Published in final edited form as:

Ticks Tick Borne Dis. 2018 February ; 9(2): 281–287. doi:10.1016/j.ttbdis.2017.11.001.

Colony Formation in Solid Medium by the Relapsing Fever Spirochetes *Borrelia hermsii* and *Borrelia turicatae*

Sandra J. Raffel^a, Brandi N. Williamson^{a,b}, Tom G. Schwan^a, and Frank C. Gherardini^{a,*}

^aLaboratory of Bacteriology, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, MT, USA

^bLaboratory of Virology, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, MT, USA

Abstract

Relapsing fever (RF) in North America is caused primarily by the spirochete *Borrelia hermsii* and is associated with the bite of its tick vector *Ornithodoros hermsi*. Although this spirochete was known long before the discovery of the Lyme disease (LD) spirochete, *Borrelia burgdorferi*, basic methods to facilitate the study of *B. hermsii* have lagged behind. One important technique to expedite the study of the molecular biology and pathogenesis of *B. hermsii* would be a reliable method to grow and clone these bacteria in solid medium, which we now describe. We have defined the solidifying agent, plating temperature, oxygen concentration, and pH for the efficient plating of two species of RF spirochetes, *B. hermsii* and *Borrelia turicatae*. Importantly, this technique allowed us to successfully isolate virulent, clonal cell lines of spirochetes, and to enumerate and isolate viable *B. hermsii* from infected mouse blood and tick tissues. Our results also demonstrate the value of testing a range of several environmental variables to increase the efficiency of bacterial isolation, which may be helpful for researchers working on other prokaryotes that are intractable for *in vitro* growth.

Keywords

Ornithodoros hermsi; plating; *in vitro* cultivation; Argasid ticks; tick-borne disease

1. Introduction

Borrelia hermsii is the primary agent of relapsing fever in western North America. Being a zoonotic pathogen with a complex maintenance cycle, *B. hermsii* resides in mammals and its tick vector, *Ornithodoros hermsi*. *Borrelia hermsii* was first grown continuously in liquid culture by Kelly in 1971 (Kelly, 1971). Kelly's medium was further improved for growth of other *Borrelia* species, including the Lyme disease (LD) spirochete, *Borrelia burgdorferi*,

*Corresponding author: Sandra J. Raffel (Sandra J. Stewart), sstewart@niaid.nih.gov, Rocky Mountain Laboratories, NIAID, NIH, 903 S. 4th Street, Hamilton, MT, USA.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

leading to the currently used medium, BSK-II (Stoenner, 1974; Burgdorfer et al., 1982; Barbour, 1984). Although the medium allows for colony formation of the LD spirochetes (Kurtti et al., 1987; Rosa and Hogan, 1992; Samuels, 1995), to date, growth of any RF spirochetes on solid medium as isolated colonies has not been successful. Currently, clonal populations of *B. hermsii* and *B. turicatae* are isolated in liquid medium by limiting dilution (Stoenner, 1974; Battisti et al., 2008; Fine et al., 2011; Lopez et al., 2013; Raffel et al., 2014). However, this method is time-consuming and does not yield large numbers of clonal isolates. Plating for colonies on solid medium would be a more efficient method for selecting and screening mutants and enumerating viable bacteria from the blood and other tissues of infected mammals and ticks. Here, we describe the development and optimization of a method for the efficient plating of RF spirochetes from liquid culture medium, infected ticks, and the blood of infected mice.

2. Materials and methods

2.1. Ethics statement

All animal work followed the guidelines of the National Institutes of Health for the care and use of laboratory animals. Protocols were approved by the Rocky Mountain Laboratories Animal Care and Use Committee. Rocky Mountain Laboratories, NIAID, NIH, are accredited by the International Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

2.2. Bacterial strains and growth conditions

Borrelia hermsii strains DAH, a DAH non-motile mutant in *fliH* (Guyard et al., 2013), and *Borrelia turicatae* 91E135 (Schwan et al., 2005) were grown in mBSK-c (Battisti et al., 2008), which is BSK-II modified with 6 g/L glucose and 12% rabbit serum (Pel-Freez, Rogers, AZ, USA). Liquid cultures were incubated at 35°C in 5% CO₂ and either 3% O₂ or atmospheric O₂ (18% in Hamilton, MT) in a Forma Series II Water Jacket CO₂/O₂ incubator (Thermo Fisher Scientific, Inc., Waltham, MA, USA) with the caps loosely attached on the tubes.

