HHS Public Access

Author manuscript

Methods Mol Biol. Author manuscript; available in PMC 2018 February 16.

Published in final edited form as:

Methods Mol Biol. 1995 ; 47: 253–259. doi:10.1385/0-89603-310-4:253.

Electrotransformation of the Spirochete *Borrelia burgdorferi*

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1. Introduction

Borrelia burgdorferi is an etiologic agent of Lyme disease, the most common arthropod-borne disease in the United States (1,2). The bacterium, a member of the spirochete phylum, has a genome predominantly composed of linear DNA molecules (3,4). Formulating a medium in which *B. burgdorferi* grows in vitro was the first step toward a genetic understanding of the physiology and pathogenesis of the organism (5,6). The growth of *B. burgdorferi* as single colonies in solid medium (7–9) has facilitated mutant isolation by selection (10,11), although a defined medium for selection of auxotrophs is not currently available. The transformation system described in this chapter will be useful for manipulating the spirochete on a molecular genetic level.

Electroporation is the use of an electric pulse to permeabilize cell membranes reversibly (12) and is an extremely efficient method of genetically transforming bacteria (13). Electrotransformation has been used to disrupt a hemolysin gene in the spirochete *Serpulina hyodysenteriae,* an etiologic agent of swine dysentery, by homologous recombination (14). The effect of electroporation buffers and capacitance on the survival of *B. burgdorferi* has been reported (15), and electrotransformation has been employed to insert point mutations conferring antibiotic resistance into the *gyrB* gene by homologous recombination (16).

This chapter provides detailed methods for introducing DNA into *B. burgdorferi* by electroporation and for the selection of transformants (or spontaneous mutants) on solid medium. We typically obtain transformation efficiencies of 10^3 transformants/μg of linear DNA. The protocol may work for other species of the genus *Borrelia.* The most critical parameter appears to be the growth phase or cell density of the culture when collected. The cells are washed extensively, twice in cold phosphate-buffered saline and three times in a cold osmotically buffered low-ionic-strength solution, and concentrated to about 10^{10} cells/mL. They are genetically transformed by adding DNA in a low-ionic-strength solution and pulsing with a short-duration, high-intensity exponential decay electric field. Transformants are selected in solid medium containing an antibiotic (or an antibody). Unfortunately, there are both biosafety and physiological limitations on the use of many antibiotics with *B. burgdorferi* species.

2. Materials

1. *B. burgdorferi* strain B31 is available from American Type Culture Collection (Rockville, MD).

2. Barbour-Stoenner-Kelly (BSK) II medium (without gelatin): 8% (v/v) 10X CMRL-1066 (without L-glutamine and sodium bicarbonate; Life Technologies, Gaithersburg, MD), 4 g/L Neopeptone (Difco, Detroit, MI), 40 g/L bovine serum albumin (BSA; fraction V, Pentex; Miles, Kankakee, IL), 1.6 g/L Yeastolate (TC; Difco), 4.8 g/L *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 4 g/L glucose, 0.56 g/L sodium citrate, 0.64 g/L sodium pyruvate, 0.32 g/L *N*-acetyl-D-glucosamine, 1.76 g/L sodium bicarbonate, and 6.6% rabbit serum (trace hemolyzed; Pel-Freez, Rogers, AR). Adjust to pH 7.6 with 1*N* NaOH, stir slowly for 2–3 h, and sterilize by filtration (successively through a prefilter, a 1.2-μm filter, a 0.45-μm filter, and a 0.22-μm filter). Store at 4°C for up to 2 mo (*see* Note 1).

3. Dulbecco's phosphate-buffered saline (dPBS): 8 g/L NaCl, 0.2 g/L KCl, 1.15 g/L Na_2HPO_4, and 0.2 g/L KH_2PO_4. Sterilize by filtration and store at 4°C.

4. dPBS with divalent cations ($dPBS^{2+}$): Add 0.1 g/L $CaCl_2$ and 0.2 g/L $MgCl_2 \cdot 6H_2O$ to dPBS.

5. Electroporation solution (EPS): 93 g/L sucrose and 15% (v/v) glycerol. Sterilize by filtration and store at 4°C.

6. Plating-BSK (P-BSK) medium: Add 83 g BSA, 8.3 g Neopeptone, 10 g HEPES, 1.2 g sodium citrate, 8.3 g glucose, 1.3 g sodium pyruvate, 0.7 g *N*-acetyl-D-glucosamine, 3.7 g sodium bicarbonate, and 4.2 g Yeastolate to 1 L of water (18 MΩcm). Adjust to pH 7.5 with 1*N* NaOH, stir slowly for 2–3 h, and sterilize by filtration (successively through a prefilter and a 0.22-μm filter). Store at 4°C for up to 6 mo.

