Genetic Manipulation of *Borrelia burgdorferi*

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Abstract

The development of robust genetic tools to manipulate *Borrelia burgdorferi*, the etiologic agent of Lyme disease, now allows investigators to assess the role(s) of individual genes in the context of experimental Lyme borreliosis. This unit is devoted to the description of experimental approaches that are available for the molecular genetic analysis of *B. burgdorferi* with an emphasis placed on the cultivation, electrotransformation, the selection of desired mutants, and the genetic complementation of the mutants acquired. The intent here is to provide a consensus protocol that encapsulates the methodologies currently employed by the *B. burgdorferi* research community and describe pertinent issues that must be accounted for when working with these pathogenic spirochetal bacteria.

Introduction

Borrelia burgdorferi, the spirochetal bacterium that causes Lyme disease, is the most common tick-borne infection in the United States with over 35,198 cases reported in 2008. This value represented a 28% and 77% increase in incidence in the previous two years of reporting, respectively, indicating that Lyme borreliosis is a re-emerging disease. Early on, the infection is characterized by a skin lesion known as erythema migrans and a non-descript flu-like illness (Nadelman and Wormser, 1998; Steere et al., 2004). Patients with untreated Lyme disease experience multisystemic symptoms with the arthritis being the defining indicator of persistent infection. *B. burgdorferi* is a highly motile organism that is organized as a Gram-negative bacterium but lacks lipopolysaccharide (LPS) in its outer membrane. Interestingly, it has the ability to scavenge complex molecules for cellular envelope biosynthesis and essential nutrients for metabolism from its environment (Gherardini et al., 2010; Boylan et al., 2008). In the laboratory, *in vitro* cultivation of *B. burgdorferi* requires highly nutrient rich and undefined media. Furthermore, it is widely established that *B. burgdorferi* responds to changing environmental conditions in the arthropod and mammalian host environment.

B. burgdorferi genome was one of the first living systems whose genome was sequenced and annotated (Casjens et al., 2000; Fraser et al., 1997); however, one of the most striking features noted upon perusal of the genetic information was the lack of similarity of borrelial gene products with any other known virulence determinant or toxin within the existing database. This, coupled with the difficulties in the genetic manipulation of this pathogen, made it impossible to test whether putative virulence determinants contributed to borrelial pathogenesis. Subsequently, several independent groups developed genetic tools and methodologies to generate and characterize mutations in targeted genes, as well as suicide and shuttle vectors for *cis* and *trans* complementation, respectively (Li et al., 2007; Rosa et al., 2005, 2010; Weening et al., 2008). In the interim, the *B. burgdorferi* mutants

characterized have provided important insight into the function of numerous borrelial gene products that affect the spirochetes pathogenic potential, physiologic capabilities, and gene regulatory systems (Skare et al., 2010; Barthold, et al., 2010; Rosa et al., 2010; Gherardini et al., 2010; Norris et al., 2010; Pal and Fikrig, 2010). Despite these important advances and continued refinement of the genetic modalities available, the ability to transform and complement infectious *B. burgdorferi* remains challenging.

This unit describes the cultivation (Basic Protocol 1), electrotransformation (Basic Protocol 1), and isolation of transformed clones (Basic Protocol 3) of *B. burgdorferi*. Subsequent complementation of confirmed mutants is outlined in Basic Protocol 3. We recognize that there are multiple methods to cultivate and genetically modify *B. burgdorferi* and have attempted to faithfully represent the "consensus" within the borrelial research community. Nevertheless, there are likely some approaches that have been unintentionally omitted within these protocols and, if so, this accidental oversight is not meant to imply that these methodologies are in any way insufficient in either their scope or utility.

> *CAUTION: Borrelia burgdorferi* is a Biosafety Level 2 (BSL-2) infectious agent and should be handled in a BSL-2 approved biosafety cabinet (BSC). This includes *in vitro* grown cultures as well as infected animal tissues. Animal necropsies should also be conducted within BSC containment.

> *IMPORTANT NOTE:* In addition to biosafety issues, the complex and nutrient rich media should be used under containment to prevent comtamination. It is important to note that the usage of antibiotic resistance in *B. burgdorferi* requires institutional approval as mandated by NIH.

Basic Protocol 1: in vitro cultivation of *Borrelia burgdorferi*

Borrelia burgdorferi requires a complex, undefined, nutrient rich media for *in vitro* cultivation. BSK-H media is available from Sigma but does not support growth of all infectious strains. As such, many researchers make their own BSK-II media. Because *B. burgdorferi* is such a fastidious organism, it is sensitive to subtle changes in the abundance of the unknown components, presumably provided by the abundant bovine serum albumin (BSA) or added rabbit serum, which may contribute to variations seen between purchased BSK-H and individually produced batches of BSK-II media. To circumvent this issue, lots of normal rabbit serum (NRS) and BSA must be screened for optimal motility and growth rate of *B. burgdorferi*. (Barbour, 1984). Because this media is rich, BSK-II is easily contaminated and should be manipulated following filtration media in a biological safety cabinet. Further safeguards for contamination include the addition of the antifungal amphotericin B, and the antimicrobials phosphomycin and rifampin, which do not adversely affect borrelial growth (Zuckert, 2007).

Materials

Complete 1X Barbour-Stoenner-Kelly II (BSK-II) medium or BSK-H medium (Sigma) (see recipes)

Filter units (0.22 μm)

CO_2 incubator

Type II A Biosafety Cabinet

Inoculation and *In vitro* Growth of *B. burgdorferi*

1. Pipet 5–12 ml fresh complete BSK into a 15 ml conical tube while working in a biological safety cabinet to avoid exposure to *B. burgdorferi* and contamination of complete BSK media.

   Complete BSK refers to the supplementation of BSK-II or BSK-H supplemented with 6% normal rabbit serum (NRS).

2. Cultures can be inoculated from frozen stock by two methods:

   a. New borrelial cultures can be started from a 1 ml frozen stock (10% DMSO final concentration) by scraping a portion of the frozen cells with a sterile spatula and transferring to complete BSK-II media.

   b. Thawing a 1 ml aliquot of late exponential cultures and transferring the entire volume to complete BSK media can start new borrelial cultures. Thawing and transfer should be done as quickly as possible in a 37°C water bath and the tube sterilized by immersion in 70% ethanol in a biosafety cabinet prior to inoculation. The aliquot should be diluted >15-fold in complete BSK media since high concentrations of DMSO do not support growth of *B. burgdorferi*.

3. *B. burgdorferi* is grown microaerobically under static conditions in with 1–5% CO_2 at 32–37°C to the appropriate cell density. On average the doubling of *B. burgdorferi* is approximately 8–10 hours for wild type strains (range is between 5–16 hours depending on strain) (Table 1). Mutants may grow at different rates and different growth conditions can adversely affect doubling times.

   In addition to their growth microaerobically, B. burgdorferi also grow anaerobically. Cultures grown under anaerobic conditions must be inoculated from a frozen stock under the same condition with media that has been equilibrated under anaerobic conditions.

