |       |         | CP018263      | 85,180    | 97   | 85      |                       |                 |       |         |
| lp17              | AE000793      | 16,821    | 19   | 14      | CP018269      | 22,953    | 27   | 22      | CP018751      | 20,915    | 20   | 19      |
| lp21              | AE001582      | 18,777    | 11   | 10      |                       |                 |       |         |                       |                 |       |         |
| lp25              | AE000785      | 24,177    | 16   | 9       |                       |                 |       |         |                       |                 |       |         |
| lp28-1            | AE000794      | 28,155    | 33   | 16      |                       |                 |       |         |                       |                 |       |         |
| lp28-2            | AE000786      | 29,766    | 33   | 29      |                       |                 |       |         |                       |                 |       |         |
| lp28-3            | AE000784      | 28,601    | 28   | 19      | CP018265      | 27,055    | 27   | 19      |                       |                 |       |         |
| lp28-4            | AE000789      | 27,323    | 25   | 19      | CP018268      | 24,507    | 21   | 17      |                       |                 |       |         |
| lp28-7            |                       |                 |       |         | CP018267      | 25,591    | 30   | 26      | CP018749      | 27,075    | 31   | 28      |
| lp28-8            |                       |                 |       |         | CP018264      | 28,972    | 29   | 25      |                       |                 |       |         |
| lp36              | AE000788      | 36,849    | 37   | 28      | CP018746      | 37,053    | 33   | 21      |                       |                 |       |         |
| lp5               | AE001583      | 27,055    | 6    | 6       |                       |                 |       |         |                       |                 |       |         |
| lp56              | AE001584      | 52,971    | 67   | 50      |                       |                 |       |         |                       |                 |       |         |
| Unassigned        |                       |                 |       |         |                       | 104,625    | 142  | 124     | 80,859       | 115       | 82   |         |
| total             |                       |                 |       |         |                       | 1,543,035  | 1,568 | 1,391   | 1,259,698    | 1,265      | 1,165 | 1,156,110 |

https://doi.org/10.1371/journal.pone.0199641.t001
Recently, Casjens et al. named plasmids in the two strains based on the sequence of their paralogous protein family 32 (PFam32) ParA partition proteins [30]. A total of 6 linear and 2 circular plasmids were assigned for B. afzelii BO23, while only 4 linear and 1 circular plasmids in B. garinii CIP 103362 (Table 1). Interestingly, B. afzelii had a larger plasmid of 85,180bp, which seems to be a fusion of lp54-lp38. Fusion of linear plasmids has previously been detected in Borrelia genomes [20, 31]. More investigation will be required to determine the exact number of plasmids as well as to confirm the fusion of the linear plasmids. Besides facilitating genetic studies on these two strains, this study provides new genomes for phylogenetic studies on B. burgdorferi s.l. strains. Indeed, fewer strains have been sequenced and annotated for B. afzelii (HLJ01 [32], Pko [19], Tom3107 [33], K78 [34] and ACA-1 [19]) or B. garinii (BgVir [35], NMJW1 [36], SZ, PBr [35] and Far04 [19]) than for B. burgdorferi s.s. (18 strains).

**B. afzelii can be transformed with the pBSV2 shuttle vector**

To assay the transformability of each strain, we attempted to transform each with the shuttle vector pBSV2 [37]. Transformation experiments were performed multiple times on each strain. B. afzelii and B. burgdorferi B31 A3 were equally transformable while numerous efforts to transform B. garinii with this vector were unsuccessful. The absence of transformants could be the result of more stringent restriction-modification (RM) systems in B. garinii [38]. It is important to remember that initial attempts to transform B. burgdorferi during the development of genetic systems for this bacterium were difficult until more genetically malleable strains were isolated.

In B. burgdorferi, disruption of the RM system by inactivation of bbe02, a type I restriction endonuclease subunit R, and bbq67, an adenine specific DNA methyltransferase, increases transformation efficiency with pBSV2 [38, 39]. An additional type I restriction endonuclease subunit R (bbh09) is annotated in B. burgdorferi but its role is unclear. Genomes of B. afzelii BO23 and B. garinii CIP 103362 also harbor a type I restriction endonuclease subunit R: BLA33_04690 in B. garinii and BLA32_04945 in B. afzelii are homologs of bbe02, and BLA32_05350 in B. afzelii. Four types of RM systems have been described in bacteria [40] and homologs of these systems have not yet been identified in *borrelia spp.*

The transformation frequency (TF) of pBSV2 was $4.31 \times 10^{-5} \pm 0.12 \times 10^{-5}$ for B. afzelii BO23 and $2.05 \times 10^{-4} \pm 0.85 \times 10^{-4}$ for B. burgdorferi B31 A3 bbe02 bgq67 (see Methods). This result was comparable to those observed by Rego et al., [38], although transformation efficiency (TE) was lower in our study for both strains ($0.07 \pm 0.01$ for B. afzelii and $1.04 \pm 0.2$ for B. burgdorferi) than previously reported [38]. These results may be explained by the high concentration of DNA used for the transformation (~70 μg per transformation) compared to ~20 μg used in the previous study. Strains survived at a similar rate to the transformation process (1.4% for B. burgdorferi B31, 1.1% for B. afzelii BO23 and 1.3% for B. garinii CIP 103362). This data suggests that B. afzelii BO23 may be more suitable for genetic study than B. garinii. More study will be required to optimize the transformation protocols for these isolates.

**B. afzelii and B. garinii grow at a similar rate to B. burgdorferi under anaerobic and microaerobic atmospheres**

For a phenotypic characterization of the two strains, we first looked at the effects of different oxygen levels on the growth rate. Oxygen levels vary between the host and the vector [41] and within different tissues of the host [42, 43]. Furthermore, the oxygen level is also an important factor for *in vitro* culture. When we monitored the growth rate in BSK-II medium under different oxygen atmospheres, we were unable to observe growth of B. afzelii or B. garinii under aerobic conditions (Fig 1). However, aerobic conditions were not lethal to these strains since...
plating these aerobically-incubated cells on solid media under 3–5% O\textsubscript{2}, 5% CO\textsubscript{2}/90%N\textsubscript{2} at 34˚C indicated a >95% survival rate. \textit{B. burgdorferi} and \textit{B. garinii} grew normally under both anaerobic and microaerobic conditions with an average doubling time of 12 h, regardless of the strain or the oxygen level (Fig 1). \textit{B. afzelii} grew normally under microaerobic conditions with a doubling time of 12 h. Interestingly, cell numbers started to decrease as cultures approached stationary phase (~1.1 10\textsuperscript{8} cell/ml). Under anaerobic conditions, \textit{B. afzelii} grew slower than under microaerobic conditions with a doubling time of 19 h. Our data show that both strains can grow under microaerobic and anaerobic conditions but not under aerobic conditions. Why these strains are sensitive to aerobic growth conditions is not known.

\textit{B. afzelii} has more robust motility than \textit{B. garinii} and \textit{B. burgdorferi}

Motility is one of the main virulence factors in bacteria \cite{44,45}. \textit{Borrelia} species have distinctive endoplasmic flagella that enable infectious strains to disseminate throughout the whole body while non-motile mutants are avirulent \cite{46}. To determine if there was a difference in the motility of the three \textit{Borrelia} species, we performed a swarm plate assay as described previously by Motaleb et al. \cite{47}. The strains were spotted in the swarm plate, grown for 4d and the swarm diameter was measured (Fig 2). \textit{B. burgdorferi} B31 (1.9 +/- 0.2 cm) and \textit{B. garinii} CIP 103362 (1.4 +/- 0.25 cm) displayed a similar motility (Fig 2). Interestingly, \textit{B. afzelii} BO23 (2.9 +/- 0.1 cm) appeared to be more motile than the other strains that were tested. It is not clear whether this difference may provide \textit{B. afzelii} BO23 any selective advantage for virulence or during transmission.