2.3. Plating of RF spirochetes from liquid medium

The plating medium consisted of a 20 ml bottom agar of the BSK-II plating medium used for *B. burgdorferi* (Rosa and Hogan, 1992; Rosa et al., 1992; Samuels, 1995) at pH 7.5 with rabbit serum increased to 10%. Bottom plates were poured 1–3 d in advance of spirochete plating and allowed to equilibrate in the CO₂/O₂ incubator at 35°C. A top agar was made with BSK-II, 10% rabbit serum, 1% SeaPlaque Low Melting Point Agarose (LMP) (Lonza Rockland, Inc., Rockland, ME, USA) and equilibrated to 37°C. *Borrelia* cells were grown in mBSK-c, enumerated using a Petroff-Hausser Counter (Hausser Scientific, Horsham, PA, USA), and diluted to provide a countable number of colonies on the plates (30–300 cells). Ten ml of top agar was added to a 15-ml tube containing the spirochetes and the suspension was poured onto the bottom agar plate. After the top agar solidified, plates were returned to the incubator. The plating efficiency (in %) was determined for each plate and was based on the number of colonies observed, divided by the number of colonies predicted from the

microscopic counts. Statistics were performed with GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA).

2.4. Gel electrophoresis and Immunoblotting

Borrelia hermsii DAH whole-cell lysates were prepared from 5 ml mBSK-c cultures. The cells were harvested by centrifugation, washed twice with Haley's Buffer (4.77 g/L HEPES, 2.92 g/L NaCl, pH 7.6), resuspended in 150 µl Laemmli sample buffer and heated at 95°C for 10 min. Lysates (8 µl, $\sim 4 \times 10^7$ cells) were electrophoresed in 4–15% Mini-PROTEAN TGX gels (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Proteins were transferred to a nitrocellulose membrane with the Trans-Blot Turbo blotting system (Bio-Rad Laboratories, Inc.). Membranes were blocked overnight in TBS-T (25 mM Tris base, 150 mM NaCl, pH 7.4, 0.1% Tween-20) containing 5% non-fat dry milk, then incubated with serum samples collected from mice at 4 wk post-infection (1:600) for 1 h in TBS-T plus 5% milk. The blots were then washed 3 times with TBS-T each for 10 min, incubated with HRP-rec-protein A (1:5000) (Thermo Fisher Scientific) for 30 min in TBS-T plus 5% milk, followed by 4 washes with TBS-T each for 10 min. The blots were incubated with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Inc.) and developed on Amersham Hyperfilm ECL (GE Healthcare, Buckinghamshire, UK).

2.5. Quantification of *B. hermsii* from infected mouse blood

Adult female RML mice were obtained from an outbred colony maintained at Rocky Mountain Laboratories that were derived from Swiss-Webster mice. Two RML mice were inoculated in the intraperitoneal (i.p.) cavity with 500 spirochetes. When the mice became spirochetemic ($\sim 10^7$ cells/ml) 4 d post-infection, blood was drawn from the mice by intracardiac puncture using a 1 ml syringe first filled and then dispelled with heparin (1000 U/ml) to coat the syringe and prevent the blood from clotting. One hundred µl of blood was added to 1.9 ml mBSK-c (1/20) and spirochetes were counted microscopically with a Petroff-Hausser Counter. A series of 10-fold dilutions was made from the same sample and 1 ml of each dilution was plated with 10 ml of top agar. To enumerate the number of spirochetes in the blood by Quantitative PCR (QPCR), two 5 µl aliquots of infected blood were placed into 95 µl of SideStep Lysis and Stabilization Buffer (Agilent Technologies, Santa Clara, CA, USA) and stored at -80°C until QPCR was performed as previously described (McCoy et al., 2010; Raffel et al., 2014).

