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Original article

Colony formation in solid medium by the relapsing fever spirochetes *Borrelia hermsii* and *Borrelia turicatae*

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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.

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*, 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% CO_2 and either 3% O_2 or atmospheric O_2 (18% in Hamilton, MT) in a Forma Series II Water Jacket CO_2/O_2 incubator (Thermo Fisher Scientific, Inc., Waltham, MA, USA) with the caps loosely attached on the tubes.

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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 CO_2/O_2 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 (4.77g/L HEPES, 2.92 g/L NaCl, pH 7.6), re-suspended 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 intra-cardiac 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% H_20_2, 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 1×10^2 cells/ml to 1×10^{10} 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.