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Generation of mammalian host-adapted *Borrelia burgdorferi* by cultivation in peritoneal dialysis membrane chamber implantation in rats

Melissa J. Caimano, Ph.D.

Departments of Medicine, Pediatrics and Molecular Biology & Biophysics, UConn Health, Farmington, Connecticut, 06030-3715

Abstract

The transmission, survival and virulence of *Borrelia burgdorferi* depend upon the spirochete's ability to modulate its transcriptome as it cycles between its arthropod vector and reservoir host. This complex adaptive process is collectively referred to as "host-adaptation". The paucibacillary nature of borrelian infections precludes the detailed analysis of host adaptation within infected mammalian tissues. To circumvent this limitation, Akins *et al.* (1) developed a model system whereby spirochetes are cultivated within dialysis membrane chambers (DMCs) surgically implanted within the peritoneal cavity of a rat. Spirochetes within DMCs are exposed to many, if not all, of the environmental signals and physiological cues required for mammalian host adaptation but are protected from clearance by the host's immune system.

Keywords

Borrelia burgdorferi; Lyme disease; spirochetes; host adaptation; gene expression; animal models

1. Introduction

Lyme disease, the most prevalent vector-borne illness in the United States (2), is a chronic, multisystem disorder caused by the spirochete *Borrelia burgdorferi* (3, 4). In nature, *B. burgdorferi* has an obligate enzootic cycle involving a mammalian reservoir host and an *Ixodes* tick vector (5). In order to persist within these markedly different milieus, spirochetes must alter their transcriptional, antigenic and metabolic profiles in response to mammalian host- and tick-specific signals, a process we refer to as "host adaptation" (5–9). While some aspects of host adaptation can be reproduced by manipulating growth conditions *in vitro* (*i.e.*, increased growth temperature and/or decreased pH)(10–13), repression of tick-phase genes, most notably *ospA*, *bba62/lp6.6* and the *glp* operon, requires one or more as-yet-undefined mammalian host signals (6, 13–16) (Figure 1). Here we describe a protocol for the

⁸Try to make the DMC as taut as possible; this will help to maneuver the chamber into the peritoneal cavity during surgery. Larger DMCs may interfere with normal intestinal or bladder functions.

⁹Sprague-Dawley rats are the preferred host animal for this procedure. In cases where extraordinarily large numbers of organisms are required, New Zealand white rabbits (3 – 5 kg) may be used. Each rabbit is implanted with three DMCs, each containing ~20–25 mLs of spirochetes diluted as described in 3.2.2. Consult your attending veterinarian for details regarding operative and post-operative details.

generation of mammalian host adapted *B. burgdorferi* by cultivation within dialysis membrane chambers (DMCs)(1). This elegant and versatile model involves the implantation of spirochetes contained within DMCs into the peritoneal cavities of rats for a 12- to 14-day incubation period. The small pore size (8000 Da molecular weight cut-off) of the dialysis tubing used for this procedure permits access to host nutrients but excludes larger molecules, including host antibodies and immune effector cells. Spirochetes cultivated in DMCs are suitable for a wide-range of routine molecular techniques, including qRT-PCR, SDS polyacrylamide gels, immunoblotting and protein localization studies. The DMC model is the only method currently available for obtaining the large number of host-adapted organisms required for genome-wide transcriptomic and proteomic analyses. Lastly, the DMC model provides a facile means of refining the virulence-related phenotypes of *B. burgdorferi* mutants that are unable to establish infection within mice (17–20). This procedure also has been used to cultivate *B. hermsii* (21), pathogenic *Leptospira interrogans* (22, 23) and avirulent (Lcr-) *Yersinia pestis*.

NOTE: All procedures using live animals must conform to governmental and institutional guidelines and regulations regarding the care and use of laboratory animals (e.g., *The Guide for the Care and Use of Laboratory Animals*, 8th edition, National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals).

CAUTION: *Borrelia burgdorferi* is a BSL-2 level pathogen. Animals exposed to *B. burgdorferi* should be handled and disposed of according to Animal Biosafety Levels (ABSL) guidelines for use of infectious agents and vertebrate animals (e.g., *The Biosafety in Microbiological and Biomedical Laboratories* manual, 5th Edition).