\textit{B. garinii} and \textit{B. afzelii} are more sensitive to gentamicin and rifaximin than \textit{B. burgdorferi}

Susceptibility to antibiotics is an important characteristic for both genetic manipulations and clinical treatment. We determined the susceptibility to different antibiotics by measuring the minimum inhibitory concentration (MIC) for the three strains. The MIC is defined as the lowest concentration of antibiotics at which the number of countable cells after 72h of incubation at 34˚C does not exceed the initial number of cells. To determine the MIC, 5 x 10\textsuperscript{6} cells/ml
were inoculated with increasing concentration of antibiotics. The final antibiotic concentration ranged from 0.015 to 32 μg/ml. We assayed 10 antibiotics targeting: cell wall synthesis (ampicillin, carbenicillin), RNA synthesis (rifaximin) and protein synthesis (chloramphenicol, doxycycline, gentamicin, kanamycin, spectinomycin, streptomycin, vancomycin) (Table 2). B. garinii was more sensitive to carbenicillin (0.015 μg/ml vs 0.03 μg/ml), doxycycline (0.03 μg/ml vs 0.06 μg/ml) and spectinomycin (0.12 μg/ml vs 0.25 μg/ml) than B. afzelii and B. burgdorferi. B. afzelii and B. garinii were more sensitive to gentamicin (0.06 μg/ml vs 0.12 μg/ml) or rifaximin (8 μg/ml vs 16 μg/ml) than B. burgdorferi. No difference in the MIC between the three strains was observed for ampicillin (0.03 μg/ml), chloramphenicol (1 μg/ml), kanamycin (4 μg/ml), streptomycin (4 μg/ml) and vancomycin (0.25 μg/ml). Taken together, our data showed

![Motility of the three strains](https://doi.org/10.1371/journal.pone.0199641.g002)

Table 2. Antibiotic susceptibility of B. burgdorferi, B. afzelii and B. garinii.

| MIC (μg/ml) | B. burgdorferi | B. afzelii | B. garinii |
|-------------|----------------|------------|------------|
|              | B31-A3         | BO23       | CIP 103362 |
| Cell wall antibiotics | | | |
| Ampicillin  | 0.03           | 0.03       | 0.03       |
| Carbenicillin  | 0.03           | 0.03       | 0.015      |
| Protein synthesis antibiotics | | | |
| Chloramphenicol  | 1              | 1          | 1          |
| Doxycycline  | 0.06           | 0.06       | 0.03       |
| Gentamicin  | 0.12           | 0.06       | 0.06       |
| Kanamycin  | 4              | 4          | 4          |
| Spectinomycin  | 0.25           | 0.25       | 0.12       |
| Streptomycin  | 4              | 4          | 4          |
| Vancomycin  | 0.25           | 0.25       | 0.25       |
| RNA synthesis antibiotics | | | |
| Rifaximin       | 16             | 8          | 8          |

Highlighted in red: the difference in antibiotic susceptibility compared to B. burgdorferi.

[https://doi.org/10.1371/journal.pone.0199641.t002](https://doi.org/10.1371/journal.pone.0199641.t002)
minor differences in the antibiotic susceptibility between the three strains with no relevant bearing on genetic engineering studies or on potential clinical treatment.

**B. garinii is more sensitive to oxidative stress than B. afzelii and B. burgdorferi**

During tick feeding, the *Borrelia* are challenged by reactive oxygen species (ROS) during the digestion of the blood meal [48]. Boylan *et al.* demonstrate that *B. burgdorferi* membrane lipids are the main targets of ROS [49]. To assay the susceptibility of these strains to ROS, we analyzed the survival of each strain at 1, 2.5, 5, 10 and 25 mM tert-butyl hydroperoxide (Fig 3). *B. burgdorferi* was sensitive to tert-butyl hydroperoxide to a similar level as observed previously [49]. While *B. afzelii* had similar resistance to ROS as *B. burgdorferi*, *B. garinii* was more sensitive. This higher susceptibility to ROS might explain why *B. garinii* was inhibited under aerobic growth conditions. Under those conditions, more ROS would be produced than would be predicted to be generated in microaerobic or anaerobic growth media. Additionally, sensitivity to ROS could affect survival in the host in response to a *Borrelia*-induced oxidative burst [50–54].

**B. afzelii has a narrower range of osmotolerance than B. garinii and B. burgdorferi**

Osmolarity fluctuates in the midgut of ticks during the feeding of *I. scapularis* [41, 55, 56], *I. ricinus* [57–59] and *D. andersonii* [60–62]. According to the current model, osmolarity fluctuates in different hosts and in different vector tissues before, during and after feeding. It varies from 300 mOsm in the mammalian host [41, 63] to 600 mOsm in the midgut of unfed or replete ticks [41] and oscillates from 300 to 500 mOsm in the midgut, saliva or in the hemolymph during the feeding [41, 56]. To define the range of osmotolerance, we monitored the growth rate of *Borrelia* sp. in various osmolarities (150 to 850 mOsm) by plating on BSK-II at 24h intervals (Fig 4). As shown previously [41], *B. burgdorferi* B31 grew normally between 250 and 550 mOsm. At 650 mOsm, the cultures did not reach $10^8$ cell/ml. At 750 mOsm,
bacteria stopped growing after only a few cycles of cell division. Cells lysed when cultures were grown at 150 or 850 mOsM. *B. garinii* CIP 103362 grew normally between 350 and 650 mOsM and only reached a density of $10^7$ cells/ml at 250 mOsM. Outside of that range (150, 750 and 850 mOsM) *B. garinii* cells appeared to lyse. Similar to *B. garinii*, *B. afzelii* BO23 grew normally at low osmolarity (350 and 450 mOsM), but only reached $2 \times 10^7$ cells/ml at 250 mOsM. Cells were unable to grow at 150 mOsM or over 650 mOsM and rapidly lysed. Taken together, these major differences in osmotolerance could impact the survival of bacteria in the tick and may reflect differences in the vectors more common to the range (Eurasia) of these pathogens.

![Fig 4. Rate of growth of *B. burgdorferi* B31-A3, *B. garinii* CIP 103362 and *B. afzelii* BO23 at various osmolarties.](https://doi.org/10.1371/journal.pone.0199641.g004)

(A) Growth curves of strains were performed in BSK-II at various osmolarities (150 to 850 mOsM) under microaerobic conditions. (B) Graph of final densities of strains at various osmolarities. Growth was monitored daily by plating on BSK-II. Asterisks indicate a significant difference compared to *B. burgdorferi* B31 using a one-way ANOVA where p < 0.05. Data represent means ± standard deviation.
**B. afzelii and B. burgdorferi** can colonize and persist in mice while **B. garinii** is unable to sustain a long-term infection

To determine if the strains were infectious in mice, naïve mice (3 per strain) were needle-inoculated with 10^6 CFU of *B. garinii* or *B. afzelii* grown to ~5 x 10^7 cells/ml under a microaerobic atmosphere at 34˚C. After six weeks, tissue samples (bladder, joint, skin, ear) were removed from each animal, cultured in BSK-II for 2 months and checked every 7 d by darkfield microscopy to determine the presence of bacteria (Table 3). After 2 wks, spirochetes were observed in cultures from tissues from *B. afzelii* and *B. burgdorferi* infected mice. *B. burgdorferi* were re-isolated from all cultured tissues while *B. afzelii* was re-isolated from skin, bladder, and ear tissues but never from joint tissue. *B. garinii* was not re-isolated from any tissues of infected mice. Interestingly, mice infected with all strains seroconverted after 6 wks (Fig 5) suggesting

