Genetic Manipulation of *Borrelia* Spp

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Abstract

The spirochetes *Borrelia* (*Borreliella*) *burgdorferi* and *Borrelia hermsii*, the etiologic agents of Lyme disease and relapsing fever, respectively, cycle in nature between an arthropod vector and a vertebrate host. They have extraordinarily unusual genomes that are highly segmented and predominantly linear. The genetic analyses of Lyme disease spirochetes have become increasingly more sophisticated, while the age of genetic investigation in the relapsing fever spirochetes is just dawning. Molecular tools available for *B. burgdorferi* and related species range from simple selectable markers and gene reporters to state-of-the-art inducible gene expression systems that function in the animal model and high-throughput mutagenesis methodologies, despite nearly overwhelming experimental obstacles. This armamentarium has empowered borreliologists to build a formidable genetic understanding of the cellular physiology of the spirochete and the molecular pathogenesis of Lyme disease.

1 Introduction

The maxim *spirochetes do it differently* has proved repeatedly to be true, from ultrastructure to motility to gene regulation (Charon and Goldstein 2002; Charon et al. 2012; Johnson 1977; Samuels and Radolf 2009; Samuels 2011), yet a few exceptions exist; one of these rule-proving exceptions regards genetically manipulating *Borrelia* spp. Except for the labyrinthine tactics required to grow these serpentine bacteria in culture and handle their Gordian genomes, which are exceedingly segmented and largely linear (Brisson et al. 2012; Fraser et al. 1997), the transformation and complementation of *Borrelia* (*Borreliella*) *burgdorferi* (and related species that cause Lyme disease) and *Borrelia hermsii* (and related species that cause relapsing fever) are not unlike that of model microorganisms (Battisti et al. 2008; Samuels 1995).

The awesome power of reverse genetics has been tapped to dissect the microbial physiology of *B. burgdorferi* and, to a lesser extent, *B. afzelii*, *B. garinii*, and *B. hermsii* as well as the molecular pathogenesis of Lyme disease and, more recently, relapsing fever (Brisson et al. 2012; Groshong and Blevins 2014; Raffel et al. 2014; Rosa et al. 2005). One good reason for the success of the genetic approach is the availability of robust animal models (Barthold et al. 2010); these experimental systems mimic the natural course of disease as well as the two-host enzootic cycles that include a vertebrate and an arthropod vector, usually a tick (Caimano et al. 2016; Piesman and Schwan 2010; Radolf et al. 2012). The genetic toolbox

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constructed since *Borrelia* was first transformed (Samuels et al. 1994a) includes genome sequences (Di et al. 2014; Fraser et al. 1997), selectable markers and gene reporters (see Sect. 3), shuttle vectors (see Sect. 6), transposon mutagenesis (see Sect. 7), inducible promoters (see Sect. 5), and a counterselectable marker (see Sect. 8). Borreliologists have mutated a multitude of genes and now molecularly probe the *Borrelia* lifestyle encompassing all things from metabolism to transcriptional regulation to host interactions.

2 Transformation

B. burgdorferi was first transformed in the early 1990s with an allele of the endogenous *gyrB* gene, encoding the B subunit of DNA gyrase, that confers resistance to coumarin antibiotics (Samuels et al. 1994a). The electrotransformation protocol is similar to that used for many bacteria (Nickoloff 1995), but low transformation efficiency and retention of the native suite of plasmids continues to challenge borreliologists. The basic protocol has remained relatively unchanged: competent cells are prepared by removing salts, purified DNA substrate is added, and a high-voltage electric current is applied to the cells. Electroporation transiently compromises the integrity of the outer membrane, visualized by electron microscopy to reveal dark-stained patches that likely represent breaches in the membrane (Fig. 1) (Samuels and Garon 1997). The membrane rapidly recovers (within 30 min) when the cells are placed in growth medium following electroporation. Substantial improvements in transformation efficiency have been wrought by increasing the amount of substrate DNA to tens of micrograms (Eggers et al. 2002; Elias et al. 2002; Hübner et al. 2001; Stewart et al. 2001; Tilly et al. 2000) and transforming with modified DNA (Chen et al. 2008; Jewett et al. 2007).

2.1 Competent Cells and Electroporation

Successful preparation of *B. burgdorferi* competent cells requires growth to mid- to late-log phase; cell density is probably the most vital parameter (Samuels et al. 1994a; Tilly et al. 2000). Certain low-passage and mutant strains are refractory to transformation due to inordinate clumping during competent cell preparation (Hübner et al. 2001; Samuels 1995; Tilly et al. 2000). Salts are removed from the growth medium by a series of cold, sterile washes and the *Borrelia* cells are resuspended in a glycerol-sucrose solution. Arcing will occur during electroporation if salts are not effectively removed, which will kill the vast majority of cells and, obviously, prevent transformation. Electrotransformation is most efficient with fresh competent cells, but cells can be stored at −80 °C and slow-thawed on ice, although there is about a 50% decrease in efficiency (Samuels 1995; Samuels and Garon 1997).