2. Materials

2.1 Preparation of DMCs

1. EDTA Stock solution: 0.5 M EDTA, pH 8.0. Add ~50 mLs of ultrapure water to a 300 mL beaker containing a stir bar. Stir vigorously on a magnetic stir plate. Weigh 18.6 grams of EDTA·Na₂·2H₂O (Molecular Weight 372.24) and transfer to the beaker containing water. Adjust pH to 8.0 using NaOH pellets or 10 N NaOH. Adjust the volume to 100 mLs with ultrapure water and continue stirring (see Note 1). Filter sterile using a 0.22 µm filter unit. Store at 15 - 25°C for several months. For preparation of sterile dialysis tubing, add 2 mLs of stock solution per liter ultrapure water; final concentration = 1 mM.
2. Dialysis membrane tubing: Regenerated cellulose dialysis membrane tubing, 6000 to 8000 MWCO, 32 mm width.
3. BSK medium: BSK growth medium (24, 25) may be produced in-house or purchased from commercial sources (see Note 2).

¹EDTA may not dissolve completely until the solution reaches pH 8.0 at 25°C. Alternatively, sterile-filtered ultrapure 0.5 M EDTA, pH 8.0 can be purchased from a commercial vendor.

²Lot to lot variations in BSK medium components, bovine serum albumin in particular, can have a significant impact on the infectivity and pathogenicity of *B. burgdorferi* isolates (26, 27). Please verify that the BSK used for DMC implantation supports the growth of virulent *B. burgdorferi* under standard *in vitro* growth conditions. We also recommend that individual batches of BSK medium are

2.2 Peritoneal Implant Procedure

1. Sprague-Dawley rats: male or females, 175 – 200 g at the time of use, purchased from approved vendors.
2. Surgical instruments: An instrument pack typically contains No. 10 scalpel blade handle, 4 in. iris scissors, 5-½ in tissue forceps (1x 2-tooth dissecting or Adson-Brown), 5-½ in. blunt-end forceps, (optional) 5-½ needle-holder forceps with built-in scissors (*e.g.*, Olsen Hegar). Instruments should be autoclaved inside self-sealing sterilization pouches just prior to the procedure. A fresh pack of sterile instruments should be used on each animal. Alternatively, instruments may be sterilized between animals using a glass bead sterilizer. Be sure to allow instruments to cool prior to use.
3. Suture: Ethicon 4-0, SH-1, 27 in., coated Vicryl, violet-braided suture or equivalent.
4. 70% ethanol: Dilute 70 mLs of absolute ethanol with 30 mLs ultrapure water. Store at room temperature in a clean spray bottle.
5. Ketamine/xylazine anesthetic cocktail: 30 mg/mL Ketamine, 3 mg/mL xylazine-HCl. Prepare a working solution of ketamine-HCl (30 mg/mL) and xylazine-HCl (3 mg/mL) from commercial veterinary-grade stock solutions using sterile ultrapure water. Based on weight, inject animals intramuscularly with an appropriate volume of anesthetic cocktail (30–50 mg per kg ketamine and 3–5 mg per kg xylazine). Store the cocktail at room temperature in a secure location. Length of storage is based on the expiration date of the original stock solutions for each drug.
6. Carprofen: Prepare a working solution of carprofen (5 mg/mL) from commercial veterinary-grade stock solution using sterile ultrapure water. Based on weight, inject animals subcutaneously with an appropriate volume of analgesia (5–10 mg per kg). Store at 4°C in a secure location. Length of storage is based on the expiration date of the original stock solution.

3. Methods

The success of this protocol is dependent on the use of strict aseptic technique during all stages of dialysis membrane chamber preparation and animal surgery.

3.1 Preparation of sterile dialysis membrane tubing

Prepare all solutions using ultrapure deionized water (18 MΩ -cm at 25°C).

1. Fill three clean, sterile 2-Liter heavy-duty Pyrex beakers as follows:

Beaker 1: 1 - 1.5 Liters ultrapure water

tested to ensure that spirochetes grown in the medium properly induce expression of OspC following a temperature-shift from 23C to 37C.