4. For expansion of a cultivated sample, cultures can be passed into fresh media of the necessary volume for the planned experiments. Cultures intended for RNA or protein samples should be inoculated no higher than 1×10^5 *B. burgdorferi*/ml to allow the cells to double numerous time prior to sampling in exponential growth phase or stationary growth phase in order to accurately reflect the experimental conditions imposed.

5. CO_2 levels alters gene expression and protein synthesis in *B. burgdorferi* and loose caps are necessary for gas exchange in the complete BSK media. When this type of cultivation is utilized, it is important to employ secondary containment to prevent spills.

6. To examine the viability of a given culture, *B. burgdorferi* are examined using a dark field microscope to enumerate the number of live cells per field. A wet mount is made with 7–10 μl of culture or of an appropriate dilution for more dense cultures.

   As with any sampling method, the calculation of cell density will be more accurate by counting more fields than less. On average at least 25 fields should be counted for an accurate density determination. If this method is used, the dark field microscope will need to be calibrated, based on limiting dilutions, in order to convert the values observed to a cellular density. Overall, >90% of the cell should be motile. As the cultures increase in density the media color will change in response to the acidification of the complete BSK media.

Cell density can also be determined using a Petroff Hausser Counting Chamber (Samuels, 1995).

7. The plasmid content of *B. burgdorferi* must be rigorously monitored since this bacterium contains a segmented genome and loses plasmids with passaging the cells into fresh media or other experimental manipulation, including transformation.

    It is essential that the plasmid content of strains is assessed prior to in vivo analyses since some plasmids are required for optimal infectivity. A plasmid profile can be performed using PCR from boiled cells or purified genomic DNA using oligonucleotide primers specific for each strain B31 plasmid (Labandeira-Rey and Skare, 2001; Purser and Norris, 2000).

Basic Protocol 2: Transformation of *B. burgdorferi*

Transformation of *B. burgdorferi* is highly inefficient and requires extremely large quantities of DNA relative to other bacteria, such as *Escherichia coli* and *Salmonella.* The borrelial cultures used for transformations should exhibit active motility and replicate at the expected rate for the given strain. Solutions used for transformation must be kept ice-cold and stored at 4°C. Pelleted *B. burgdorferi* must be handled carefully and quickly during these steps to ensure recovery of the bacteria and enhance the yield and viability of the samples prior to electroporation. It is important to note that this procedure is effectively identical to that published by Samuels et al. (1995) except that the one of the buffers used are different. The intention here is to provide an alternative protocol to an already effective procedure.

Materials

Dulbecco's phosphate-buffered saline (DPBS)

8 mM HEPES, 272 mM Sucrose buffer, pH 7.4

Complete BSK

Sterile centrifuge bottles, conical tubes, pipets, and pipetman tips (aerosol-resistant)

0.2 cm cuvettes

Electroporator (Bio-rad Gene Pulser)

Prepare Electrocompetent B. burgdorferi Cells

1. Grow 500 ml of *B. burgdorferi* in complete 1X BSK media until it reaches a density of 5×10^7 spirochetes/ml. Viability (scored indirectly by the motility of the culture) should fall between 90–95%. Solutions used in step 3 and beyond must be ice-cold prior to use.

2. Split culture and centrifuge at $4000 \times g$ for 20 minutes at 4°C in pre-chilled sterile bottles.

    Make sure all subsequent centrifugations occur in pre-chilled sterile bottle or tubes.

3. Remove supernatants and keep pellets on ice.

4. Resuspend the pellets in 40 ml of ice-cold dPBS and transfer to 50 ml pre-chilled conical tubes. It is important to quickly but gently resuspend the pellets. Spin at $4000 \times g$ for 20 minutes at 4°C.

5. Remove supernatants and resuspend pellets in 40 ml of ice-cold dPBS. Spin at $4000 \times g$ for 20 minutes at 4°C.

6. Remove supernatants and resuspend pellets in 10 ml of ice-cold HEPES/Sucrose buffer. Spin at $4000 \times g$ for 20 minutes at 4°C.

7. Remove supernatants and pool pellets in 400 μl of ice-cold HEPES/Sucrose buffer.

8. Pipet cells into 100 μl aliquots containing approximately 1×10^9 *B. burgdorferi* cells and place on ice.

    Cells need to be used the same day as prepared for transformation.

    The efficiency of snap frozen competent B. burgdorferi cells is greatly reduced; always use freshly made cells.

Transform cells

9   Use 0.2 cm pre-chilled cuvettes. Keep cuvettes on ice.

10 Add between 4–10 μl of your DNA (5 μg/μl) to each cell aliquot and incubate on ice for 1 minute. An aliquot should be used as a negative control (i.e., no added DNA) to assess the quality of the competent cells and to determine background transformants as addressed in the plating and screening section.

    30 μg of precipitated DNA consistently results in the greatest number of positive transformants for shuttle vector and allelic exchange in wildtype B. burgdorferi. Multiple electroporations are performed with DNA concentrations ranging from 20 μg to 50 μg for infectious wildtype B. burgdorferi.

11 Transfer cells to a chilled cuvette. Make sure that there are no bubbles in the cuvette (shake or tap gently). Wipe off excess moisture from outside of cuvette.

12 Use the following settings: 2.5 kV, 25 μF, and 200 Ω. Time constants should fall between 4.0 and 5.0 milliseconds (ms).

13 Add 1 ml of complete BSK to the cuvette and re-suspend the cells. Transfer to 9 ml of complete BSK media to allow cells to recover for 18–20 hours at 1–5% CO_2 and 32–34°C prior to antibiotic selection.

Basic Protocol 2: Antibiotic selection of *B. burgdorferi* transformants

Once *B. burgdorferi* has been electroporated with the desired construct and recovered overnight, it is necessary to distinguish an isolate that contains the desired genetic lesion or shuttle vector construct relative to non-transformed cells. This is facilitated by selection with an antibiotic resistance determinant that functions in *B. burgdorferi*. Currently there are 4 antibiotic resistant genes that are widely used for this spirochete; they confer resistance to kanamycin (kan[R] via *aphI*; (Bono et al., 2000)), streptomycin (str[R] via *aadA* [also confers resistance to spectinomycin in *E. coli*]; (Frank et al., 2003)), gentamicin (gent[R] via *aacC1*; ref's), and erythromycin (erm[R] via *ermC*; (Sartakova et al., 2000)). Here we outline two different methods to obtain transformants with advantages and caveats indicated for each approach described.

A. Semisolid plating for *B. burgdorferi* transformed cells

Plating borrelial cells in a semisolid media is a traditional technique for isolating individual clones. Colonies appear, after approximately 10–17 days, as diffuse white spots in the red overlay. This approach is more problematic since it requires that the agarose overlay be within in a small temperature range in order to remain molten but not too hot to kill the borrelial cells and/or inactivate the antibiotics being used. It is also possible that the desired

mutation may render the borrelial cell more sensitive to the osmotic tension of a semisolid matrix that would not be seen for cells grown in liquid broth.