2.6. Enumeration of viable *B. hermsii* from infected *O. hermsi*

Ornithodoros hermsi SIS ticks were from a colony reared at Rocky Mountain Laboratories (McCoy et al., 2010) that originated from ticks collected from Siskiyou County, CA. Unfed adult ticks were infected with *B. hermsii* DAH as nymphs 3 years previously (Raffel et al., 2014), molted, and had not received another blood meal. Fed nymphs were third-stage nymphs that just fed on an infected mouse. Tick infections were performed as previously described (Raffel et al., 2014) but the nymphs were infected on mice that were anesthetized with 21 mg/ml Ketamine and 3 mg/ml Xylazine at a dosage of 0.1 ml per 25 g body weight. To maintain sedation, an additional i.p. injection with 21 mg/ml Ketamine at a dosage of 0.05 ml per 25 g body weight was given. The nymphs did not become fully engorged. The unfed adult and partially fed nymphal ticks were placed individually in a 1.5 ml microfuge

tube and surface-sterilized by washing sequentially in 3% H₂O₂, 70% ethanol, then two rinses of sterile water, each for 3 min. The ticks were suspended in 100 µl mBSK-c medium, ground with a sterile pestle, and the pestle was rinsed with 900 µl of mBSK-c. The spirochetes were plated from the 1 ml suspension in plating medium that contained borrelia-resistant antibiotics (0.04 mg Phosphomycin, 0.1 mg Rifampicin, and 5 µg Amphotericin B per liter) (Antibiotic Mixture for *Borrelia*, HiMedia Laboratories, LLC, West Chester, PA, USA) to control bacterial contaminants. Some unfed adult ticks were dissected so that spirochetes from the midgut, salivary glands, and the remainder of the tick could be individually plated and enumerated. Each sample was placed into a 1.5 ml microfuge tube containing 200 µl mBSK-c, ground with a sterile pestle, the pestle rinsed with 800 µl of mBSK-c and plated to determine the spirochete load in each tissue.

2.7. Quantitative PCR

The quantification of *B. hermsii* DAH in infected mouse blood was performed by QPCR with the Taqman Universal PCR Master Mix (Applied Biosystems, Waltham, MA, USA) on a Roche LightCycler 480 II (Roche, Pleasanton, CA, USA) using a Taqman primer and probe mix to the *B. hermsii* flagellin gene *flaB* (Integrated DNA Technologies, Inc., Coralville, IA, USA). The primer and probe sequences to the *flaB* gene were: Primer Forward (AAGTCAGCTGCTCAAAATGTAAAAAC), Primer Reverse (CAGCTAGTGATGCTGGTGTGTTAAT) and the ZEN Double-Quenched Probe (FAM-TTTGCGGGT/ZEN/TGCATTCCAAGCTCTT-IBFQ). To estimate the number of spirochetes per ml of blood, a standard curve was generated as described (McCoy et al., 2010; Raffel et al., 2014). Briefly, 10 µl of uninfected blood and 10 µl of spirochetes from a series of ten-fold dilutions ranging from 1×10² cells/ml to 1×10¹⁰ cells/ml were put into 180 µl of SideStep Lysis and Stabilization Buffer and stored at -80 °C. When thawed, the standards and blood samples were diluted 1/10 in sterile water and 3 µl were used in the QPCR and performed in triplicate.

3. Results and discussion

3.1. Development of a plating medium for *B. hermsii*

We set out to develop and optimize a method that would permit *B. hermsii* to grow and produce single colonies on a solid medium. Initially, we attempted to plate *B. hermsii* using the method developed for *B. burgdorferi* but these pilot experiments were disappointing. When colonies did form on the BSK-II solid agar, they were very diffuse and recoveries were very poor (<10%). Our thought was that *B. hermsii* moved through the medium efficiently and therefore would not make a single colony. Analysis of the plating method also suggested that the temperature of the plating medium may affect plating outcomes. *Borrelia burgdorferi* is often plated using medium that is held at 42–45°C prior to plating (Rosa and Hogan, 1992; Samuels, 1995). Therefore, we modified the protocol for plating *B. hermsii* by lowering the temperature of the medium to 37°C before plating by using low melting point agarose (LMP). We also included a non-motile flagellar mutant (*fliH* mutant) (Guyard et al., 2013) to test the effects of *B. hermsii* motility on colony formation.