Beaker 2: 1 - 1.5 Liters 1 mM EDTA, pH 8.0 (prepared using 0.5 M stock solution)

Beaker 3: 1 - 1.5 Liters ultrapure water

2. Add a 2–3 inch magnetic stir bar to each beaker, cover with heavy-duty aluminum foil and autoclave at 121°C for 45–50 min.
3. Place each beaker on stirrer hot plate and bring to a rolling boil with constant stirring.
4. Wearing sterile gloves, cut dialysis tubing into 7–9 inch strips using sterile scissors (one strip per animal). Gently tie one end of the tubing using an overhand knot. Trim away excess tubing from the knotted end using sterile scissors (*see* Note 3).
5. Place the knotted tubing into Beaker 1. Boil for 15–20 min. with constant stirring. (*see* Note 4). Turn off heat and stirring.
6. Using sterile extra-long forceps, transfer tubing to Beaker 2 and boil for 15–20 min. with constant stirring. Turn off heat and stirring.
7. Transfer tubing to Beaker 3 and boil for 15–20 min. with constant stirring. Turn off heat and stirring, and allow the beaker containing tubing to cool.
8. Once the outside is cool to the touch, transfer Beaker 3 to a Biological Safety Cabinet (BSC) (*see* Note 5).

3.2 Preparation of DMCs

To ensure sterility, prepare and handle all materials in the Biological Safety Cabinet. Movement around the BSC should be restricted during the procedure.

1. Grow spirochetes to mid-logarithmic phase at 33–37°C in BSK medium. Count by dark field microscopy using a Petroff-Hauser counting chamber (*see* Note 6).
2. Prior to beginning work in the BSC, the entire surgical field, along with the surfaces of equipment used to fill the tube (*e.g.*, automatic pipette) should be cleaned using 70% alcohol (*see* Note 7).
3. Working in the BSC, dilute culture to 3000 organisms per mL using fresh BSK medium. Prepare 10 mLs of diluted culture per DMC and aliquot into individual sterile 50-mL conical tubes. Loosen the caps of each tube for easy removal.

³It is a good idea to prepare 1–2 extra strips of tubing. Discard any unused tubing at the end of the procedure.

⁴The strips of tubing should remain submerged at all times. Beakers should remain loosely covered throughout the procedure to allow steam to escape. Lift lids carefully.

⁵Dialysis tubing may be prepared several days in advance and stored at 4°C. After boiling, transfer tubing to the bottom portion of a 0.22 µm filter unit using sterile blunt end forceps. Replace the filter unit top and filter ~100 mLs of ultrapure water from Beaker 3 into the bottom. Once capped, the container should be opened only within a BSC.

⁶The background strain used for DMC studies must contain *bbe22* (*pncA*), which is encoded on the endogenous 25 kilobase linear plasmid lp25. Moreover, we highly recommend that a wild-type strain known to host-adapt properly be included as a positive control in each experiment.

⁷The sterile inner packaging of individually wrapped surgical gloves, opened inside the BSC, can be used as a work surface.

4. After donning sterile surgical gloves, carefully remove a strip of tubing from Beaker 3 using blunt end forceps. Hold the tubing upside down to allow any excess water to drip off. Grasp the open end between your index finger and thumb, turn open side-up and twist gently to open. With your free hand, remove the cap of one 50-mL conical tubes and transfer up to 9 mLs of diluted bacteria to the tubing using a 10-mL disposable serological pipette (Figure 2A). While continuing to hold the open end, flatten the unfilled tubing using the thumb and index finger of your free hand to eliminate air bubbles and gently twist the tubing to remove any remaining void volume. Tie-off the top of the tubing strip using an overhead knot (Figure 2B). Using sterile scissors, remove excess tubing. Properly filled DMC should be ~1.5 – 2 in. (see Video 1)
5. Place each DMC into a separate sterile 50-mL conical tube containing ~5 mLs of fresh BSK medium and proceed immediately to the Peritoneal Implant procedure (Section 3.3).