![Immunoblot of B. burgdorferi B31-A3, B. garinii CIP 103362 and B. afzelii BO23 strains incubated with mouse sera. Borrelia strains were grown in BSK-II to mid-log phase and cell lysates were analyzed by immunoblotting. Cell lysates were incubated with serum obtained from mice either before (Pre-bleed, PB) or 6 weeks after needle inoculation (Post-infection, PI) with B. burgdorferi (lanes 1 to 3), B. garinii (lanes 4 to 6) and B. afzelii (lanes 7 to 9).](https://doi.org/10.1371/journal.pone.0199641.g005)
that B. garinii could produce an initial infection leading to an adaptive immune response but was unable to persist in the mouse. This finding led us to re-examine the genome of B. garinii with a focus on identifying genes which had been shown to affect virulence of B. burgdorferi in mice (e.g. sodA, bosR, pdeA, pdeB, etc.) [4, 24]. One notable difference was that the B. garinii CIP 103362 genome lacked the gene encoding a cyclic di-GMP phosphodiesterase (PdeA) which has been shown to affect virulence in B. burgdorferi [29]. Additionally, we have shown that this strain is more sensitive to ROS which also might affect its ability to infect experimental animals. Currently, we do not know the exact reason that this strain presented reduced virulence but, based upon these data, B. afzelii BO23 is more suitable for infectious studies than B. garinii CIP 103362.

Discussion

The increasing number of cases of Lyme disease in Eurasia, an area of 5 billion human beings, should motivate the scientific community to find an appropriate bacterial model to study this disease. Publications of the last 30 years demonstrate that B. afzelii and B. garinii are the two-main species responsible for Lyme disease in Eurasia [2, 16]. Differences in clinical features are observed between the different species: arthritis for American species and acrodermatitis chronica atrophicans for Eurasian species [2, 3, 16]. Additionally, climate change expands the geographic distribution of the vector by allowing ticks to colonize new areas [64–67].

In this study, we investigated if B. garinii CIP 103362 and B. afzelii BO23 could be used to study Eurasian Lyme disease via a murine model. Both strains are available and maintained by the ATCC. We first sequenced and partially annotated the genome of both strains in order to make them usable for genetic and phylogenetic studies. Genomes were deposited in the NCBI database to be publicly available. Although the strains showed some minor differences in the numbers of protein-encoding genes (Table 1, S1 Table), none of these genes seem to impart new metabolic capabilities or virulence capacities that would offer a selective advantage in the natural infectious cycle.

Knowing the appropriate conditions to grow Borrelia species is a prerequisite to any investigation of changing physicochemical factors or virulence analyses. Previously, Veinović and colleagues showed that B. afzelii and B. garinii have an optimal growth temperature of 33˚C [68]. Our initial studies of in vitro growth conditions of B. afzelii and B. garinii indicated that both species were unable to grow under aerobic conditions. Since, Lyme spirochetes do not metabolize oxygen, it seems likely that the sensitivity to high oxygen reflects a corresponding sensitivity to ROS. Indeed, this seemed to be the case for B. garinii which was more sensitive to ROS and aerobic growth conditions than B. afzelii or B. burgdorferi. It seems quite likely that these differences in aerotolerance and sensitivity to ROS, might reflect differences in the expression of genes encoding proteins which function to degrade ROS (e.g., SodA, CoADR, etc.) [4, 24]. Importantly, we demonstrated that both species prefer to grow under limited oxygen (microaerobic or anaerobic) and these growth conditions should facilitate in vitro studies of the spirochetes (Fig 1).

We further characterized the two strains by assessing their respective motilities. B. garinii CIP 103362 has the same capacity as B. burgdorferi B31-A3 (Table 4, Fig 2), while B. afzelii B023 was more motile. Initially, we believed that this feature would allow for a more efficient colonization of the host, compared to B. burgdorferi. Despite this observation, in our hands, we were unable to re-isolate these spirochetes from all the murine tissues tested (Fig 5, Table 3) which suggested that increased motility did not enhance virulence of B. afzelii. However, it is also possible that the infection by B. afzelii has already switched from a systemic infection to a localized infection after 6 wks [16]. Another explanation could be that Borrelia species are well
known to have some organotropism [2, 3, 13, 16]. B. afzelii or B. garinii have been observed to reside in the skin or the nervous system while B. burgdorferi s.s. also invades the joints [2, 3, 16]. The initial hypothesis by Hubálek and colleagues was that a difference in the optimal growth temperature is the reason for this organotropism [69]. Recently, a study by Veinović and colleagues didn’t support this hypothesis [68]. Instead, they suggest that organotropism could be explained by the ability to grow at different oxygen levels. Indeed, the dissolved oxygen concentrations fluctuate in mammalian tissues [42, 43]. However, our data doesn’t support this hypothesis since the three strains grew similarly under microaerobic conditions (Fig 1).

Since water flux and osmolarity fluctuate during the infectious cycle [41, 55–62], we also compared the osmotolerance of the three strains. All strains were able to grow at low osmolarity, i.e. blood-like osmolarity (Fig 4, Table 4). Interestingly, only B. burgdorferi and B. garinii were able to survive and grow at higher osmolarity, i.e. midgut osmolarity (Fig 4, Table 4). This result is of interest because Jacquet and colleagues showed recently that B. afzelii cell numbers dramatically decrease in I. ricinus [70] while B. burgdorferi numbers stayed constant between two feedings [71]. Sensitivity to osmolarity could explain, in part, this decrease in the number of B. afzelii in the midgut of the vector. As shown previously, the main effect of increased osmolarity is an increase in the pressure on the cell wall with corresponding inhibition of growth and/or cell death [72, 73]. However, Bourret and colleagues showed that specific mutations in genes encoding DNA repair enzymes in B. burgdorferi dramatically affect cell growth in the tick midgut but did not affect transmission [74]. This study demonstrated that robust growth and high spirochete loads are not an absolute requirement for successful transmission. Taken together, these data suggest that the transmission of B. afzelii, despite its sensitivity to “high” osmolarity, may not be incumbered by osmotic stress.