Materials

1.7% molecular grade agarose

2X BSK-II (see solutions)

Normal Rabbit Serum (NRS)

Antibiotics (filter sterilized)

Autoclave

Water bath

Disposable Petri dish (sterile, 100 mm O.D. × 15 mm)

Disposable sterile pipets, pipet tips (aerosol resistant) and sterile 15 ml conical tubes

Parafilm

1. Autoclave 100 ml of 1.7% molecular grade agarose in a wide mouth bottle. Allow molten agarose to cool in a 48°C water bath.

2. Add 100 ml of prewarmed 2X BSK-II and 14 ml of prewarmed NRS to have enough mixture for 10 plates. Add the appropriate antibiotics and gently mix the solution; return to 48°C water bath. Once the antibiotics are added to media the temperature should not exceed 52°C.

3. To pour the underlay, pipet 10 ml of the complete BSK agarose mixture into a sterile petri dish promptly. Allow underlay to cool and polymerize in a sterile environment for approximately 15 minutes.

4. Spin 10 ml of the recovered transformed *B. burgdorferi* culture at 4000 × *g* for 10 minutes. Remove 9 ml of the resulting supernatant and re-suspend the cells in the remaining 1 ml of medium.

5. Split the 1 ml concentrate of cells between two 15 ml conical tubes.

6. Pipet 10 ml of complete BSK agarose mixture and quickly mix by pipeting with the 0.5 ml aliquot of transformed cells in the 15 ml conical tubes.

7. Transfer cells in the complete BSK agarose mixture to a sterile petri dish containing a polymerized underlay. Gently swirl the overlay to allow an even spread of cell on the underlay.

   NOTE: the agarose overlay will solidify rapidly so it is important to evenly distribute it over the surface of the existing overlay as quickly as possible.

8. Allow the plates to completely solidify for 15 minutes in a sterile environment. Close plates and wipe the outer edge of plate with ethanol. Wrap the plates with parafilm.

9. Incubate inverted plates for borrelial growth. Depending on the strain and/or mutation introduced, colonies will appear as early as 7 days and out to 21 days or longer.

10. Individual colonies can be removed by using a sterile barrier tip to pull agarose plugs from the semisolid matrix. Transfer the plug to 5 ml of fresh complete media with the appropriate selection.

11. Following expansion in complete BSK, an aliquot of the putative mutant or shuttle vector transformant should be frozen in complete BSK with 10% DMSO at –80°C and subsequently verified by appropriate molecular approaches (i.e., PCR, Southern and/or Western blotting [if an antibody reagent is available]).

B. Liquid plating of transformed *B. burgdorferi* cells

Liquid plating of transformed *B. burgdorferi* cells is widely used because of the ease of the technique (i.e., no molten agarose and temperature issue to contend with). Effectively, the total volume of the culture is distributed in small volumes over 96-well plates (Yang et al., 2004). This, coupled with the inefficiency of the borrelial transformation generally assures that, if a transformant is obtained, it is likely to be clonal. Nevertheless, there is a real possibility that an individual liquid sample may contain multiple transformants.

Materials

Complete 1X BSK

0.5% Phenol red (filter sterilized)

Antibiotics (filter sterilized)

Multichannel pipet

Sterile 96-well plate, basin, pipet tips (aerosol resistant)

Plate tape (Petri Seal™)

1. Following transformation and 16–18 hours of recovery, 35 ml of complete BSK supplemented with Phenol red (0.002% final concentration) and the appropriate antibiotics is added.

2. Transfer 40 ml into a sterile basin and, using a multichannel pipet, transfer 180 μl aliquots per well over two 96-well plates.

3. Incubate the remaining 5 ml of the transformed culture, along with the 96 well plates, at 1–5% CO_2 and 32–34°C.

4. Generally speaking, if the transformation is successful, live, motile cells should be visible in the 5 ml culture approximately 10 days after electroporation. A few days following their visualization in the 5 ml culture, individual wells should begin to change color, due to borrelial growth and concomitant acidification of the complete BSK media. This generally occurs 12–16 days after transformation, but may take up to (or longer than) 21 days. Positive wells can then be verified by dark field microscopy to confirm the presence of live motile cells.

5. Transfer 10 μl of culture from a positive well into 10 ml of complete BSK media and grow under appropriate selection. Add DMSO to a final concentration of 10% to the remaining volume of the well (i.e., 170 μl) and store at –80°C. Alternatively, a final concentration of 20–25% glycerol can used in place of DMSO.

6. Following expansion in complete BSK, the mutant or shuttle vector transformant should be verified by appropriate molecular approaches (i.e., PCR, Southern and/or Western blotting [if an antibody reagent is available]).

Basic Protocol 3: *B. burgdorferi* Complementation—recombinase based method

Once the mutants have been isolated and confirmed, it is important to genetically complement them to ensure that the mutation isolated is attributable to the mutation

introduced and not the result of a second site mutation. There are several methods to carry out genetic complementation that involve conventional restriction digested and ligated constructs, either of which can be designed to complement in both a *trans* or *cis* acting sites or at a heterologous location within the genome (Li et al., 2007; Rosa et al., 2005; Weening et al., 2008). This approach is dependent on specific details that are key to any recombinant construct (i.e., presence or absence of restriction sites on the gene of interest relative to the cloning backbone) and, as such, will not be described here. Instead, the focus of this protocol will be to outline how existing recombinase-dependent constructs, which require no restriction digestion or ligation steps, have been used to complement *B. burgdorferi* mutants (Figure 1 and (Weening et al., 2008).

Borrelial Gateway *trans* Complementation

Materials

PCR fragment of a gene of interest

Entry Vector pCR8 or one of many pENTR vectors (Invitrogen)

Borrelial destination shuttle vector (Table 2)

LR Clonase™ II (Invitrogen)

Proteinase K

ccdB survival T1R *E. coli* competent cells (Invitrogen)

DH5α competent cells

1. Transformed destination vectors, like pBBE22gate, must be maintained in *ccdB* survival T1R *E. coli* cells (Invitrogen).

2. Generate an entry vector by TOPO cloning a PCR fragment of a gene of interest into pCR8 or one of many pENTR vectors available from Invitrogen.

3. Combine 150 ng of the entry vector and destination vector into an eppendorf tube and incubate at 55°C for 10 minutes. Quickly chill the sample on ice.

   Entry and destination vectors should not have the same selectable marker to prevent recovering cells with the entry vector and the newly recombined plasmid.

4. Add 2 μl of LR Clonase™ II enzyme mix to the sample. Briefly vortex and spin. Incubate for 1 hour at room temperature followed by an overnight incubation at 15°C.

5. Add 1μl of proteinase K (2 μg/μl) to end the reaction. Incubate at 37°C for 10 minutes.

6. Transform into DH5α *E. coli* strain. Select for positive transformants with antibiotic resistance encoded on the destination vector. Positive transformants can be screened by PCR and for sensitivity to chloramphenicol.

7. Purify plasmid from E. coli.

8. Sequence insert to make sure correct.

9. Transform B burgdorferi.

Support Protocol: DNA methylation of transformed DNA

The borrelial genome contains two restriction modification systems, *bbe02* and *bbq67,* that inhibit the recovery of positive transformants. Methylation of DNA prior to transformation improves transformation efficiency of shuttle vectors in strains encoding *bbq67* (Chen et al.,

2008). In theory the methylation of suicide vectors should also have a increased transformation efficiency relative to unmethylated DNA. For reasons that are not entirely clear, it appears that DNA methylation does not overcome barriers imposed by *bbe02* (Chen et al., 2008).