3.3 Peritoneal implantation procedure

1. Working outside of the BSC, anesthetize rats by intramuscular injection with the ketamine/xylazine cocktail. Once sedate, apply a small amount of ophthalmic ointment to each eye. Shave the abdomen using a commercial beard trimmer and remove loose fur using successive washes with 70% alcohol.
2. Prepare the surgical site using successive washes with a topical broad-spectrum antiseptic solution (e.g., Betadine®, Surgisrub) followed by 70% alcohol and then cover with sterile 4 × 4 in. gauze.
3. Administer pre-operative analgesia (e.g., Carprofen).
4. Transfer the animal to the BSC and place on a sterile drape in a supine position.
5. Prior to beginning, assess the animal's level of anesthesia by performing a 'toe pinch' and remove the gauze covering the surgical site.
6. Using a sterile No. 10 scalpel, make a 2 in. incision through the skin, starting ~1/2 in. below the xiphoid process (Figure 3A). Using tissue forceps (5-1/2 in. [~14 cm], 1x 2-tooth dissecting or Adson-Brown), lift the skin on either side of the incision and gently trim the fascia connecting the skin to the abdominal wall using a No. 10 scalpel. Repeat on the other side. Using the same scalpel (or iris scissors), make a small incision (~1.5 in.) in the abdominal wall, using the *linea alba* as guide (see Video 2). The incision should be clean and straight.
7. Using tissue forceps, raise one side of the abdominal incision and position a DMC inside rat peritoneal cavity (Figure 3B and Video 2). Gently push the DMC towards either side of the peritoneal cavity to prevent it from being nicked/ruptured while suturing the incision. Be sure to position the DMC so that it does not interfere with intestine or bladder function.
8. Close the abdominal incision by suturing each end with double knots. Working upwards, place additional sutures ~2–3 mm apart.

9. Close the skin incision using a contiguous line of 9 mm AutoClip® wound clips (Figure 3C). Alternatively, the skin excision may be closed using subcuticular sutures followed by liquid skin adhesive (*e.g.*, Nexaband).
10. Transfer the rat to a recovery chamber. Alternatively, animals may be placed on top of a clean surgical drape inside a cage containing fresh bedding. To maintain the appropriate animal core body temperature, the cage should be placed over a circulating water thermal pad. Monitor continuously until animals are alert and responsive.
11. Administer analgesia (*e.g.*, Carprofen) for at least two days post-operatively.
12. Animals should be monitored at least once daily for the first week post-operatively and then every third day thereafter. If animals show any sign of distress or discomfort, consult institutional veterinary staff immediately.

3.4 DMC explantation and recovery of host adapted organisms

1. Twelve to 14 days post-implantation, euthanize rats by an approved method (*e.g.*, CO₂ narcosis). Once death has been confirmed, place the animal inside a BSC in a supine position.
2. Using tissue forceps, lift one end of the incision site and cut through the skin and abdominal wall using sterile scissors. Using the same forceps, locate and remove the DMC and transfer to a sterile 50-mL conical tube.
3. Make a small cut in the tubing just below the knot. Slowly remove the DMC contents using a sterile, disposable serological pipet and transfer to a sterile 15-mL conical tube. Alternatively, DMC fluid can be recovered using an 18-G, 1 in. needle attached to a sterile 10-mL syringe. However, aspiration by syringe may damage the organism's fragile outer membrane.
4. Examine a small aliquot of DMC fluid from rat by dark field microscopy and determine cell density. Proceed to downstream applications (*e.g.*, RNA isolation, preparation of whole cell lysates for SDS-PAGE and/or immunoblot).