Finally, survival in the host is also dependent on the ability to survive and proliferate in the presence of the antagonistic host immune responses. Here, we showed that B. garinii CIP 103362 cannot be isolated from tissues of an infected mouse (Tables 3 and 4, Fig 5). The result

| Table 4. Summary of the results. |
|----------------------------------|
|                                  |
| **B. burgdorferi**               |
| **B. garinii**                   |
| **B. afzelii**                   |
|                                  |
| **B31-A3**                       |
| **CIP 103362**                   |
| **BO23**                         |
|                                  |
| **Oxygen level**                 |
| Anaerobic ++                     |
| Microaerobic ++                  |
| Aerobic +                        |
| Anaerobic ++                     |
| Microaerobic ++                  |
| Aerobic +                        |
| Anaerobic +                      |
| Microaerobic ++                  |
| Aerobic -                         |
|                                  |
| **Motility**                     |
| +                                |
| +                                |
| +                                |
|                                  |
| **Antibiotic**                   |
| More sensitive to: Carbenicillin, Doxycycline, Gentamicin, Spectinomycin, Rifaximin |
| More sensitive to: Gentamicin, Rifaximin |
|                                  |
| **ROS**                          |
| t-BH: 5/10mM                     |
| t-BH: 1/2.5 mM                   |
| t-BH: 5 mM                       |
|                                  |
| **Osmotolerance**                |
| 250–550 mOsM                     |
| 350–650 mOsM                     |
| 350–450 mOsM                     |
|                                  |
| **Mouse infectivity**            |
| +                                |
| -                                |
| +                                |
|                                  |
| **Transformation**               |
| +                                |
| -                                |
| +                                |

"A: Ability of B. burgdorferi to grow in aerobic conditions was reported previously [10].
B: Motility was defined based on B. burgdorferi: +: motile like B. burgdorferi, ++: more motile than B. burgdorferi.
C: Antibiotic susceptibility was defined based on B. burgdorferi susceptibility (see Table 2).
D: t-BH: tert-butyl hydroperoxide.
E: Osmotolerance was defined as ‘able to grow up to high density, i.e. 10^8 cells/ml’.
F: Strain was considered infectious if bacteria were re-isolated from mouse tissue (see Table 3).
G: Cell was (+) or was not (-) transformed with the pBSV2 shuttle vector.

https://doi.org/10.1371/journal.pone.0199641.t004
was surprising since Xu and Johnson reported that the strain was virulent in hamsters at a high infective dose (10^8 cells/animal) [27]. In our study, the strain was not able to establish a long-term infection but was able to trigger an adaptive immune response in mice (Fig 5) suggesting that the strain could possibly establish an acute infection but not persist in the mammalian host. The inability to colonize/persist in a mouse could also be the consequence of the loss of pdeA from the in B. garinii CIP 103362 genome. Indeed, a similar phenotype is observed in a B. burgdorferi B31-A3 pdeA mutant that could not be re-isolated from the tissue of an infected mouse but could trigger seroconversion when a high infective dose (10^7 cells/mouse) was used [29]. Additionally, Borrelia species are known to induce an oxidative burst during an infection [50–54]. Based upon our analyses, it seems plausible that the sensitivity to ROS demonstrated by B. garinii CIP 103362 might also affect survivability in infected animals (Fig 3). As previously mentioned, the B. garinii genome harbors the genes encoding oxidative stress related proteins identified in B. burgdorferi [75, 76]. Finally, it has been shown in multiple studies that plasmid loss can dramatically impact the infectivity of Lyme and Eurasian Lyme disease spirochetes [27, 77]. Using our genome and plasmid sequences, Casjens et al. cataloged some of the paralogous plasmid groups from B. garinii but not all of the plasmids have been accounted for. So we cannot eliminate plasmid loss as a factor in the phenotype of this strain. Currently, we do not know which of these factors are contributing to the reduced virulence of observed for B. garinii.

In summary, our data, summarized in Table 4, demonstrates that B. afzelii BO23 is currently the most useful model organism to study protein function, gene regulation and the pathogenesis of Eurasian Lyme disease spirochetes. Furthermore, phenotypic differences between B. afzelii and B. burgdorferi provide a first step in understanding the observed clinical and ecological dissimilarities between these different forms of Lyme disease.

Materials and methods

Bacterial strains, media and growth conditions

The bacterial strains used in this study are B. garinii (CIP 103362 / 20047 / ATCC 51383), B. afzelii (BO23 / ATCC 51992), B. burgdorferi (B31-A3 [78], but also available at ATCC 35210 as B31). Borrelia strains were grown in Barbour-Stoenner-Kelly II medium (BSK-II) (Table 5). Cultivation was performed at 34˚C under microaerobic condition (3% O2, 5% CO2), and when indicated, under anaerobic (5% CO2, 5%H2, balance N2) or aerobic (18% O2 in
Hamilton, MT, USA) conditions. Cell densities were determined by dark-field microscopy. The osmolarity of the BSK-II medium is 450 mOsM. To generate high-osmolarity media, NaCl was added. To obtain low-osmolarity medium, ddH2O was added to dilute 1.8-fold the BSK-II. Low-osmolarity BSK-II media support normal growth [41]. Osmolarity of growth media (mOsM) was measured with a vapor pressure osmometer (model 3320, Advanced Instruments, Inc., MA, USA). Growth rates were defined during the exponential phase and defined as 1/doubling time and expressed in 1/h.

**Genome sequencing, assembling and annotation**

The genomes of *B. afzelii* and *B. garinii* were sequenced at ACGT, Inc. (Wheeling, IL, USA) using Illumina MiSeq and the NextSeq 500 platform.

DNA was extracted by the Phenol/Chloroform method [79] from cells grown in BSK-II medium.

Sequencing was performed on the Illumina NGS platform with PE150 reads. Both paired-end and mate-pair libraries were used for the assembly. For *B. afzelii*, a total of 4,627,524 and 7,034,705 read pairs were generated from the paired-end (average 550 bp fragment size) and mate-pair libraries (average 600 bp fragment size), respectively. For *B. garinii*, a total of 20,718,626 and 4,487,254 read pairs were generated from the paired-end (average 550 bp fragment size) and mate-pair libraries (average 600 bp fragment size), respectively.

The final assembled genome of *B. afzelii* consists of 32 contigs, including 1 linear chromosome (905,394 bp) and 31 contigs with lengths ranging from 1,117 to 85,180 bp.

The final assembled genome of *B. garinii* consists of 12 contigs, including 1 linear chromosome (905,638 bp) and 11 contigs with lengths ranging from 1,085 to 57,532 bp.

Prediction of protein-coding sequences and annotation of the chromosome and plasmids were performed by the Prokaryotic Genome Annotation Pipeline v3.1 (http://www.ncbi.nlm.nih.gov/genome/annotation_prok/).

**Nucleotide sequence accession numbers**

The genome sequence of *B. garinii* strain CIP 103362 has been deposited in the GenBank database under accession numbers: chromosome (CP018744) and plasmids (cp26: CP018750, cp32 partial: CP018754 and CP018755, lp28-7: CP018749, lp54: CP018745, unassigned: CP018746 to CP018748 and CP018751 to CP018753).

The genome sequence for *B. afzelii* strain BO23 has been deposited in the GenBank database under accession numbers: chromosome (CP018262), and plasmids (cp26: CP018266, lp28-3: CP018265, lp54-lp38 fusion: /CP018263, unassigned: CP018264, CP018267 to CP018293).

**Transformation of pBSV2 in Borrelia spp.**

pBSV2 [37] was transformed into *B. burgdorferi* B31-68-LS [80], *B. afzelii* BO23 and *B. garinii* CIP 103362 as described previously by Samuels, D.S. [81].

To prepare competent cells, bacteria were grown in BSK-II to a density of 5 x 10^7 cells/mL. Cells were centrifuged at 9,600xg, 10 min at 20˚C and resuspended in 0.25 volumes of ice-cold EPS (93g/L of sucrose, 15% Glycerol). Cells were pelleted again at 7,000xg, 10 min, 10˚C and resuspended in 0.1 volume of ice-cold EPS. Cells were centrifuged at 7,700g, 10 min, 10˚C and resuspended in 0.0025 volume ice-cold EPS. Cells were transferred into a microtube. Cells were examined by darkfield microscopy for a suspension without clumping of bacteria.