Cultures of *B. hermsii* DAH and the non-motile mutant were grown in mBSK-c with 12% rabbit serum in 3% O₂/5% CO₂. The cells were counted on a Petroff-Hausser Counter and plated in a top agar layer of BSK-II with 12% rabbit serum and 1.35% LMP agarose (SeaPlaque, Lonza Rockland), and incubated at 3% O₂/5% CO₂. With both strains, colonies started to appear 5 to 7 d later and all colonies were visible by 10–14 d. Both wild-type DAH and the *fliH* mutant had plating efficiencies of ~90% (Fig. 1). The *fliH* mutant had very small colonies compared to the wild type (Fig. 1.), similarly to those colonies described for a *B. burgdorferi* non-motile mutant (Motaleb et al., 2000). Motility clearly played a role in colony morphology and spreading but did not contribute to plating efficiency. Perhaps, the lower temperature of the top agar or the LMP agarose itself contributed to growing *B. hermsii* DAH on solid medium. LMP agarose concentrations tested between 0.83% and 1.35% did not change the plating efficiencies, although in the higher agarose concentrations colony formation was delayed by 1–2 d. Different rabbit serum concentrations from 10–15% had no effect on colony formation. Therefore, we now routinely plate with medium that contains 10% rabbit serum and 1% LMP agarose.

To determine how long *B. hermsii* cells remained viable in the solid medium, plates were sealed with parafilm and stored at 4°C or room temperature (RT). During initial optimization of the plating method, we noticed that plates with large numbers of colonies or plates that had been incubated for 14 d began to turn yellow, indicating the plates were turning acidic (Fig. 1 panel A, Fig. 5). Therefore, we not only tested long-term survival of cells on plates at 4°C versus RT but also tested the effects of the acidification of the plating medium on cell viability. Colonies picked from plates that were red or orange but had not yet turned yellow (maximum acidification) were still viable after 1 mo when inoculated into mBSK-c broth regardless of whether the plates were stored at RT or 4°C. However, colonies picked from plates stored at 4°C took slightly longer to recover. In contrast, colonies picked from bright yellow, acidic plates were not viable after a few days. This solid medium allowed *B. hermsii* to form single colonies, resulting in the ability to obtain a greater number of clonal isolates over the current method of limiting dilution. This technical advance could further the study of *B. hermsii* by increasing the ease in selecting genetic or phenotypic clonal variants.

3.2 Effects of pH on plating efficiency

To further test how pH might affect the plating efficiency, we included another species of RF spirochete, *B. turicatae* 91E135. This species produced colonies following our new protocol but had a plating efficiency of ~30% compared to ~80% for *B. hermsii* when grown in parallel (Fig. 2). *Borrelia* spirochetes experience a range of pH during their enzootic cycle in ticks and mammalian blood. Thus, we proceeded to examine pH as a variable to increase plating efficiencies of *B. hermsii* and *B. turicatae*. Both species were grown in liquid mBSK-c pH 7.5 in 3% O₂ -5% CO₂ and plated at pH 7.0, (closer to the pH of the tick midgut (as determined for *Ornithodoros moubata* (Grandjean, 1984)), pH 7.35 (pH of blood) and pH 7.5 (pH of the medium) (Fig. 3). *Borrelia hermsii* had a significantly better plating efficiency at pH 7.35 than pH 7.0, but not significantly better than at pH 7.5. *Borrelia turicatae* had its highest plating efficiency at pH 7.5, significantly higher than at either pH 7.0 or 7.35. Therefore, these results showed that pH can influence the plating efficiency of *B. hermsii* and *B. turicatae* but the plating medium at pH 7.5 was adequate.

3.3 Effects of oxygen concentration on plating efficiency

The conditions used to culture the RF spirochetes in liquid medium may have significant impact on the plating efficiencies of these bacteria in solid medium. Possibly *B. hermsii* and *B. turicatae* had different plating efficiencies due to the medium growth conditions to which they were adapted. For example, we routinely grow *B. hermsii* in mBSK-c at pH 7.5 at 35°C in microaerobic conditions (3% O₂) because in vivo the bacteria are not exposed to high levels of oxygen. Many labs working with *B. hermsii* or *B. turicatae* incubate the growth tubes in a CO₂ incubator with atmospheric oxygen (18–20%) rather than low oxygen (Fine et al., 2011; James et al., 2016; Neelakanta et al., 2017), which was true for our *B. turicatae* isolate. Even though *B. turicatae* was passed 2–3 times in low oxygen and achieved similar densities to *B. hermsii* before plating, it may not have completely adapted to the conditions required for efficient plating. Therefore, we evaluated the effects of O₂ concentration on plating efficiencies for the two species. *Borrelia turicatae* and *B. hermsii* cells were grown in mBSK-c (pH 7.5) in 5% CO₂ with 3% or 18% O₂, and then plated and incubated under those same oxygen conditions. *Borrelia hermsii* had equal plating efficiencies when grown in either oxygen concentration (Fig. 4A). In contrast, *B. turicatae* had a significantly higher plating efficiency when grown in 18% oxygen compared to 3% O₂ (80% versus 44%, respectively) (Fig. 4A). Interestingly, although more colonies developed in 18% O₂, the colonies took longer to appear. On day 7, there were more colonies on the plates incubated in 3% O₂ while by day 10–12, there were more colonies on the plates incubated in 18% O₂. Clearly, the higher concentration of O₂ increased the plating efficiency of *B. turicatae*, and indicates that optimizing the conditions is required for each species of spirochete.