Materials

CpG Methyltransferase (M.SssI; New England Biolabs)

NEBbuffer 2 (New England Biolabs)

S-adenosylmethionine (SAM)

Phenol-chloroform-isoamyl alcohol

1. Add 1unit/μg M.SssI, 1X NEBbuffer 2 and 160μM *S*-adenosylmethionine (SAM) to the DNA for electroporation.

2. Incubate at 37°C for 4 hours.

3. Heat inactivate at 65°C for 20 minutes

4. Purify and isolate DNA by phenol-chloroform-isoamyl alcohol extraction

5. Resuspend precipicated at 5 μg/μl final concentration

Solutions

Barbour-Stoenner-Kelly (BSK) media

Materials

10X CMRL 1066 (without L-glutamine)

Neopeptone

Yeastolate

Glucose

HEPES

Citric Acid Trisodium Salt

Sodium Pyruvate

N-acetyl-D-glucosamine

Sodium bicarbonate

Bovine Serum Albumin (BSA) – Invitrogen

Normal Rabbit Serum (NRS) – Pel-Freez Biologicals, Inc.

5M NaOH

Rifampicin

Phosphomycin

Amphotericin B

Filter units (0.22 μm)

Recipe for 1 liter of BSK-II media:

9.82g 10X CMRL 1066 (without L-glutamine)

5.0g	Neopeptone
2g	Yeastolate
6g	HEPES
5g	Glucose
0.7g	Citric Acid Trisodium Salt
0.8g	Sodium Pyruvate
0.4g	N-acetyl-D-glucosamine
2.2g	Sodium bicarbonate
50g	BSA

The recipe for BSK-H was developed by Pollack et al and is more cumbersome to produce in the laboratory relative to BSK-II media (Pollack et al., 1993). It can be purchased from Sigma.

1. Mix components of BSK media into 500 ml of glass distilled H$_2$O with the BSA added to the solution last. Allow solution to mix well until BSA has dissolved into solution.

    Several lots of BSA should be screened for optimal motility and growth rate. Dramatic differences can be observed by microscopic and growth curve analysis between different lots of BSA.

2. Adjust pH to 7.6 with 5M NaOH.

3. Add rifampicin (50 μg/ml final concentration), phosphomycin (100 μg/ml final concentration), and amphotericin B (5 μg/ml final concentration).

4. Incubate media in a 45°C water bath and allow media to equilibrate for approximately 1 hour to ensure the BSA is completely dissolved.

5. Filter media with a 0.22 μm filter unit.

6. Media can be stored at room temperature for 4–6 weeks and at 4°C for at least 1–2 years without diminished growth capacity (if frozen the media should be good indefinitely).

7. Prior to cultivation of *B. burgdorferi* add NRS to media in a 6% final concentration. This is referred to as complete BSK media (either BSK-II or BSK-H depending on the recipe used).

    Note: 2X media can be made by reducing the total volume to 500 ml and used in semi-solid plating of *B. burgdorferi* (see Basic Protocol 3).

Dulbecco's phosphate-buffered saline (dPBS

8 g NaCl

0.2 g KCl

1.15 g Na$_2$HPO$_4$

0.2 g KH$_2$PO$_4$

1L glass distilled water

sterilize by autoclaving under slow, liquid exhaust

Commentary

Background Information

The development of genetic tools and analyses in *B. burgdorferi* in recent years has provided the means to evaluate the roles of individual gene in borrelial physiology and pathogenesis. The construction and characterization of antibiotic resistance determinants that functioned in *B. burgdorferi* was key in the development of shuttle vectors and allelic exchange mutagenesis that revolutionized our ability to apply molecular Koch's postulates to Lyme borreliosis (Rosa et al., 2005, 2010). This, coupled with the identification of restriction/ modification systems, *bbe02* and *bbq67*, respectively, that mapped to plasmids lp25 and lp56 in the *B. burgdorferi* strain B31 genome, resulted in the isolation of plasmid-less strains that lacked these enzymes and were more readily transformable (Lawrenz et al., 2002). Unfortunately, strains missing lp25 are non-infectious in mice unless the lp25-encoded nicotinamidase (*bbe22* or *pncA*) is added back to these strains (Labandeira-Rey et al., 2003; Labandeira-Rey and Skare, 2001; Purser et al., 2003; Purser and Norris, 2000). In addition, a gene required for tick colonization, *bbe16* or *bptA*, also maps to lp25 (Revel et al., 2005). Therefore, to complete the infectious lifecycle, both *bbe22* (*pncA*) and *bbe16* (*bptA*) are needed.

Based on these observations, one approach is to isolate a mutant in an lp25 deficient strain via allelic exchange and then complement back the *bbe22* region to assess the role of the inactivated gene in Lyme borreliosis and, separately, together with intact version of the inactivated gene on a shuttle vector to genetically complement the mutant (Labandeira-Rey et al., 2003; Labandeira-Rey and Skare, 2001; Purser et al., 2003; Seshu et al., 2006). While effective, these strains cannot be used for tick studies unless the *bptA* gene can be added back. This limitation is addressed in the strain containing the *bbe02*::kan^R mutation since it exhibits the high transformation frequency seen for the strains lacking lp25 and contains the other lp25 genes (Kawabata et al., 2004). Another alternative is to complement back the entire lp25 containing resistance to gentamicin or streptomycin (Gilbert et al., 2007; Grimm et al., 2004) after making a mutant in the lp25 deficient background and complementing the inactivating gene either in *cis* or in *trans.* In addition to the use of the aforementioned strains, other investigators have described additional infectious strains from strains B31, 297, and N40 that are competent for *B. burgdorferi* transformation (Rosa et al., 2010). Although transformable, it is not clear how these strains differ from wild type strain B31 in regard to their restriction/modification activity. Taken together, investigators have numerous antibiotic resistance markers and strains to utilize in order to mutagenize borrelial genes of interest.

There are several different ways that one can genetically alter *B. burgdorferi*. They include insertional disruption, randomized or "targeted" transposition, site-specific mutagenesis, and genetic deletion (Figure 2). A less widely used but potential effective way to inactivate a gene is to clone an incomplete and internal fragment of a gene of interest into a suicide vector containing a selectable and functional antibiotic resistance determinant in *B. burgdorferi* and to select for a single crossover event (Figure 2, panel A). Although single crossovers have been used successfully in *B. burgdorferi* (Stevenson et al., 1998), this approach is presumed to be less efficient than double crossover based modalities probably due to the smaller size of the homologous fragment required for the single crossover event (see below).