To transform the cells, 70µg of purified vector DNA were mixed with 100 µL of freshly prepared competent cells. The mixture was transferred to an electroporation cuvette and cells
were transformed by electroporation using a Gene Pulser Xcell Electroporator (Bio-Rad, Hercules, CA, USA) using the following settings: 2.5 kV, 25 μF, and 200 Ω. Then, cells were resuspended in 5 mL of warm BSK-II. Prior to plating, cells were allowed to recover for 12h at 34˚C under microaerobic conditions. A no antibiotic control plate was performed to assess viability of the spirochetes after transformation.

To plate the cells, BSK-II containing 0.68% Ultrapure™ agarose (16500500, Thermo Scientific, Rockford, IL, USA) was warmed-up to 55˚C and 200 μg/ml kanamycin (1355006, Millipore Sigma, St. Louis, MO, USA) was added to the medium. As a bottom layer, 20 ml of medium was poured in the Petri dish. When the bottom plate was solidified, 30 ml of BSK-II with antibiotic were poured containing 1 ml of the transformation. Plates were then incubated at 34˚C under microaerobic conditions. Transformants appeared after 7 to 10 d of incubation.

Kanamycin-resistant colonies were analyzed by PCR to confirm the construction using Kan-F (CGAGGCCGCGATTAAATTCC) and Kan-R (AGCCGTTTCTGTAATGAAGA) as primers.

Transformation frequency and efficiency
Transformation frequency (TF) and transformation efficiency (TE) were calculated as described previously by Rego et al. [38].

Motility assay
The motility assay was performed as described before by Motaleb and colleagues [47]. Swarm plate assays were tested on BSK-II medium diluted 1:10 in PBS plates containing 0.35% agarose (16500–500, Thermo Scientific, Rockford, IL, USA). Cells were washed twice in HN buffer (50 mM HEPES pH 7.5, 50 mM NaCl) and resuspended in BSK-II. 5 x 10^5 cells in 5 μl were spotted onto plates containing BSK-II medium diluted 1:10 in PBS without divalent cations (21-040-CV, Corning, Corning, NY, USA). Plates were incubated 4 d at 34˚C under microaerobic conditions. Swarm diameters were measured in centimeters.

Statistical analysis
Prism 7 software (GraphPad Software, Inc., La Jolla, CA, USA) was used to analyze data using a One-Way ANOVA with a Geisser-Greenhouse correction; a value of p < 0.05 was considered significant.

Antibiotic susceptibility
Antibiotic susceptibility was performed as described previously by Sicklinger and colleagues [82]. Briefly, antibiotics (Table 6) were dissolved in warm BSK-II medium, pH 7.6, and supplemented with 6% Rabbit Serum. The final concentration of antibiotics ranged from 0.015 to 32 μg/ml. To determine the MIC, the initial cell density was adjusted to 5 x 10^6 cells/ml. After 72h of incubation at 34˚C, cells were counted again.

ROS susceptibility assays
Susceptibility to ROS was performed as described previously [49]. Strains were grown in BSK-II medium under microaerobic conditions at 34˚C starting at 1 x 10^5 cells/ml to early stationary phase of growth at 5 x 10^7 cells/ml. Cells were exposed to the indicated concentration of Luperox®, TBH70X, tert-Butyl hydroperoxide (458139, Millipore Sigma, St. Louis, MO, USA) for 1.5h in BSK-II. Samples were plated on BSK-II and incubated at 34˚C under microaerobic conditions for 7-14d to allow enumeration of CFUs.
Immunoblots
For analysis of cell lysates by Western blot, bacteria were grown to mid-log phase at 34˚C under microaerobic conditions. The cells were harvested by centrifugation, washed twice in HN buffer (50 mM HEPES pH7.5, 50 mM NaCl), resuspended in 0.25M Tris-HCl pH 6.8 and lysed by sonication. The protein concentration was determined with Take3 micro-volume plates in a Synergy 2 Multi-Mode plate reader (BioTek Instruments, Winooski, VT, USA). 40 μg of protein was loaded in a 4–15% pre-cast SDS-PAGE gel (Bio-Rad, Hercules, CA, USA) and transferred to a nitrocellulose membrane using a Trans-Blot TurboTM blotting system (Bio-Rad, Hercules, CA, USA) with a pre-programmed protocol (2.5A, up to 25V, 3 min). Immunoblotting was performed using standard protocols: membranes were blocked for 1hr in 5% nonfat milk in TBS-T (0.1% Tween 20), then incubated for 1h in TBS-T with infected mouse serum (1:200) by using a Mini-Protean II multiscreen apparatus (Bio-Rad, Hercules, CA, USA), washed in TBS-T and then incubated for 1hr in TBS-T with an anti-Mouse IgG (Fab specific)–peroxidase antibody produced in goat (1:2,000, A2304, Millipore Sigma, St. Louis, MO, USA). Blots were imaged by chemiluminescent detection using Super Signal Pico chemiluminescent substrate kit (34080, Thermo Scientific, Rockford, IL, USA) on a ChemiDoc MP system (Bio-Rad, Hercules CA, USA).

Mouse infection
Immune-competent RML mice are derived from a colony of Swiss-Webster mice established at NIH in 1935 and maintained at the Rocky Mountain Laboratories with an outbreeding program designed to intentionally maintain genetic diversity. RML mice (six to eight weeks old) were inoculated with 1 x 10^6 cells in BSK-II (500 μl intraperitoneal and 250 μl sub-cutaneous) with B. burgdorferi, B. afzelii and B. garinii strains in triplicate. Six weeks post-infection, the mice were sacrificed and tissues (ankle joint, bladder, skin, and ear) were cultured in BSK-II supplemented with 50 μg/ml rifampicin, 2.5 μg/ml amphotericin B and 20 μg/ml phosphomycin (FD179, HiMedia, West Chester, PA, USA). Blood was collected for serology prior to inoculation by retro-orbital bleeding using a microhematocrit capillary and post-infection bleedings were performed by cardiac puncture.

Ethics statement
Mouse infection studies were carried out in accordance with the Animal Welfare Act (AWA1990), the guidelines of the National Institutes of Health, Public Health Service Policy on Humane Care (PHS 2002) and Use of Laboratory Animals and the United States Institute
of Laboratory Animal Resources, National Research Council, Guide for the Care and Use of Laboratory Animals. All animal work was done according to protocols approved by the Rocky Mountain Laboratories, NIAID, NIH Animal Care and Use Committee (Protocol Number 2014–021). The Rocky Mountain Laboratories are accredited by the International Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

All infection studies were performed in an Animal Biosafety Level 2 facility according to protocols reviewed and approved by the RML Institutional Biosafety Committee and the RML IACUC. All work in this study adhered to the institution’s guidelines for animal husbandry, and followed the guidelines from the Guide for the care and use of laboratory animals. Animals were monitored at least once a day during the study. All efforts were made to minimize animal suffering. Mice were anesthetized by isoflurane inhalation prior to inoculation or blood withdrawal. Mice were euthanized by isoflurane inhalation followed by cervical dislocation. Mice showed no sign of illness and none of them died without euthanasia. A completed ARRIVE guidelines checklist is included in S1 Checklist.

Supporting information
S1 Checklist. Completed “The ARRIVE Guidelines Checklist” for reporting animal data in this study.
(PDF)
S1 Table. Genes of the chromosome found only in one or two strains: B. burgdorferi B31, B. afzelii BO23 and B. garinii CIP 103362.
(XLSX)

Acknowledgments
We would like to thank Drs Patricia Rosa and Phil Stewart (RML/NIH) for critical discussions and reviewing of the manuscript, Dr Craig Martens (RTS/RML/NIH) for in silico analysis, Sandy Stewart and Dr. Karin Aistleitner (RML/NIH) for technical assistance and reviewing the manuscript, Anita Mora and Austin Athman (VMA/RML/NIH) for figure preparation, Taylor Robinson (RML/NIH) for grammar and plagiarism checking.