3.5 Processing mammalian host-adapted organisms for SDS-PAGE

1. Centrifuge fluid collected from DMCs at 8000 g for 15 minutes at 4°C.
2. Gently wash pellet with an equal volume of cold PBS and centrifuge as above.
3. Resuspend the cell pellet in 1 mL cold PBS and transfer to a sterile microfuge tube. Centrifuge at 4°C for 10 minutes at 12–14,000 g.
4. Resuspend the pellet in 30–50 µL PBS.
5. Solubilize cells using protein sample buffer (*e.g.*, Laemmli buffer) and separate by SDS-PAGE. Typically, 2×10^7 organisms is sufficient for a mini-PROTEAN gel (Figure 1).
6. Store remaining solubilized sample at –20°C.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Akins DR, Bourell KW, Caimano MJ, Norgard MV, Radolf JD. A new animal model for studying Lyme disease spirochetes in a mammalian host-adapted state. *J Clin Invest.* 1998; 101:2240–2250. [PubMed: 9593780]
2. Bacon RM, Kugeler KJ, Mead PS. Surveillance for Lyme disease--United States, 1992–2006. *MMWR Surveill Summ.* 2008; 57:1–9.
3. Steere AC, Glickstein L. Elucidation of Lyme arthritis. *NatRevImmunol.* 2004; 4:143–152.
4. Steere AC, Grodzicki RL, Kornblatt AN, Craft JE, Barbour AG, Burgdorfer W, Schmid GP, Johnson E, Malawista SE. The spirochetal etiology of Lyme disease. *N Engl J Med.* 1983; 308:733–740. [PubMed: 6828118]
5. 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; 10:87–99. [PubMed: 22230951]
6. Iyer R, Caimano MJ, Luthra A, Axline D Jr, Corona A, Iacobas DA, Radolf JD, Schwartz I. Stage-specific global alterations in the transcriptomes of Lyme disease spirochetes during tick feeding and following mammalian host adaptation. *Mol Microbiol.* 2015; 95:509–538. [PubMed: 25425211]
7. Pal, U., Fikrig, E. Tick Interactions. In: Samuels, DS., Radolf, JD., editors. *Borrelia Molecular Biology, Host interactions and Pathogenesis.* Caister Academic Press; Norfolk, UK: 2010. p. 279–298.
8. Skare, JT., Carroll, JA., Yang, XF., Samuels, DS., Akins, DR. Gene Regulation, Transcriptomics, and Proteomics. In: Samuels, DS., Radolf, JD., editors. *Borrelia Molecular Biology, Host Interactions and Pathogenesis.* Calister Academic Press; Norfolk, UK: 2010.
9. Groshong AM, Blevins JS. Insights into the biology of *Borrelia burgdorferi* gained through the application of molecular genetics. *Adv Appl Microbiol.* 2014; 86:41–143. [PubMed: 24377854]
10. Yang X, Goldberg MS, Popova TG, Schoeler GB, Wikel SK, Hagman KE, Norgard MV. Interdependence of environmental factors influencing reciprocal patterns of gene expression in virulent *Borrelia burgdorferi*. *Mol Microbiol.* 2000; 37:1470–1479. [PubMed: 10998177]
11. Stevenson B, Schwan TG, Rosa PA. Temperature-related differential expression of antigens in the Lyme disease spirochete, *Borrelia burgdorferi*. *Infect Immun.* 1995; 63:4535–4539. [PubMed: 7591099]
12. Ojaimi C, Brooks C, Casjens S, Rosa P, Elias A, Barbour A, Jasinskas A, Benach J, Katona L, Radolf J, Caimano M, Skare J, Swingle K, Akins D, Schwartz I. Profiling of temperature-induced changes in *Borrelia burgdorferi* gene expression by using whole genome arrays. *Infect Immun.* 2003; 71:1689–1705. [PubMed: 12654782]
13. Revel AT, Talaat AM, Norgard MV. DNA microarray analysis of differential gene expression in *Borrelia burgdorferi*, the Lyme disease spirochete. *Proceedings of the National Academy of Sciences.* 2002; 99:1562–1567.
14. Samuels DS. Gene regulation in *Borrelia burgdorferi*. *Annu Rev Microbiol.* 2011; doi: 10.1146/annurev.micro.112408.134040
15. Caimano MJ, Eggers CH, Gonzalez CA, Radolf JD. Alternate sigma factor RpoS is required for the *in vivo*-specific repression of *Borrelia burgdorferi* plasmid lp54-borne *ospA* and *lp6.6* genes. *J Bacteriol.* 2005; 187:7845–7852. [PubMed: 16267308]
16. Caimano MJ, Iyer R, Eggers CH, Gonzalez C, Morton EA, Gilbert MA, Schwartz I, Radolf JD. Analysis of the RpoS regulon in *Borrelia burgdorferi* in response to mammalian host signals