More commonly employed are insertional mutations that exploit pre-existing (if present) or engineered restriction enzyme sites within a gene of interest whereby one inserts an antibiotic resistance determinant that is expressed in *B. burgdorferi* (Figure 2, panel B). In this case, homologous sequences that are upstream and downstream of the disrupted gene

(between 1–2 kb in length) are included to promote the desired double crossover event. A disadvantage of this experimental design is the potential (albeit rare) for the synthesis of truncated protein that exhibits reduced but detectable activity. Also, if the targeted gene is within an operon, polar effects may occur that could contribute to the phenotype(s) being tracked.

Similar to insertional mutants, transposon mutagenesis inserts an antibiotic cassette into DNA randomly and, when done in the conventional "global" manner, requires extensive characterization to map the insertions to an individual locus (Botkin et al., 2006; Lin et al., 2009; Rosa et al., 2010). Mutant libraries in both non-infectious and infectious *B. burgdorferi* have been constructed using this type of transposon-based mutagenesis (Botkin et al., 2006; Morozova et al., 2005; Stewart et al., 2004). Alternatively, one can target discrete regions for in vitro-based transposon mutagenesis to specifically target a specific genetic region (Akerley et al., 2002; Seshu et al., 2004). In the latter example, a fragment containing the region to be mutagenized is amplified by PCR and incubated with the transposon and transposable antibiotic resistance marker. Various transposon insertions are then mapped using molecular based techniques. As with insertional inactivation, this method can also result in truncated proteins and be subject to polarity issues.

To isolate a gene deletion, the gene in question is replaced with an antibiotic cassette. As with insertional inactivation, 1–2 kb of the upstream and downstream regions of the deleted gene are amplified by PCR and a antibiotic resistance determinant is cloned to replace the gene of interest (Figure 2, panel C). If the construct is designed such that orientation is defined, the antibiotic cassette can be placed in the same orientation of the deleted gene, generating a non-polar mutant (if relevant to the gene of interest). One potential limitation is the loss of sequences via the deletion event that unintentionally affect the expression of adjacent genes. This can be evaluated by transcript quantification of the linked genes, whereby the deletion mutant is compared to the parental strain.

Site-directed mutagenesis of the coding sequence in the gene of interest can be used to characterize specific functional domains within a given protein. Specifically, this method can be used to inactivate one function of a multi-function protein particularly when one function is absolutely required for viability. A specific example of this is the *rrp2* locus that could not be deleted in *B. burgdorferi* (Yang et al., 2003). However, if the ATP binding domain was mutated (a single residue was mutagenized) the resulting Rrp2 protein could no longer direct the synthesis of *B. burgdorferi* RpoS, indicating that this domain is required for *rrp2* activation (Yang et al., 2003). The complication of this approach is that a selectable marker must be genetically linked to the mutagenized gene, which may hinder expression of adjacent genes. Also, the crossover event must be carefully evaluated since it is possible that recombination events other than those needed to obtain the desired mutation may occur, thereby retaining the wild type allele (Figure 2, panel D).

Equally important to mutagenesis and, in some circumstances, more challenging is the complementation of mutant borrelial strains with the wild type allele. Complementation is absolutely essential to ensure that that the phenotype being tracked is due to the genetically modified gene and not the result of a second site mutation or lack of a borrelial plasmid. As with other bacteria, genetic complementation is commonly accomplished in *trans* on one of several available shuttle vectors containing an *E. coli* origin of replication and the region required for autonomous replication and cell partitioning from *B. burgdorferi* (Table 2) (Elias et al., 2003; Frank et al., 2003; Stewart et al., 2001). Specifically, shuttle vectors have been developed using the autonomous replication region from cp9, cp26, cp32, lp17, lp25, and lp28-1 (Table 2 and (Beaurepaire and Chaconas, 2005; Byram et al., 2004; Eggers et al., 2002; Elias et al., 2003; Frank et al., 2003; Stewart et al., 2003, 2001) and, in some studies,

have been used to promote the loss of a targeted plasmid due to plasmid incompatibility (Grimm et al., 2004; Eggers et al., 2002). The recovery rate of positive transformants with an autonomous replicating plasmid is greater than of those requiring allelic exchange, but if there is no selective pressure to maintain the complemented gene, it is possible that the plasmid could be lost during the infectious process. An example of this type of selective pressure is seen for the pBBE22 shuttle vector, which contains the *bbe22/pncA* locus from lp25 (Table 2) (Purser et al., 2003). The pBBE22 plasmid is maintained in *B. burgdorferi* clones missing lp25 during the infection of mice since the nicotinamidase encoded by the *bbe22/pncA* gene is absolutely required for survival in the mammalian host.

An alternative method to generating *trans* complementation constructs is a recombinase-based system that does not require traditional restriction enzyme digestion and ligation. Currently, the most commonly used system is the Gateway™ system from Invitrogen. Our laboratory has modified existing borrelial shuttle vectors to contain attachment (*att*) sites to generate a new collection of borrelial shuttle/destination vectors (Figure 1 and Table 2). The cloned *att* sites promote homologous recombination with an entry vector that contains any gene(s) of interest when combined in the presence of the LR recombinase. Following transformation of *E. coli* with the resulting in vitro recombination mixture only cells containing the recombined fragment will survive. Gateway™-based complementation of a *B. burgdorferi dbpBA* deletion strain was recently reported, whereby intact *dbpBA* was delivered into a customized borrelial shuttle/destination vector, designated pBBE22gate (Table 2), confirming the utility of this approach (Weening et al., 2008). Since the Gateway™ recombinase-based approach requires no restriction digestion, the isolation of the desired recombinant construct is sequence independent (past the addition of *att* sites during the construction of the destination vector). As such, the isolation of the desired recombinant is not hindered by the absence of restriction sites, the presence of multiple restriction sites in the borrelial genomic fragment being cloned, or the lack of restriction sites available for cloning into preexisting borrelial shuttle vectors.

Recent publications suggest that shuttle vectors such as pBSV2 are present in multiple copies in *B. burgdorferi*. This may result in altered regulation or the overexpression of genes relative to their native genomic arrangement, which may contain important *cis*-acting elements. For these reasons, *cis* complementation might be preferred since the same upstream regulatory sequences would be retained. The limitation of this type of construct is the requirement for a linked antibiotic resistance marker (and distinct from the one used to initially mutagenize the gene of interest) that might alter the expression of genetically linked genes, particularly if the mutated gene is an internal gene in an operon. Alternatively, the intact complement gene can be introduced at a heterologous site within the genome. Examples of this include integration between the *bb0445* and *bb0446* genes in the borrelial chromosome (Li et al., 2007) and within a non-coding region in cp26 (Dunham-Ems et al., 2009; Kenedy et al., 2009).