Author Contributions
Conceptualization: Sébastien Bontemps-Gallo, Kevin A. Lawrence, Crystal L. Richards, Frank C. Gherardini.
Funding acquisition: Frank C. Gherardini.
Investigation: Sébastien Bontemps-Gallo, Kevin A. Lawrence, Crystal L. Richards.
Methodology: Sébastien Bontemps-Gallo, Kevin A. Lawrence, Crystal L. Richards, Frank C. Gherardini.
Project administration: Sébastien Bontemps-Gallo.
Resources: Frank C. Gherardini.
Validation: Sébastien Bontemps-Gallo.
Writing – original draft: Sébastien Bontemps-Gallo.
Writing – review & editing: Sébastien Bontemps-Gallo, Kevin A. Lawrence, Crystal L. Richards, Frank C. Gherardini.
References

1. Barbour AG, Adeolu M, Gupta RS. Division of the genus Borrelia into two genera (corresponding to Lyme disease and relapsing fever groups) reflects their genetic and phenotypic distinctiveness and will lead to a better understanding of these two groups of microbes (Margos et al. (2016) There is inadequate evidence to support the division of the genus Borrelia. Int. J. Syst. Evol. Microbiol. doi: 10.1099/ijsem.0.001717). Int J Syst Evol Microbiol. 2017; 67(6):2058–67. https://doi.org/10.1099/ijsem.0.001815 PMID: 28141502.

2. Stanek G, Wormser GP, Gray J, Strie F. Lyme borreliosis. Lancet. 2012; 379(9814):461–73. https://doi.org/10.1016/S0140-6736(11)60103-7 PMID: 21903253.

3. Steere AC, Grodzicki RL, Kornblatt AN, Craft JE, Barbour AG, Burgdorfer W, et al. The spirochetal etiology of Lyme disease. N Engl J Med. 1983; 308(13):733–40. https://doi.org/10.1056/NEJM19830331081301 PMID: 6828118.

4. Stewart PE, Rosa PA. Physiologic and Genetic Factors Influencing the Zoonotic Cycle of Borrelia burgdorferi. Curr Top Microbiol Immunol. 2017. https://doi.org/10.1007/82_2017_43 PMID: 28864829.

5. Burgdorfer W, Barbour AG, Hayes SF, Benach JL, Grunwaldt E, Davis JP. Lyme disease—a tick-borne spirochete disease? Science. 1982; 216(4552):1317–9. PMID: 7043737.

6. Kuehn BM. CDC estimates 300,000 US cases of Lyme disease annually. JAMA. 2013; 310(11):1110. https://doi.org/10.1001/jama.2013.279331 PMID: 24045727.

7. Bacon RM, Kugeler KJ, Mead PS, Centers for Disease Control and Prevention. Surveillance for Lyme disease—United States, 1992–2006. MMWR Surveill Summ. 2008; 57(10):1–9. PMID: 18830214.

8. Lindgren E, Jaenson TGT. Lyme borreliosis in Europe: Influences of Climate and Climate change, Epidemiology, Ecology and Adaptation Measures. World Health Organization, 2006.

9. Brownstein JS, Holford TR, Fish D. Effect of Climate Change on Lyme Disease Risk in North America. Environ Health. 2005; 2(1):38–46. https://doi.org/10.1007/s10399-004-0139-x PMID: 19008966.

10. Barbour AG. Isolation and cultivation of Lyme disease spirochetes. Yale J Biol Med. 1984; 57(4):521–5. PMID: 6393604.

11. Barbour AG, Burgdorfer W, Hayes SF, Péter O, Aeschlimann A. Isolation of a cultivable spirochete from Ixodes ricinus ticks of Switzerland. Current Microbiology. 1983; 8(2):123–6. https://doi.org/10.1007/bf01566996.

12. Baranton G, Postic D, Saint Girons I, Boerlin P, Piffaretti JC, Assous M, et al. Delineation of Borrelia burgdorferi sensu stricto, Borrelia garinii sp. nov., and group VS461 associated with Lyme borreliosis. Int J Syst Bacteriol. 1992; 42(3):378–83. https://doi.org/10.1099/00207713-42-3-378 PMID: 1380285.

13. Pritt BS, Mead PS, Johnson DK, Neitzel DF, Respicio-Kingry LB, Davis JP, et al. Identification of a novel pathogenic Borrelia species causing Lyme borreliosis with unusually high spirochaetaemia: a descriptive study. Lancet Infect Dis. 2016; 16(5):556–64. https://doi.org/10.1016/S1473-3099(15)00464-8 PMID: 26856777.

14. Jaulhac B, de Martino S, Boulanger N. Centre National de référence des Borrelia—Rapport annuel d’activité. Strasbourg, France: Hôpitaux Universitaires de Strasbourg, 2013 2013. Report No. 2013.

15. Cerar T, Strie F, Stupica D, Ruzic-Sabljic E, McHugh G, Steere AC, et al. Differences in Genotype, Clinical Features, and Inflammatory Potential of Borrelia burgdorferi sensu stricto Strains from Europe and the United States. Emerg Infect Dis. 2016; 22(5):818–27. https://doi.org/10.3201/eid2205.151806 PMID: 27088349.

16. Steere AC, Strie F, Wormser GP, Hu LT, Branda JA, Hovius JW, et al. Lyme borreliosis. Nat Rev Dis Primers. 2016; 2:16090. https://doi.org/10.1038/nrdp.2016.90 PMID: 27976670.

17. Casjens S, Palmer N, van Vugt R, Huang WM, Stevenson B, Rosa P, et al. A bacterial genome in flux: the twelve linear and nine circular extrachromosomal DNAs in an infectious isolate of the Lyme disease spirochete Borrelia burgdorferi. Mol Microbiol. 2000; 35(3):490–516. PMID: 10672174.

18. Fraser CM, Casjens S, Huang WM, Sutton G, Clayton R, Lathigra R, et al. Genomic sequence of a Lyme disease spirochaete, Borrelia burgdorferi. Nature. 1997; 390(6660):580–6. https://doi.org/10.1038/37551 PMID: 9403685.

19. Casjens SR, Mongolin EF, Qiu WG, Dunn JJ, Luft BJ, Fraser-Liggett CM, et al. Whole-genome sequences of two Borrelia afzelii and two Borrelia garinii Lyme disease isolate agents. J Bacteriol. 2011; 193(24):6995–6. https://doi.org/10.1128/JB.05951-11 PMID: 22123755.

20. Casjens SR, Mongolin EF, Qiu WG, Luft BJ, Schutze SE, Gilcrease EB, et al. Genome stability of Lyme disease spirochetes: comparative genomics of Borrelia burgdorferi plasmids. PLoS One. 2012; 7 (3):e33280. https://doi.org/10.1371/journal.pone.0033280 PMID: 22423010.

21. Schutze SE, Fraser-Liggett CM, Casjens SR, Qiu WG, Dunn JJ, Mongolin EF, et al. Whole-genome sequences of thirteen isolates of Borrelia burgdorferi. J Bacteriol. 2011; 193(4):1018–20. https://doi.org/10.1128/JB.01158-10 PMID: 20935092.
22. Schutzer SE, Fraser-Liggett CM, Qiu WG, Kraicz P, Mongodin EF, Dunn JJ, et al. Whole-genome sequences of Borrelia bissettii, Borrelia valaisiana, and Borrelia spielmani. J Bacteriol. 2012; 194(2):545–6. https://doi.org/10.1128/JB.06263-11 PMID: 22207749.