Also, plates incubated in 18% oxygen produced a second colony type at a higher frequency than when incubated in 3% oxygen. Plates incubated in 3% O₂ produced mostly tight, compact colonies with an occasional diffuse colony while plates incubated in 18% O₂ had a greater percentage of diffuse colonies for both *B. hermsii* and *B. turicatae* (Fig. 4B). We do not know why 18% O₂ triggered these changes in colony morphology, although this may have resulted from oxygen stress or genotypic or phenotypic changes in these relapsing fever spirochetes.

3.4 Colony morphology does not alter infectivity

Borrelia hermsii cells from both colony types were picked and grown in liquid medium and plated again in solid medium. Regardless of the original colony type, a high percentage of the resulting colonies had the tight colony morphology and a small percentage had the diffuse colony morphology. This result is in contrast to *B. burgdorferi*, which also demonstrated different colony morphologies. When the colony types of *B. burgdorferi* were picked, grown in liquid medium and plated again, the colony phenotypes were reproducible in solid medium (Rosa and Hogan, 1992; Elias et al., 2002). Whole-cell lysates of *B. hermsii* from the two colony types showed no major protein changes on a PAGE gel (Fig. S1 panel A). Therefore, to determine if colony morphology reflected a difference in infectivity, 18 colonies (9 tight and 9 diffuse) were picked, grown in liquid medium, and 200 µl of a 10⁻⁴ dilution (100–1000 spirochetes, determined by plating) of each colony were inoculated individually into RML mice. At 4 wk post-inoculation, mouse serum samples were collected and evaluated on immunoblots to confirm infection and to assess any differences in antibody

response to the lysates of the two colony types. All 18 colonies were infectious in mice, and sera from mice infected with either colony type were positive and showed only slight differences in antibody response against lysates of both colony types (Fig. S1 panels B, C). These data suggest that distinct colony morphologies are not due to genetic differences because the colony phenotype was not reproducible when replated, and there was no effect on infectivity.

3.5 Plating *B. hermsii* from infected mouse blood

Borrelia hermsii can achieve cell densities as high as 10^8 spirochetes per ml in the blood of an infected mammal during an infection. Plating directly from an infected mouse would allow for the isolation of individual clones directly from blood. Also, the number of viable spirochetes could be quantified, especially when comparing spirochetemic levels of viable mutant and wild type cells during the primary infections and relapses. Therefore, we attempted to produce colonies of *B. hermsii* when plated directly from blood, and to determine if plating was an effective way to enumerate viable spirochetes compared to current methods that are used such as counts on a Petroff-Hausser Counter and QPCR (McCoy et al., 2010; Raffel et al., 2014). Colonies were formed from each of the ten-fold dilutions of blood that were plated (Fig. 5) and the blood did not inhibit bacterial growth even at the lowest dilution tested (10^{-1}). Comparing the three methods of quantifying spirochetes from blood, QPCR estimated more spirochetes per ml of blood than either plating or direct microscopic enumeration with a Petroff-Hausser Counter (Table 1). QPCR indicated 7–8-fold more spirochetes than plating, while the Petroff-Hausser counts indicated about 2-fold more spirochetes than direct plating (Table 1). The higher estimates of cell numbers measured by QPCR likely represented the detection of DNA from lysed and dead cells as well as viable spirochetes. Likewise, direct counting probably detected significant numbers of non-viable, intact spirochetes. The number of lysed or intact, non-viable cells may be more prevalent in the blood after peak spirochetemia. However, the plating efficiency from blood may be significantly lower than the >80% plating efficiency achieved from liquid medium. Nonetheless, plating *B. hermsii* will produce single clonal isolates directly from the blood, and allow for comparing spirochete quantities in the blood of infected hosts between wild type and mutant strains. Quantifying spirochetes by plating also has an advantage over QPCR and counting cells on the Petroff-Hausser Counter due to the lower limit of detection. The limit of detection for QPCR is 3×10^4 cell/ml and for the Petroff-Hausser Counter is 5×10^4 cells/ml, whereas plating can detect as little as 1 cell.