Critical Parameters and Troubleshooting

Transformation—Improved efficiency of borrelial transformation over the years stems from the refinement of existing protocols, the isolation of strains with decreased ability to eliminate introduced DNA, and the development of antibiotic resistance determinants that function in *B. burgdorferi* (Bono et al., 2000; Elias et al., 2003; Frank et al., 2003; Lawrenz et al., 2002; Sartakova et al., 2000, 2003). The protocol for the electrotransformation of *B. burgdorferi* by Samuels is effectively unchanged and is widely used for the past 15 years (Samuels, 1995). The only difference in the protocol listed here is the substitution of a HEPES/sucrose buffer relative to the initially suggested phosphate buffered and glycerol solutions (Seshu et al., 2004). The electroporation conditions imposed for *B. burgdorferi* are

similar to other bacterial organisms with the exception that extremely large amounts of DNA (micrograms) are used. This requirement can complicate transformation because the increase in DNA concentration may result in the unintended concomitant concentration of salt and subsequent arcing of the transformation mix. To avoid this, it is imperative that the DNA used be as pure (devoid of salt) and concentrated as is possible. Linearization of suicide vectors increases the likelihood of allelic exchange by double crossover in the genome. *In vitro* methylation of DNA can also improve transformation efficiency due to protection against the restriction of the transformed DNA (Chen et al., 2008).

Maintenance of Segmented Genome—The segmented genome of *B. burgdorferi* (21 plasmids; 12 linear and 9 circular, and a single linear chromosome for the prototypical strain B31) can be difficult to maintain in its entirety during *in vitro* cultivation in complete BSK media. BSK media (either BSK-II or commercially available BSK-H) is an undefined, nutrient rich media that supports *in vitro* growth. The rich nature of the BSK media often results in unintentional contamination; as such, careful sterile technique is critical. **Since *B. burgdorferi* is a BSL-2 organism, work with this agent requires a type II A biosafety cabinet (BSC).** In addition to maintaining proper BSL-2 practices, this level of containment is also important in reducing the exposure to unwanted contaminants.

Another important issue experienced by most borrelial researchers is the loss of plasmids following *in vitro* cultivation. To address this concern, plasmid content should be vigilantly monitored following long-term cultivation (passage) *in vitro* and genetic manipulation (after electrotransformation and selection *in vitro*). This issue is a by-product of the segmented borrelial genome, whereby daughter cells must receive the full complement of plasmids to retain their entire genetic compendium. It is likely that the loss of plasmids observed in some labs, and the increased rate of plasmid loss, is again due to subtle differences in cultivation methods between research groups. In summary, it is clear that many research labs are successful in cultivating and maintaining *B. burgdorferi* even though different practices are used between groups, as indicated in a previous Current Protocols by Zuckert (Zuckert, 2007). This is due, in all probability, to the fact that most of these differences have negligible effects. The intent here is to provide a perspective so that new investigators are cognizant of issues relevant to borrelial cultivation and transformation and can account for them accordingly.

Complex BSK media—It is possible that the reproducibility of experimentation between laboratories may be affected by a number of parameters that are associated with laboratory-specific differences in the cultivation of *B. burgdorferi*. One issue of note involves the source and composition of the BSK media used. As indicated herein, BSK media can be made on site, as either BSK-II or BSK-H media, or can be purchased commercially as BSK-H media. BSK media contains several undefined components, including bovine serum albumin (BSA), rabbit serum, neopeptone, and yeastolate. While these compounds are likely to be similar from different vendors, it is possible that compositional differences may exist that may affect the growth of an identical strain of *B. burgdorferi* between different research labs. For example, it is well established that *B. burgdorferi* does not synthesize its own fatty acids and is dependent on the media (or infected host) to provide these essential components so the borrelial cell can generate lipids. Because of this requirement, *B. burgdorferi* incorporates both saturated and polyunsaturated fatty acids present with the BSA and rabbit serum, as well as other components, provided in the BSK-II or BSK-H media. When making ones own media it is important to screen lots of BSA to find one that supports the growth of infectious *B. burgdorferi*. The exact reason for this screening step is not clear but may be due to different lots being rich in fatty acids that support the growth of the laboratory strain being tested.

It is also possible to culture adapt *B. burgdorferi* to different BSK media that does not initially support the growth of a particular strain. However one should use caution with this approach since it is possible that unintended mutations and/or plasmid loss (see below for more on plasmid composition) may occur during this process resulting in a strain with an altered phenotype. If adaptation is attempted, investigators are advised to test the strain for plasmid content and altered infectivity potential to determine if any overt (and unwanted) changes have been acquired during this process.