23. Gherardini FC, Boylan JA, Lawrence K, Skare J. Metabolism and Physiology of Borrelia. In: Samuels DS, Radolf J, editors. Borrelia: Molecular Biology, Host Interaction and Pathogenesis. Norfolk, UK: Caister Academic Press; 2010. p. 103–38.

24. Radolf JD, Caimano MJ, Stevenson B, Hu LT. Of ticks, mice and men: understanding the dual-host lifestyle of Lyme disease spirochaetes. Nat Rev Microbiol. 2012; 10(2):87–99. https://doi.org/10.1038/nrmicro2714 PMID: 22230951.

25. Verhaegh D, Joosten LAB, Oosting M. The role of host immune cells and Borrelia burgdorferi antigens in the etiology of Lyme disease. Eur Cytokine Netw. 2017; 28(2):70–84. https://doi.org/10.1684/ecn.2017.0396 PMID: 28840838.

26. Pal U, Buyuktanir O. Borrelia burgdorferi: Methods and Protocols. Humana Press; 2018. 361 p.

27. Xu Y, Johnson RC. Analysis and comparison of plasmid profiles of Borrelia burgdorferi sensu lato strains. J Clin Microbiol. 1995; 33(10):2679–85. PMID: 8567905.

28. Novak EA, Sultan SZ, Motaleb MA. The cyclic-di-GMP signaling pathway in the Lyme disease spirochete, Borrelia burgdorferi. Front Cell Infect Microbiol. 2014; 4:56. https://doi.org/10.3389/fcimb.2014.00056 PMID: 24822172.

29. Sultan SZ, Pitzer JE, Miller MR, Motaleb MA. Analysis of a Borrelia burgdorferi phosphodiesterase demonstrates a role for cyclic-di-guanosine monophosphate in motility and virulence. Mol Microbiol. 2010; 77(1):128–42. Epub 2010/05/07. https://doi.org/10.1111/j.1365-2958.2010.07191.x PMID: 20444101.

30. Casjens SR, Di L, Akther S, Mongodin EF, Luft BJ, Schutzer SE, et al. Primordial origin and diversification of plasmids in Lyme disease agent bacteria. BMC Genomics. 2018; 19(1):218. Epub 2018/03/28. https://doi.org/10.1186/s12864-018-4597-x PMID: 29580205.

31. Casjens SR, Gilcrease EB, Vujadinovic M, Mongodin EF, Luft BJ, Schutzer SE, et al. Plasmid diversity and phylogenetic consistency in the Lyme disease agent Borrelia burgdorferi. BMC Genomics. 2017; 18(1):165. https://doi.org/10.1186/s12864-017-3553-5 PMID: 28201991.

32. Jiang BG, Zheng YC, Tong YG, Jia N, Huo QB, Fan H, et al. Genome sequence of Borrelia afzelii Strain HLJ01, isolated from a patient in China. J Bacteriol. 2012; 194(24):7014–5. https://doi.org/10.1128/JB.01863-12 PMID: 23209254.

33. Kurilshikov AM, Fomenko NV, Stronin OV, Tikunov AY, Kabilov MR, Tupikin AE, et al. Complete Genome Sequencing of Borrelia valaisiana and Borrelia afzelii isolated from Ixodes persulcatus Ticks in Western Siberia. Genome Announc. 2014; 2(6). https://doi.org/10.1128/genomeA.01315-14 PMID: 25540343.

34. Schuler W, Bunikis I, Weber-Lehman J, Comstedt P, Kutschman-Bunikis S, Stanek G, et al. Complete genome sequence of Borrelia afzelii K78 and comparative genome analysis. PLoS One. 2015; 10(3):e0120548. https://doi.org/10.1371/journal.pone.0120548 PMID: 25798594.

35. Brenner EV, Kurilshikov AM, Stronin OV, Fomenko NV. Whole-genome sequencing of Borrelia garinii BgVir, isolated from Taiga ticks (Ixodes persulcatus). J Bacteriol. 2012; 194(20):5713. https://doi.org/10.1128/JB.01460-12 PMID: 23012288.

36. Jiang B, Yao H, Tong Y, Yang X, Huang Y, Jiang J, et al. Genome sequence of Borrelia garinii strain NMJW1, isolated from China. J Bacteriol. 2012; 194(23):6660–1. https://doi.org/10.1128/JB.01844-12 PMID: 23144006.

37. Stewart PE, Thalken R, Bono JL, Rosa P. Isolation of a circular plasmid region sufficient for autonomous replication and transformation of infectious Borrelia burgdorferi. Mol Microbiol. 2001; 39(3):714–21. PMID: 11169111.

38. Rego RO, Bestor A, Rosa PA. Defining the plasmid-borne restriction-modification systems of the Lyme disease spirochete Borrelia burgdorferi. J Bacteriol. 2011; 193(5):1161–71. https://doi.org/10.1128/JB.01176-10 PMID: 21193609.

39. Lawrenz MB, Kawabata H, Purser JE, Norris SJ. Decreased electroporation efficiency in Borrelia burgdorferi containing linear plasmids lp25 and lp56: impact on transformation of infectious B. burgdorferi. Infect Immun. 2002; 70(9):4798–804. Epub 2002/08/17. https://doi.org/10.1128/IAI.70.9.4798-4804.2002 PMID: 12183522.

40. Loenen WA, Dryden DT, Raleigh EA, Wilson GG, Murray NE. Highlights of the DNA cutters: a short history of the restriction enzymes. Nucleic Acids Res. 2014; 42(1):3–19. https://doi.org/10.1093/nar/gkt990 PMID: 24141096.

41. Bontemps-Gallo S, Lawrence K, Gherardini FC. Two Different Virulence-Related Regulatory Pathways in Borrelia burgdorferi Are Directly Affected by Osmotic Fluxes in the Blood Meal of Feeding
42. Siegmun M, van Bommel J, Ince C. Assessment of regional tissue oxygenation. Intensive Care Med. 1999; 25(10):1044–60. PMID: 10551958.

43. Venkatesh B, Morgan TJ, Lipman J. Subcutaneous oxygen tensions provide similar information to ileal luminal CO2 tensions in an animal model of haemorrhagic shock. Intensive Care Med. 2000; 26(5):592–600. PMID: 10923735.

44. Motaleb MA, Liu J, Wooten RM. Spirochetal motility and chemotaxis in the natural enzootic cycle and development of Lyme disease. Curr Opin Microbiol. 2015; 28:106–13. https://doi.org/10.1016/j.mib.2015.09.006 PMID: 26519910.

45. Charon NW, Goldstein SF. Genetics of motility and chemotaxis of a fascinating group of bacteria: the spirochetes. Annu Rev Genet. 2002; 36:47–73. https://doi.org/10.1146/annurev.genet.36.041602.134359 PMID: 12429686.

46. Sultan SZ, Manne A, Stewart PE, Bestor A, Rosa PA, Charon NW, et al. Motility is crucial for the infectious life cycle of Borrelia burgdorferi. Infect Immun. 2013; 81(6):2012–21. https://doi.org/10.1128/IAI.01228-12 PMID: 23529620.

47. Boylan JA, Lawrence KA, Downey JS, Gherardini FC. Borrelia burgdorferi membranes are the primary targets of reactive oxygen species. Mol Microbiol. 2008; 68(3):786–99. https://doi.org/10.1111/j.1365-2958.2008.06204.x PMID: 18373524.