3.6 Plating *B. hermsii* from infected *O. hermsi*

We next determined if spirochetes could be plated directly from infected ticks. Seven unfed adult *O. hermsi* ticks infected with *B. hermsii* DAH 3 years previously and four 3rd-stage nymphal ticks that had just taken a blood meal on a spirochetemic mouse produced colonies when plated, with 10^2 – 10^3 viable spirochetes isolated per tick (Fig. 6). Therefore, both fasting and recently fed whole ticks were assayed successfully by plating and the number of spirochetes enumerated. Also striking was the finding of nearly 10,000 viable spirochetes in individual ticks that had not fed for three years.

RF spirochetes reside in multiple tissues and organs (e.g. salivary glands, midgut, ovaries) in *Ornithodoros* ticks (Herms and Wheeler, 1936; Lopez et al., 2013; Policastro et al., 2013; Krishnavajhala et al., 2017) and the production of important virulence factors, such as the major outer surface proteins (e.g. the variable tick protein, Vtp) in *B. hermsii*, are differentially expressed in different tick tissues (Schwan and Hinnebusch, 1998; Raffel et al., 2014). Therefore, we tested the plating method for isolating and enumerating *B. hermsii* from specific tick tissues. We surface-sterilized 3 infected, unfed adult ticks and dissected out the midgut and salivary glands and retained the remainder of the tick carcass for processing (Table 2). The salivary glands were difficult to triturate because of their small size (< 1.0 mm) and gel-like consistency. Nevertheless, spirochetes were plated and quantified from each of the tissues assessed and suggests that *B. hermsii* are dispersed throughout much of the adult tick. These findings are consistent with results from Krishnavajhala et al. (2017) that showed *B. turicatae* spirochetes in the *O. turicata* tick persistently colonized the tick midgut and salivary glands. We suspect that spirochetes detected in the “remainder” represent spirochetes that had colonized other portions of the tick digestive tract, the reproductive organs, and the nervous system. Therefore, we are confident that this method will allow for the isolation of individual clones of spirochetes from numerous tick tissues and may prove valuable in assessing mutants that may have altered abilities to disseminate within the tick.

4. Conclusions

We developed a procedure and plating medium to obtain isolated clonal colonies of the relapsing fever spirochetes *B. hermsii* and *B. turicatae*. Low melting point agarose, temperature, oxygen concentration, and pH were key variables in obtaining high plating efficiencies. This method permitted the isolation of individual *B. hermsii* clones from liquid culture that retained their infectivity and also allowed enumeration and isolation of spirochetes from infected mouse blood, entire ticks, and specific tick tissues. This technique should further aid in the study of relapsing fever spirochetes by providing an efficient method to obtain clonal isolates when developing and assaying mutants, searching for phenotypic variants, based on various phenotypes such as colony morphology or promoter activity of individual clones with β -galactosidase and fluorescent markers, and to efficiently study environmental stresses on spirochete viability.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Phil Stewart, Dan Dulebohn, Crystal Richards, Sebastien Bontemps-Gallo, Kendal Cooper and Patti Rosa at the Laboratory of Bacteriology, Rocky Mountain Laboratories, NIAID, NIH, for comments on the manuscript, and Anita Mora at the Research Technologies Branch, Rocky Mountain Laboratories, NIAID, NIH, for assistance with the figures. This work was funded by the Division of Intramural Research, National Institute for Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA. Funders had no involvement in study design, data collection, analysis, or interpretation, writing of the manuscript or decision on where to submit for publication.