Time Considerations

The process of transforming *B. burgdorferi* is a time intensive process. First, it takes ample time to grow the large volume of cells needed for transformation. Next, following transformation, it takes the mutants between 2–3 weeks before putative transformants appear. Confirmation of the desired mutant via molecular techniques can take an additional 1–2 weeks depending on the approach being used. Once the mutant is confirmed, the process of transforming the genetic complement ensues, which, from start to finish, can take an additional 3–4 weeks. As such, although the ability to make mutants in *B. burgdorferi* represents an important advance, the process itself is still cumbersome. Regardless of these temporal limitations, transformants must be carefully screened and validated to confirm that antibiotic resistance (and desired mutation) are present and not the result of spontaneous secondary mutations that can afford antibiotic resistance to *B. burgdorferi*. It is ideal to verify PCR screening results by Southern or Western analysis (assuming in the latter case that an antibody reagent exists for the targeted gene product). Finally, once a mutation is confirmed, the plasmid profile should be determined to ensure that plasmids critical for tick and mammalian infections are present.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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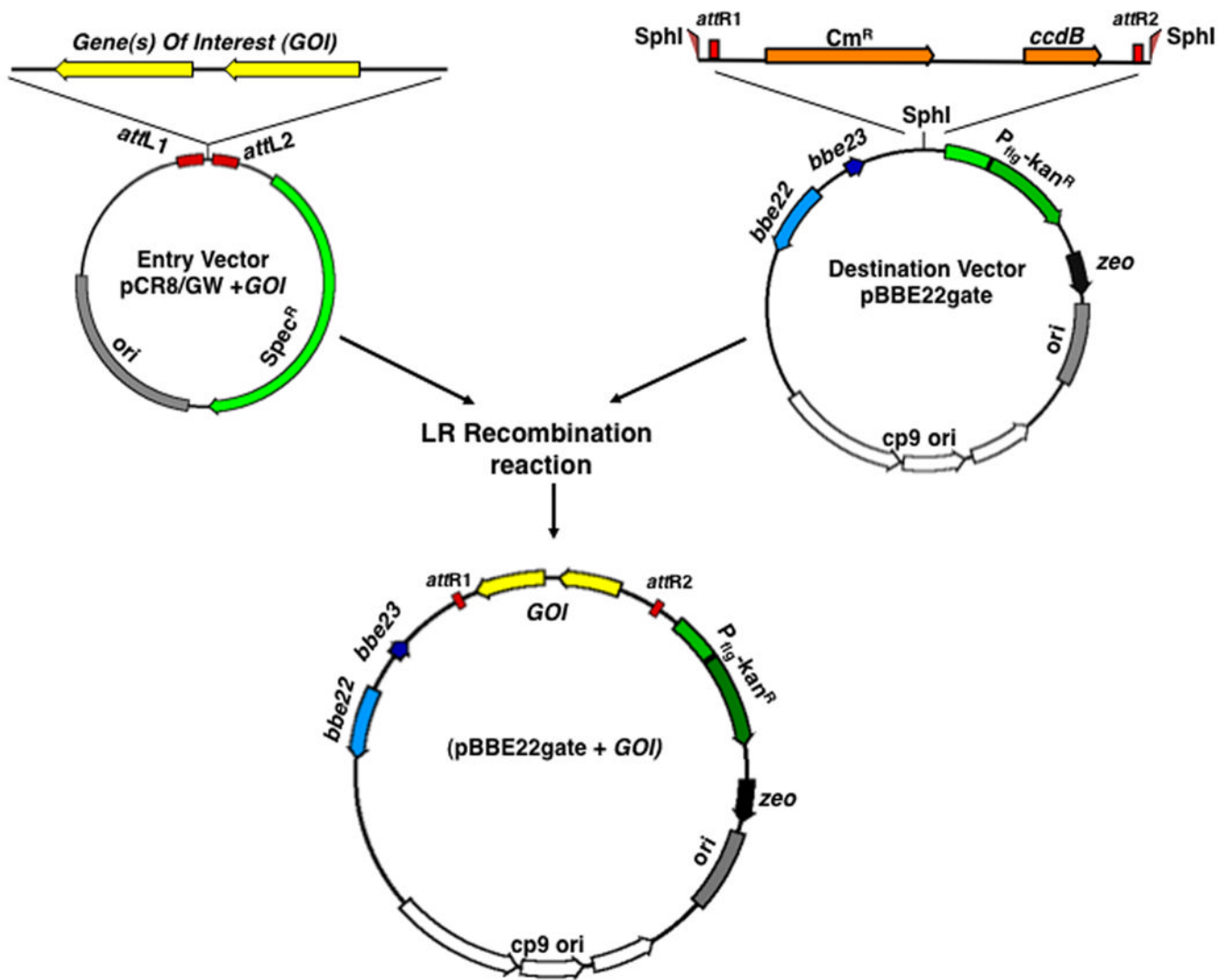
Figure 1.
B. burgdorferi adapted Gateway recombinase cloning (Invitrogen). The gene(s) of interest (*GOI*) are cloned into an entry vector (kan^R) and, via a ligation-independent, recombinase-specific reaction at the indicated *att* sites, recombine with a chloramphenicol resistant destination vector. The desired recombined construct carries the gene(s) of interest, retains kan^R, and loses the chloramphenicol resistance (Cm^R) determinant.
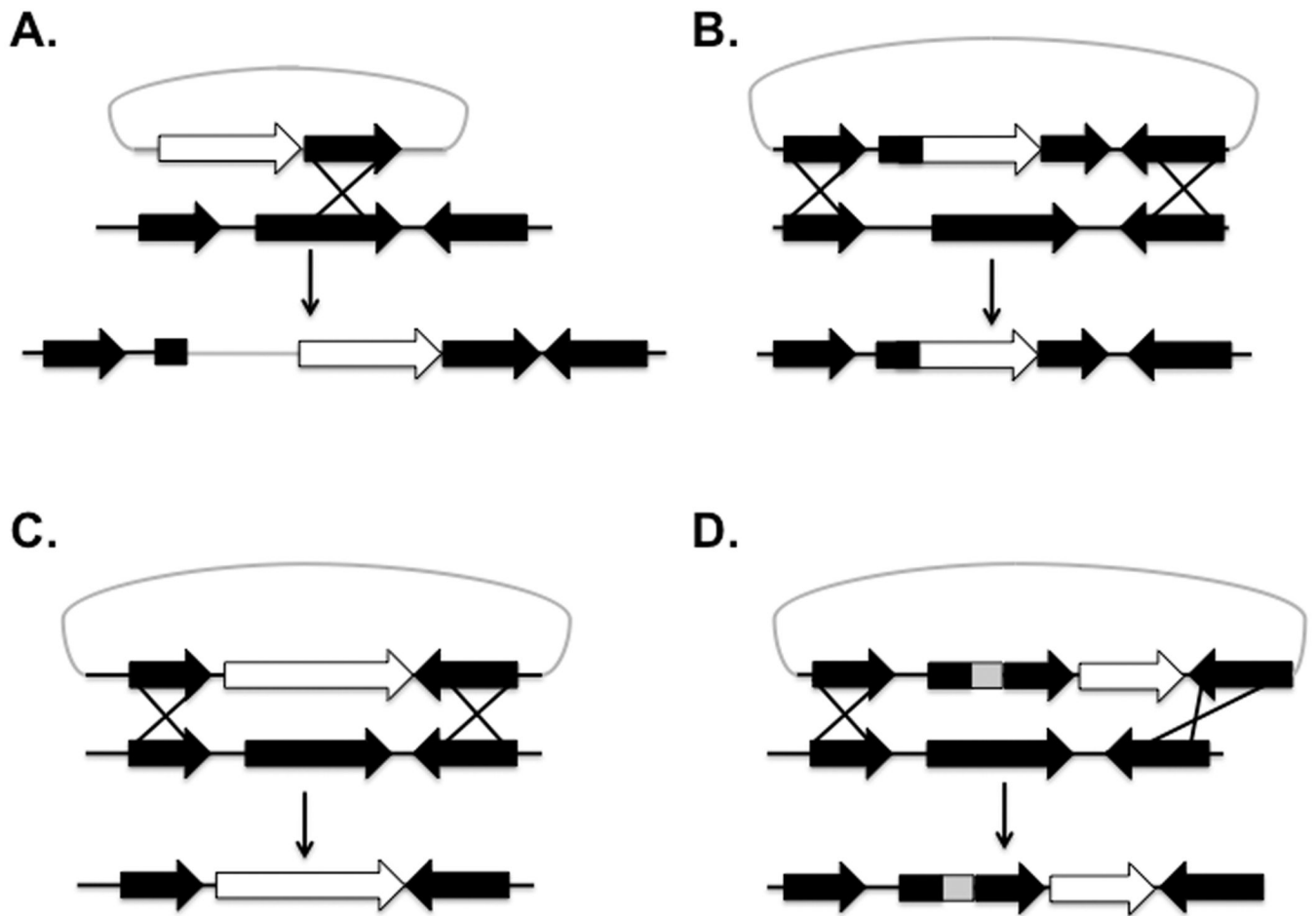
Figure 2.
Genetic Mutagenesis Schematic. In all of the panels, the white arrow represents the antibiotic resistance marker used for selection in *B. burgdorferi*. A. *Single crossover mutants*. A single crossover in the gene of interest occurs by homologous recombination, resulting in the incorporation of the entire suicide vector (shown above) including the antibiotic resistance determinant. Here, the suicide vector contains a truncated segment of the gene to be mutagenized (the middle gene) that mediates the single crossover event. B. *Insertional inactivation*. Generation of an insertional disruption mutation whereby recombination occurs between homologous sequences carried on the suicide vector (above) and the genetic target (again the middle gene). C. *Deletion mutant*. Double crossover requires homologous DNA regions flanking the gene of interest and generates a deletion mutation whereby allelic exchange replaces the target gene (middle gene) with an antibiotic resistant cassette. D. *Site directed mutagenesis*. In this case, a specific mutation is introduced in the gene of interest (middle gene), as indicated by a "gray box", and is genetically linked to a selectable marker. As with the insertional inactivation and deletion mutagenesis, the flanking regions provided in the suicide vector (above) facilitates allelic exchange via double crossover recombination.