7. 1.7% agarose: Use high-strength, analytical-grade.

8. Antibiotic solution (for selection of transformants): 50 mg/mL of coumermycin A_1 in dimethyl sulfoxide (*see* Note 2). Store at −20°C for up to 6 mo. 25 mg/mL Novobiocin (a less expensive coumarin antibiotic, can be used in place of coumermycin A_1 (Sigma, St. Louis, MO). Make fresh or store in small fractions at −20°C in the dark for up to 6 mo.

9. 5% sodium bicarbonate: prepared fresh and filter-sterilized.

[1]The quality of BSA varies by source and lot. We have found Miles to be a reliable source. However, we reserve 5- or 10-kg batches and test samples for the ability to support the growth of *B. burgdorferi*. Pretested BSK II medium without gelatin can be purchased from Sigma (BSK-H), but it is expensive. We have stored BSK-H medium (without serum) at 4°C for up to 2 yr and found that it can support the growth of highly passaged strain B31 on the addition of fresh serum.

[2]The only antibiotic that is not clinically useful and has been shown to be effective for selection of resistant mutants is coumermycin A_1 (11,17). However, a preliminary report suggests that *B. burgdorferi* can be electrotransformed with a gene that confers chloramphenicol resistance (D. Persing and D. Podzorski, personal communication), and the spirochete *S. hyodysenteriae* has been electrotransformed with a gene that confers kanamycin resistance (14). In addition, bactericidal antibodies have been used to select for mutants of *B. burgdorferi* (10,18,19).

3. Methods

3.1. Preparation of Competent Cells

1. Inoculate 500 mL of BSK II medium in a 500-mL screw-top bottle with 1 mL of a late-log-phase culture (*see* Note 3). Incubate at 32–34°C (without agitation) until the culture reaches a density of about 5×10^7 cells/mL (*see* Note 4). This requires 36–96 h.
2. Transfer culture to two sterile 250-mL screw-top centrifuge bottles and cap.
3. Centrifuge at 4000*g* for 20 min at 4°C. Decant the supernatant fraction and resuspend each cell pellet in 30 mL of cold dPBS (*see* Note 5).
4. Transfer cells to two sterile 50-mL screw-top centrifuge tubes and cap.
5. Centrifuge at 3000*g* for 10 min at 4°C. Decant the supernatant fraction, and resuspend each cell pellet in 30 mL of cold dPBS.
6. Centrifuge at 3000*g* for 10 min at 4°C. Decant the supernatant fraction, and resuspend each cell pellet in 10 mL of cold EPS.
7. Transfer cells to two sterile 14-mL polypropylene tubes and cap.
8. Centrifuge at 2000*g* for 10 min at 4°C. Decant the supernatant fraction, and resuspend each cell pellet in 10 mL of cold EPS. Repeat.
9. Centrifuge at 2000*g* for 10 min at 4°C. Decant the supernatant fraction, and pool the cell pellets in 0.6 mL of cold EPS (*see* Note 6).
10. Distribute 50-μL aliquot fractions of the cell suspension into sterile 1.7-mL tubes on ice (*see* Notes 7 and 8).

3.2. Electroporation

1. Cool electroporation cuvets (0.2-cm electrode gap) to 4°C.

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[3] *B. burgdorferi* is a class 2 human pathogen and therefore should be handled in a class II biological safety cabinet (laminar flow hood). In addition, BSK II medium is rich, and all procedures should be performed aseptically. Introduction of recombinant DNA into a class 2 pathogen requires permission from the Institutional Biosafety Committee before initiation of the experiments according to Section IIIB of the Guidelines for Research Involving Recombinant DNA Molecules (Federal Register).

[4] The cell density (or growth phase) is a significant factor for successful electrotransformation, as is the case with other bacteria (12,20). The cells will not transform efficiently if the cell density is too high (when the color of the medium changes). We have had success electrotransforming cultures harvested at $1–7 \times 10^7$ cells/mL, although a low cell density ($1–2 \times 10^7$ cells/mL) requires pelleting the cells at a higher *g* force (up to 5000*g*) and adjusting the final volume of the cell suspension (*see* Note 6). Cell density should be determined using a Petroff Hausser Counting Chamber (Hausser Scientific Partnership, Horsham, PA). Dilute 0.1 mL of the culture with 0.9 mL of cold $dPBS^{2+}$ and place in the counting chamber. Count cells over all 25 groups of 16 small squares in all planes using a dark-field microscope. Multiply the number of cells counted by 5×10^5 to calculate cells/mL. Alternatively, cell density can be determined by spectrophotometry (17). Centrifuge 10 mL of the culture at 5000*g* for 10 min. Decant the supernatant fraction, and resuspend the cell pellet in 1 mL of $dPBS^{2+}$. Centrifuge at 8000*g* for 5 min. Decant the supernatant fraction, resuspend the cell pellet in 1 mL of $dPBS^{2+}$, and measure the A_{600}. Multiply the A_{600} by 1.4×10^8 to calculate cells/mL in the culture.