References

- Barbour AG. Isolation and cultivation of Lyme disease spirochetes. *Yale J Biol Med.* 1984; 57:521–525. [PubMed: 6393604]
- Battisti JM, Raffel SJ, Schwan TG. A system for site-specific genetic manipulation of the relapsing fever spirochete *Borrelia hermsii*. *Methods Mol Biol.* 2008; 431:69–84. [PubMed: 18287748]
- Burgdorfer W, Barbour AG, Hayes SF, Benach JL, Grunwaldt E, Davis JP. Lyme disease--a tick-borne spirochetosis? *Science.* 1982; 216:1317–1319. [PubMed: 7043737]
- Elias AF, Stewart PE, Grimm D, Caimano MJ, Eggers CH, Tilly K, Bono JL, Akins DR, Radolf JD, Schwan TG, Rosa P. Clonal polymorphism of *Borrelia burgdorferi* strain B31 MI: implications for mutagenesis in an infectious strain background. *Infect Immun.* 2002; 70:2139–2150. [PubMed: 11895980]
- Fine LM, Earnhart CG, Marconi RT. Genetic transformation of the relapsing fever spirochete *Borrelia hermsii*: stable integration and expression of green fluorescent protein from linear plasmid 200. *J Bacteriol.* 2011; 193:3241–3245. [PubMed: 21551306]
- Grandjean O. Blood digestion in *Ornithodoros moubata* Murray sensu stricto Walton (Ixodoidea: argasidae) females I. Biochemical changes in the midgut lumen and ultrastructure of the midgut cell, related to intracellular digestion. *Acarologia.* 1984; 25:147–165.
- Guyard C, Raffel SJ, Schrupf ME, Dahlstrom E, Sturdevant D, Ricklefs SM, Martens C, Hayes SF, Fischer ER, Hansen BT, Porcella SF, Schwan TG. Periplasmic flagellar export apparatus protein, FliH, is involved in post-transcriptional regulation of FlaB, motility and virulence of the relapsing fever spirochete *Borrelia hermsii*. *PLoS One.* 2013; 8:e72550. [PubMed: 24009690]
- Hermes WB, Wheeler CM. *Ornithodoros hermsi* Wheeler as a vector of relapsing fever in California. *J Parasitol.* 1936; 22:276–282.
- James AE, Rogovskyy AS, Crowley MA, Bankhead T. Characterization of a DNA adenine methyltransferase gene of *Borrelia hermsii* and its dispensability for murine infection and persistence. *PLoS One.* 2016; 11:e0155798. [PubMed: 27195796]
- Kelly R. Cultivation of *Borrelia hermsii*. *Science.* 1971; 173:443–444. [PubMed: 5557322]
- Krishnavajhala A, Wilder HK, Boyle WK, Damania A, Thornton JA, Pérez de León AA, Teel PD, Lopez JE. Imaging of *Borrelia turicatae* producing the green fluorescent protein reveals persistent colonization of the *Ornithodoros turicata* midgut and salivary glands from nymphal acquisition through transmission. *Appl Environ Microbiol.* 2017; 83:e02503. [PubMed: 27986725]
- Kurtti TJ, Munderloh UG, Johnson RC, Ahlstrand GG. Colony formation and morphology in *Borrelia burgdorferi*. *J Clin Microbiol.* 1987; 25:2054–2058. [PubMed: 3693538]
- Lopez JE, Wilder HK, Hargrove R, Brooks CP, Peterson KE, Beare PA, Sturdevant DE, Nagarajan V, Raffel SJ, Schwan TG. Development of genetic system to inactivate a *Borrelia turicatae* surface protein selectively produced within the salivary glands of the arthropod vector. *PLoS Negl Trop Dis.* 2013; 7:e2514. [PubMed: 24205425]
- McCoy BN, Raffel SJ, Lopez JE, Schwan TG. Bloodmeal size and spirochete acquisition of *Ornithodoros hermsi* (Acari: Argasidae) during feeding. *J Med Entomol.* 2010; 47:1164–1172. [PubMed: 21175068]
- Motaleb MA, Corum L, Bono JL, Elias AF, Rosa P, Samuels DS, Charon NW. *Borrelia burgdorferi* periplasmic flagella have both skeletal and motility functions. *Proc Natl Acad Sci U S A.* 2000; 97:10899–10904. [PubMed: 10995478]
- Neelakanta G, Sultana H, Sonenshine DE, Marconi RT. An in vitro blood-feeding method revealed differential *Borrelia turicatae* (Spirochaetales: Spirochaetaceae) gene expression after spirochete acquisition and colonization in the soft tick *Ornithodoros turicata* (Acari: Argasidae). *J Med Entomol.* 2017; 54:441–449. [PubMed: 28399292]
- Polcastro PF, Raffel SJ, Schwan TG. *Borrelia hermsii* acquisition order in superinfected ticks determines transmission efficiency. *Infect Immun.* 2013; 81:2899–2908. [PubMed: 23716615]
- Raffel SJ, Battisti JM, Fischer RJ, Schwan TG. Inactivation of genes for antigenic variation in the relapsing fever spirochete *Borrelia hermsii* reduces infectivity in mice and transmission by ticks. *PLoS Pathog.* 2014; 10:e1004056. [PubMed: 24699793]