- provides insight into RpoS function during the enzootic cycle. *Mol Microbiol.* 2007; 65:1193–1217. [PubMed: 17645733]
17. Purser JE, Lawrenz MB, Caimano MJ, Howell JK, Radolf JD, Norris SJ. A plasmid-encoded nicotinamidase (PncA) is essential for infectivity of *Borrelia burgdorferi* in a mammalian host. *Mol Microbiol.* 2003; 48:753–764. [PubMed: 12694619]
18. Grimm D, Eggers CH, Caimano MJ, Tilly K, Stewart PE, Elias AF, Radolf JD, Rosa PA. Experimental assessment of the roles of linear plasmids lp25 and lp28-1 of *Borrelia burgdorferi* throughout the infectious cycle. *Infect Immun.* 2004; 72:5938–5946. [PubMed: 15385497]
19. Ristow LC, Miller HE, Padmore LJ, Chettri R, Salzman N, Caimano MJ, Rosa PA, Coburn J. The beta(3)-integrin ligand of *Borrelia burgdorferi* is critical for infection of mice but not ticks. *Mol Microbiol.* 2012; 85:1105–1118. [PubMed: 22758390]
20. 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]
21. Colombo MJ, Abraham D, Shibuya A, Alugupalli KR. B1b lymphocyte-derived antibodies control *Borrelia hermsii* independent of Fc α /mu receptor and in the absence of host cell contact. *Immunol Res.* 2011; 51:249–256. [PubMed: 22139824]
22. Caimano MJ, Sivasankaran SK, Allard A, Hurley D, Hokamp K, Grassmann AA, Hinton JC, Nally JE. A model system for studying the transcriptomic and physiological changes associated with mammalian host-adaptation by *Leptospira interrogans* serovar Copenhageni. *PLoS Pathog.* 2014; 10:e1004004. [PubMed: 24626166]
23. Grassmann AA, McBride AJ, Nally JE, Caimano MJ. Generation of mammalian host-adapted *Leptospira interrogans* by cultivation in peritoneal dialysis membrane chamber implantation in rats. *Bio Protoc.* 2015:5.
24. Barbour AG. Isolation and cultivation of Lyme disease spirochetes. *Yale J Biol Med.* 1984; 57:521–525. [PubMed: 6393604]
25. Pollack RJ, Telford SR, Spielman A. Standardization of medium for culturing Lyme disease spirochetes. *J Clin Microbiol.* 1993; 31:1251–1255. [PubMed: 8501226]
26. Wang G, Iyer R, Bittker S, Cooper D, Small J, Wormser GP, Schwartz I. Variations in Barbour-Stoenner-Kelly culture medium modulate infectivity and pathogenicity of *Borrelia burgdorferi* clinical isolates. *Infect Immun.* 2004; 72:6702–6706. [PubMed: 15501807]
27. Yang X, Popova TG, Goldberg MS, Norgard MV. Influence of cultivation media on genetic regulatory patterns in *Borrelia burgdorferi*. *Infect Immun.* 2001; 69:4159–4163. [PubMed: 11349092]

Figure 1.

Representative whole cell lysates of *B. burgdorferi* wild-type strain 297 cultivated to late-logarithmic phase in BSK-II medium at 23°C and 37°C following temperature-shift *in vitro* and within dialysis membrane chambers (DMC) implanted into the peritoneal cavities of female Sprague-Dawley rats. Lysates were loaded according to the numbers of organisms ($\sim 2 \times 10^7$ per lane), separated on a 12.5% SDS polyacrylamide gel, and then stained with silver. Molecular mass markers (kDa) are indicated on the left.

Figure 2.

A. Fill dialysis tubing using a sterile serological pipette. **B.** Close the DMC using an overhand knot.

Figure 3.
Peritoneal Implant Procedure. **A.** Abdominal incision site. **B.** Placement of DMC within peritoneal cavity. **C.** Closure of the skin surrounding the incision site using wound clips.