[5] Thorough washing is important to remove components of the medium (*see* Note 10). Cell pellets are resuspended in both dPBS and EPS by pipeting followed by vortex mixing. These treatments do not appear to affect cell viability.

[6] The final cell concentration should be $1–5 \times 10^{10}$ cells/mL (with a final volume of about 0.9 mL). The volume of EPS used to resuspend the final cell pellet may have to be adjusted to account for initial cell number and efficiency of decanting.

[7] We find that use of presterilized aerosol-resistant pipet tips (with aerosol barriers) helps to maintain sterility when handling small volumes of liquid.

[8] We have not examined the effect of temperature on transformation efficiency, but maintaining the competent cells at 4°C is generally considered to yield optimal efficiencies (12,20). As with other bacterial species (20), competent cells can be stored at −70°C without a significant loss of transformation efficiency.

2. Transfer 1–5 μL of a solution containing 0.3–1 μg of DNA in water (*see* Notes 9 and 10) to the cell suspension, mix gently, and incubate on ice for about 1 min.

3. Transfer the cell/DNA mixture to a chilled electroporation cuvet. Cap the cuvet, and shake the cell/DNA mixture to the bottom of the cuvet so that it spans the two electrodes.

4. Place the cuvet in the pulse generator, and deliver a single exponential decay pulse of 2.5 kV, 25 μF, and 200 Ω. This should produce a time constant of 4–5 ms (*see* Notes 10 and 11).

5. Immediately (within 1 min), add 1 mL of BSK II medium (at room temperature) without antibiotics, and mix the cell suspension by pipeting up and down.

6. Transfer the entire mixture to a sterile 14-mL tube that contains an additional 9 mL of BSK II medium (at room temperature) and incubate (without agitation) at 32–34°C for 20 h.

3.3. Selection of Transformants

1. Mix 240 mL of P-BSK medium, 38 mL of 10X CMRL-1066, and 12 mL of rabbit serum. Equilibrate the mixture at 55°C in a water bath. Autoclave 200 mL of 1.7% agarose, equilibrate to 55°C, and combine with the medium mixture. Add 20 mL of fresh 5% sodium bicarbonate with antibiotics (the final volume is 510 mL) (*see* Notes 2 and 12).

2. Transfer 15 mL of the molten medium into 12–14 100-mm dishes, and allow to solidify at room temperature. Equilibrate the remainder of the molten medium at 42°C.

3. Transfer 0.1 mL of BSK II medium containing the electroporated cells to a 50-mL tube. Add 20 mL of the molten medium (at 42°C), and mix by pipeting up and down once. Transfer the mixture to the plates containing the solidified bottom agarose medium and allow to solidify at room temperature.

4. Centrifuge the remaining 9.9 mL of culture at 8000*g* for 5 min, resuspend in 1 mL of supernatant fraction, and plate as above.

5. Incubate the plates at 32–34°C in a humidified 5% CO_2 atmosphere. Colonies will appear in about 14 d.

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[9]We routinely obtain 1000 or more transformants/μg of DNA with strain B31, although we have only used linear DNA generated by PCR as an electrotransformation substrate (16). Linear molecules are 1000-fold less efficient in electrotransformation of *Escherichia coli* than circular molecules (12), and we are currently constructing circular replicons for use in *B. burgdorferi*. We have recently shown that *B. burgdorferi* can be transformed with oligonucleotides.

[10]Electroporation in the presence of high-ionic-strength solutions causes arcing (and a lower time constant). Two arcs will kill all of the *B. burgdorferi* cells. We use the Wizard DNA purification system (Promega, Madison, WI) and elute the DNA at a high concentration in water. Transformation efficiency generally increases with DNA concentration (12,20).

[11]Preliminary studies suggest that one pulse effected higher transformation efficiencies than multiple pulses and that varying the resistance from 100–400 Ω affected the time constant, but did not significantly alter the transformation efficiency.

[12]An antibiotic concentration that inhibits bacterial growth in liquid culture by 80–90% relative to growth in the absence of antibiotics has been used to select for spontaneous mutants and transformants in solid medium (11,16,17). We currently use 0.2 μg/mL coumermycin A_1 or 5 μg/mL novobiocin for selection.

6. Isolate single colonies by picking with a plugged 15-cm Pasteur pipet (with bulb). Transfer to 10 mL of BSK II in the presence of antibiotics. Cultures will reach late-log phase in 6–9 d.

Acknowledgments

I thank Claude Garon for support and Kit Tilly for manuscript review.

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