- Rosa, PA., Hogan, D. Colony formation by *Borrelia burgdorferi* in solid medium: clonal analysis of *osp* locus variants. First International Conference on Tick-Borne Pathogens at the Host-Vector Interface: An Agenda for Research; St. Paul, MN: University of Minnesota; 1992. p. 95-103.
- Rosa PA, Schwan T, Hogan D. Recombination between genes encoding major outer surface proteins A and B of *Borrelia burgdorferi*. Mol Microbiol. 1992; 6:3031–3040. [PubMed: 1479892]
- Samuels DS. Electrotransformation of the spirochete *Borrelia burgdorferi*. Methods Mol Biol. 1995; 47:253–259. [PubMed: 7550741]
- Schwan TG, Hinnebusch BJ. Bloodstream- versus tick-associated variants of a relapsing fever bacterium. Science. 1998; 280:1938–1940. [PubMed: 9632392]
- Schwan TG, Raffel SJ, Schrumph ME, Policastro PF, Rawlings JA, Lane RS, Breitschwerdt EB, Porcella SF. Phylogenetic analysis of the spirochetes *Borrelia parkeri* and *Borrelia turicatae* and the potential for tick-borne relapsing fever in Florida. J Clin Microbiol. 2005; 43:3851–3859. [PubMed: 16081922]
- Stoenner HG. Biology of *Borrelia hermsii* in Kelly medium. Appl Microbiol. 1974; 28:540–543. [PubMed: 4472988]

Fig. 1. Colonies of *B. hermsii* DAH. (A) *B. hermsii* wildtype 8 d after plating and (B) a non-motile *fliH* mutant 12 d after plating.

Fig. 2. Plating efficiencies of relapsing fever isolates *B. hermsii* and *B. turicatae* in 5% CO₂/3% O₂. Data represents the mean and standard deviation from 3 independent experiments.

Fig. 3.

Plating efficiencies of *B. hermsii* and *B. turicatae* at pH 7.0, 7.35, and 7.5 in 5% CO₂/3% O₂. Data represents the mean and standard deviation from one experiment. Statistical significance determined by two-way ANOVA with Tukey's Multiple Comparison Test, * P< 0.05, **** P< 0.0001.

Fig. 4.

Fig. 4A. Plating efficiency of *B. hermsii* and *B. turicatae* in low (3%) or atmospheric (18%) oxygen and 5% CO₂. Data represents the mean and standard deviation from 2 independent experiments for *B. hermsii* and 3 independent experiments for *B. turicatae* (**** P < 0.0001 (t-test)).

Fig. 4B. *Borrelia turicatae* and *B. hermsii* DAH plated at two different oxygen concentrations of 3% versus 18% O₂ and 5% CO₂. Note the varied colony morphologies at higher oxygen concentrations: black arrow, tight colony, and white arrow, diffuse colony. Plates are representative from 3 independent experiments for *B. turicatae* and 2 independent experiments for *B. hermsii*.

Fig. 5.
Plating of *B. hermsii* DAH from infected mouse blood from four 10-fold serial dilutions.

Fig. 6.

Quantification of *B. hermsii* DAH spirochetes in infected *O. hermsi* ticks by plating in solid medium. Infected unfed adults had not had a blood meal in 3 years, while the fed nymphs had just received a blood meal from an infected mouse.

Table 1Comparison of methods for quantifying *Borrelia hermsii* in infected mouse blood

Counting Method	Mouse 1 (Spirochetes/ml)	Mouse 2 (Spirochetes/ml)
QPCR	2.3×10^8	1.6×10^8
Petroff-Hausser Counter	5.5×10^7	4.8×10^7
Plating (CFU)	3.1×10^7	2.0×10^7

Table 2Colony counts of *B. hermsii* from infected *O. hermsi* tick tissues

Tick	Tissue	# of Colonies	% of Total
1	Midgut	203	22.8
	Salivary Glands	199	22.3
	Remainder	489	54.9
	Total	891	100
2	Midgut	108	70.1
	Salivary Glands	10	6.5
	Remainder	36	23.4
	Total	154	100
3	Midgut	6	9.5
	Salivary Glands	19	30.2
	Remainder	38	60.3
	Total	63	100