Table 1

Commonly used *B. burgdorferi senus stricto* strains.

Strain	Description and Reference
B31	Infectious low-passage wildtype strain isolated from *Ixodes dammini* and cloned by limiting dilution (Barbour, 1984; Burgdorfer et al., 1982).
297	Infectious low-passage wildtype strain isolated from human spinal fluid (Steere et al., 1983).
cN40	Infectious clonal low-passage wildtype strain isolated from adult *I. dammini* (Barthold et al., 1990, 1988)
CA-2-87	*I. pacificus* isolate from Northern California (Schwan et al., 1988)
JD-1	Isolated and maintained in *Ixodes* ticks (Piesman et al., 1987)
B31 derivatives	
B31 MI	Derived from tick isolated B31 and passed through mouse (Casjens et al., 1997).
B31-A3	Clonal B31 infectious and low-passage; plasmids and infectivity maintained with transformation of shuttle or suicide vectors (Elias et al., 2002)
MSK5	Clonal infectious low-passage strain isolated from C3H/HeN mouse skin (Labandeira-Rey and Skare, 2001).
MSK7	Clonal infectious low-passage strain isolated from C3H/HeN mouse skin, lacking lp28-4 (Labandeira-Rey and Skare, 2001).
MSK10	Clonal low-passage strain unable to establish persistant infection; lacking lp28-1 isolated from C3H/HeN mouse skin (Labandeira-Rey and Skare, 2001).
ML23	Clonal noninfectious low-passage strain lacking lp25 isolated from nonclonal P48 B31 culture (Labandeira-Rey and Skare, 2001).
5A#	Clonal isolates with variable plasmid composition and infectivity (Purser and Norris, 2000).
5A4	Clonal infectious isolate containing all plasmids (Purser and Norris, 2000).
5A10 & 5A14	Clonal infectious isolate lacking lp25 and lp56 (Purser and Norris, 2000).
5A18	Clonal infectious isolate lacking lp28-4 and lp56 (Purser and Norris, 2000).
5A4 NP1	Clonal infectious isolate containing all plasmids(5A4) and *bbe02* disrupted with P_{flgB}-kan^R (Kawabata et al., 2004)
5A18 NP1	5A18 with *bbe02* distrupted by a P_{flgB}-kan^R cassette (Kawabata et al., 2004)
297 derivatives	
BbAH130	Clonal infectious low-passage isolate from plating polyclonal 297 (Yang et al., 2004).
PL133	Clonal infectious lowpassage strain isolated from ear punch biopsies of mice infected with polyclonal 297 (Revel et al., 2005).
BbDTR630	Derived from PL133 with a P_{flgB}-kan^R cassette cloned into lp25 (Blevins et al., 2007).
297-LK	Non-infectious clonal isolate derived from AH130 and is lacking lp28-1; *lacI* and P_{flgB}-*kan* cloned into *bbe02* on lp25 (Gilbert et al., 2007)
c155	Infectious low-passage isolated from nonclonal 297 by two rounds of plating and colony extraction (Eggers et al., 2002).
CE162	Clonal infectious low-passage derived from wildtype 297 (Caimano et al., 2004).
cN40 derivatives	
D10/E9	Infectious low-passage clonal isolate (Parveen et al., 2006).

Table 2

Plasmids for genetic manipulation of *B. burgdorferi*

Plasmid	Selection[a]	Description and Source
Shuttle vectors		
pBSV2	kan[R]	Shuttle vector containing three ORF's from cp9 required for plasmid replication and distribution (Stewart et al., 2001).
pBBE22	kan[R]	pBSV2 containing *bbe22*/*pncA* fragment to restore infectivity to strains lacking lp25 (Purser et al., 2003).
pBBE22gate	kan[R]	Modified pBBE22 containing Gateway destination vector *attR1* and *attR2* sites from Invitrogen's pDEST17 (Weening et al., 2008).
pKFSS1	strep[R]	Streptomycin resistant borrelial shuttle vector derived from pBSV2 (Frank et al., 2003).
pKFSS1gate	strep[R]	Modified pKFSS1 containing Gateway destination vector *attR* sites coned into the SphI site within the MCS (unpublished)
pCADDY	strep[R]	Shuttle vector derived from pBBE22; replacing the kanamycin resistance with streptomycin resistant gene (Hyde et al., 2009).
pCADDYgate	strep[R]	Derived from pBBE22gate (Weening et al., 2008); replacing the kanamycin resistance with streptomycin resistant determinant (unpublished)
pJD1	strep[R]	pKFSS1 derivative with reduced P_{flgB}-*aadA* region (Blevins et al., 2007).
pJD7	strep[R]	pJD1 derivative with terminator inserted and P_{lac} removed (Blevins et al., 2007).
pJD44	kan[R]	pJD7 derived plasmid replaced spec[R]/strep[R] with kan[R] (Blevins et al., 2007)
pBSV2G	gent[R]	Gentamicin resistant shuttle vector derived from pBSV2 (Elias et al., 2003).
pBSV2Ggate	gent[R]	Modified pBSV2G containing Gateway destination vector *attR* sites at SphI site in MCS (unpublished)
pBSV2SynG	cour[R]	Shuttle vector derived from pBSV2 encoding a synthetic *gyrB* (Elias et al., 2003)
pCE310	kan[R]	Shuttle vector with cp32 loci required for replication and distribution (Eggers et al., 2002).
pCB52	kan[R]	Contains replicon and telomere ends from lp17; replicates as circular plasmids in *E. coli* and as a linear plasmid in *B. burgdorferi* (Beaurepaire and Chaconas, 2005).
pBSV17	kan[R]	Shuttle vector derived from pBSV2 with the gene required for replication from lp17 (Beaurepaire and Chaconas, 2005).
pGK12	erm[R]	Broad-host plasmid able to propagate in Gram-positive and Gram-negative bacteria including *B. burgdorferi* (Sartakova et al., 2003).
Allelic exchange vectors		
pJH333	gent[R]	A multicloning site and P_{flgB}-gent[R] cassette flanked by DNA regions for allelic exchange into the borrelial chromosome between *bb0445* and *bb0446*; based on the construct reported by Li et al., 2007 (unpublished)
pMC1667	none[b]	Suicide vector with multicloning site flanked by cp26 regions forallelic exchange (Dunham-Ems et al., 2009).
pMC1916	gent[R]	Suicide vector derived from pMC1667 with P_{flgB}-gent[R] and P_{flaB}-*gfp* in divergent orientation are flanked by cp26 regions for allelic exchange (Dunham-Ems et al., 2009).
pSPC-G	gent[R]	Suicide vector with P_{flaB}-gent[R] and P_{flaB}-*gfp* in divergent orientation are flanked by cp26 regions for allelic exchange (Kenedy et al., 2009).

[a]Selection is in reference to antibiotic resistance in *Borrelia burgdorferi* only; antibiotic resistance for usage in *E. coli* is not indicated

[b]No selectable marker is incorporated for usage in *B. burgdorferi;* plasmid does confer resistance to kanamycin and ampicillin for selection in *E. coli*