50. Benoit JB, Denlinger DL. Meeting the challenges of on-host and off-host water balance in blood-feeding arthropods. J Insect Physiol. 2010; 56(10):1366–76. https://doi.org/10.1016/j.jinsphys.2010.02.014 PMID: 20206630.

56. Kim D, Urban J, Boyle DL, Park Y. Multiple functions of Na/K-ATPase in dopamine-induced salivation of the Blacklegged tick, Ixodes scapularis. Sci Rep. 2016; 6:21047. https://doi.org/10.1038/srep21047 PMID: 26961075.

57. Lees AD. The water balance in Ixodes ricinus L. and certain other species of ticks. Parasitology. 1946; 37(1–2):1–20.

58. Lees AD. Transpiration and the Structure of the Epicuticle in Ticks. Journal of Experimental Biology. 1947; 23(3–4):379–410. PMID: 20292371

59. Lees AD. The Sensory Physiology of the Sheep Tick, Ixodes Ricinus L. Journal of Experimental Biology. 1948; 25(2):145–207.

60. Kaufman WR, Phillips JE. Ion and Water Balance in the Ixodid Tick Dermacentor Andersoni. Journal of Experimental Biology. 1973; 58(2):537–47.

61. Kaufman WR, Phillips JE. Ion and Water Balance in the Ixodid Tick Dermacentor Andersoni. Journal of Experimental Biology. 1973; 58(2):549–64.

62. Kaufman WR, Phillips JE. Ion and Water Balance in the Ixodid Tick Dermacentor Andersoni. I Routes of Ion and Water Excretion. 1973; 58(2):523–36.

63. Waymouth C. Osmolality of mammalian blood and of media for culture of mammalian cells. In Vitro. 1970; 6(2):109–27. PMID: 4943053.

64. Dolgin E. Climate change: As the ice melts. Nature. 2017; 543(7647):S4–S5. https://doi.org/10.1038/543S54a PMID: 28355191.
65. Monaghan AJ, Moore SM, Sampson KM, Beard CB, Eisen RJ. Climate change influences on the annual onset of Lyme disease in the United States. Ticks Tick Borne Dis. 2015; 6(5):615–22. https://doi.org/10.1016/j.ttbdis.2015.05.005 PMID: 26025268.

66. Simon JA, Marrotte RR, Desrosiers N, Fiset J, Gaitan J, Gonzalez A, et al. Climate change and habitat fragmentation drive the occurrence of Borrelia burgdorferi, the agent of Lyme disease, at the northeastern limit of its distribution. Evol Appl. 2014; 7(7):750–64. https://doi.org/10.1111/eva.12165 PMID: 25469157.

67. Stone BL, Tourand Y, Brissette CA. Brave New Worlds: The Expanding Universe of Lyme Disease. Vector Borne Zoonotic Dis. 2017; 17(9):619–29. https://doi.org/10.1089/vbz.2017.2127 PMID: 28727515.

68. Veinovic G, Ruzic-Sabljic E, Strle F, Cerar T. Comparison of Growth of Borrelia afzelii, Borrelia garinii, and Borrelia burgdorferi Sensu Stricto at Five Different Temperatures. PLoS One. 2016; 11(6): e0157706. https://doi.org/10.1371/journal.pone.0157706 PMID: 27310556.

69. Hubalek Z, Halouzka J, Heroldova M. Growth temperature ranges of Borrelia burgdorferi sensu lato strains. J Med Microbiol. 1998; 47(10):929–32. https://doi.org/10.1099/00222615-47-10-929 PMID: 9788818.

70. Jacquet M, Genee D, Berti A, Maluenda E, Sarr A, Voordouw MJ. The abundance of the Lyme disease pathogen Borrelia afzelii declines over time in the tick vector Ixodes ricinus. Parasit Vectors. 2017; 10(1):257. https://doi.org/10.1186/s13071-017-2187-4 PMID: 28545520.

71. Piesman J, Schneider BS, Zeidner NS. Use of quantitative PCR to measure density of Borrelia burgdorferi in the midgut and salivary glands of feeding tick vectors. J Clin Microbiol. 2001; 39(11):4145–8. https://doi.org/10.1128/JCM.39.11.4145-4148.2001 PMID: 1168244.

72. Wood JM. Osmosensing by bacteria. Sci STKE. 2006; 2006(357):pe43. https://doi.org/10.1126/stke.3572006pe43 PMID: 17047223.

73. Wood JM. Bacterial osmoregulation: a paradigm for the study of cellular homeostasis. Annu Rev Microbiol. 2011; 65:215–38. https://doi.org/10.1146/annurev-micro-091010-102815 PMID: 21663439.

74. Bourret TJ, Lawrence KA, Shaw JA, Lin T, Norris SJ, Gherardini FC. The Nucleotide Excision Repair Pathway Protects Borrelia burgdorferi from Nitrosative Stress in Ixodes scapularis Ticks. Front Microbiol. 2016; 7:1397. https://doi.org/10.3389/fmicb.2016.01397 PMID: 27656169.

75. Ramsey ME, Hyde JA, Medina-Perez DN, Lin T, Gao L, Lundt ME, et al. A high-throughput genetic screen identifies previously uncharacterized Borrelia burgdorferi genes important for resistance against reactive oxygen and nitrogen species. PLoS Pathog. 2017; 13(2):e1006225. Epub 2017/02/18. https://doi.org/10.1371/journal.ppat.1006225 PMID: 28212410.

76. Boylan JA, Posey JE, Gherardini FC. Borrelia oxidative stress response regulator, BosR: a distinctive Zn-dependent transcriptional activator. Proc Natl Acad Sci U S A. 2003; 100(20):11684–9. https://doi.org/10.1073/pnas.2032956100 PMID: 12975527.

77. Xu Y, Kodner C, Coleman L, Johnson RC. Correlation of plasmids with infectivity of Borrelia burgdorferi sensu stricto type strain B31. Infect Immun. 1996; 64(9):3870–6. Epub 1996/09/01. PMID: 8751941.

78. Elias AF, Stewart PE, Grimm D, Caimano MJ, Eggers CH, Tilly K, et al. Clonal polymorphism of Borrelia burgdorferi strain B31 MT: implications for mutagenesis in an infectious strain background. Infect Immun. 2002; 70(4):2139–50. https://doi.org/10.1128/IAI.70.4.2139-2150.2002 PMID: 11895980.

79. Simpson WJ, Garon CF, Schwan TG. Analysis of supercoiled circular plasmids in infectious and non-infectious Borrelia burgdorferi. Microb Pathog. 1990; 8(2):109–18. PMID: 2348778.

80. Chu CY, Stewart PE, Besor A, Hansen B, Lin T, Gao L, et al. Function of the Borrelia burgdorferi FtsH Homolog Is Essential for Viability both In Vitro and In Vivo and Independent of HflK/C. MBio. 2016; 7(2):e00404–16. https://doi.org/10.1128/mBio.00404-16 PMID: 27094329.

81. Samuels DS. Electrophoretic characterization of the spirochete Borrelia burgdorferi. Methods Mol Biol. 1995; 47:253–9. https://doi.org/10.1385/0-89603-310-4:253 PMID: 7550741.

82. Sicklinger M, Wienceke R, Neubert U. In vitro susceptibility testing of four antibiotics against Borrelia burgdorferi: a comparison of results for the three genospecies Borrelia afzelii, Borrelia garinii, and Borrelia burgdorferi sensu stricto. J Clin Microbiol. 2003; 41(4):1791–3. https://doi.org/10.1128/JCM.41.4.1791-1793.2003 PMID: 12682190.