2.2 DNA Source, Form and Modification

Many different substrates have been transformed into *B. burgdorferi*, from short oligonucleotides (Samuels and Garon 1997) to an entire native 28-kb linear plasmid (Grimm et al. 2004a). The original allelic exchange transformation used PCR products to mutate the *gyrB* gene and confer antibiotic resistance (Samuels et al. 1994a). More recently, linear segments of cloned DNA containing a selectable marker are typically used for allelic exchange to generate mutants. In addition, circular suicide plasmids have been integrated

into the genome by recombination (Stevenson et al. 1998). An important advance in the *Borrelia* genetic toolbox was the development of circular shuttle vectors that autonomously replicate in both *B. burgdorferi* and *Escherichia coli* (Eggers et al. 2002; Sartakova et al. 2000; Stewart et al. 2001). These plasmid vectors enhanced the number and sophistication of genetic experiments in *B. burgdorferi*, including *trans*-complementation, ectopic gene expression, and inducible gene expression. Linear shuttle vectors based on lp25, lp28-1, and lp17 have also been built using a similar strategy to the circular shuttle vector, although they have not been used frequently (Beaurepaire and Chaconas 2005; Stewart et al. 2003).

The low frequency of transformation is likely due to restriction-modification systems (Chen et al. 2008; Kawabata et al. 2004; Lawrenz et al. 2002; Rego et al. 2011). *B. burgdorferi* putatively possesses two type-IV restriction-modification enzymes: BBE02 on lp25 and BBQ67 on lp56 (Kawabata et al. 2004; Lawrenz et al. 2002). The gene products of *bbe02* and *bbq67* are predicted to methylate certain adenines and cleave foreign DNA lacking this modification. The presence of these two plasmids is correlated with low transformation efficiency of shuttle vectors isolated from *E. coli*. Transformants often lose lp25 and transformation efficiency increases about 40-fold in *bbe02* mutants (Kawabata et al. 2004). To complicate matters, lp25 also carries genes essential for vertebrate and tick infectivity (Grimm et al. 2004a; Labandeira-Rey and Skare 2001; Purser and Norris 2000; Purser et al. 2003; Revel et al. 2005; Strother and de Silva 2005). Thus, low-passage, infectious strains are more difficult to transform than high-passage, attenuated strains. BBE02 is not required for in vitro cultivation, so replacing *bbe02* with a selectable marker both increased transformation efficiency and provided a selection for lp25, allowing transformants to retain infectivity (Chan et al. 2015; Gilbert et al. 2007; Kasumba et al. 2015; Rego et al. 2011). Clever tactics to subvert the restriction-modification systems have been developed over the years. Transformation efficiency can be increased by first transforming the DNA into a strain carrying only one of the putative restriction-modification systems and using this partially methylated DNA for subsequent transformation (Jewett et al. 2007). Alternatively, in vitro CpG methylation of substrate DNA increases transformation efficiency (Chen et al. 2008). Both approaches have facilitated genetic manipulation of *B. burgdorferi*.

2.3 Relapsing Fever *Borrelia*

B. hermsii, one of the species of *Borrelia* that causes relapsing fever in humans (Barbour and Guo 2010), has also been successfully transformed (Battisti et al. 2008), but the history of genetic manipulation is considerably younger. The protocol for preparation of competent cells, electrotransformation and selection are basically the same as for *B. burgdorferi*. The notable changes include using large amounts of DNA for transformation (25 μg) and selecting for transformants in antibiotics for up to a week in batch before plating to isolate clones (Battisti et al. 2008; Fine et al. 2011; Raffel et al. 2014). The shuttle vector pBhSV2, based on the *B. burgdorferi* shuttle vector pBSV2, functions in *B. hermsii*, but only the antibiotics gentamicin and kanamycin have been effective for selection (Battisti et al. 2008). So far, three genes have been mutated: *vtp*, which encodes variable tick protein, an outer surface lipoprotein synthesized when the spirochete is resident in its tick vector (Battisti et al. 2008; Raffel et al. 2014), *vmp*, which is the expression locus for the genes encoding the variable major proteins that are synthesized in the mammalian host and allow the spirochete

to evade the immune response (Raffel et al. 2014), and *dam*, which encodes DNA adenine methyltransferase (James et al. 2016). In addition, the green fluorescent protein (GFP) gene has been expressed in *B. hermsii* (Fine et al. 2011) and its cousin *Borrelia turicatae* (Krishnavajhala et al. 2017). With these advances in hand, we are beginning to see the dawn of the age of reverse genetics in relapsing fever spirochetes.

3 Selectable Markers and Assayable Reporters

Selectable markers allow for the preferential isolation of genetically altered microorganisms, including *B. burgdorferi*. Antibiotic resistance markers have proved to be remarkably utilitarian tools for negative selection, the inhibition of the unaltered parental cells. *B. burgdorferi* is susceptible to many antibiotics and the genes conferring resistance to antibiotics such as streptomycin, kanamycin, gentamicin, and erythromycin are paramount for genetic studies. The antibiotics ampicillin and tetracycline are used to treat patients with Lyme disease, so their use in genetic experiments is discouraged.

The challenges, both in cost and time, in culturing *B. burgdorferi* to study gene expression have made reporter genes also incredibly useful tools. Reporter gene products provide both a convenient readout to measure transcriptional regulation when fused to promoters and a signal for visualization of tagged proteins or entire cells in vitro and in vivo. Advances in imaging of live mice and ticks infected with *B. burgdorferi* have provided an unprecedented vista of the interaction of the spirochete with the host (Dunham-Ems et al. 2009; Moriarty et al. 2008).

3.1 Native Antibiotic Resistance

The first selectable marker used for genetic manipulation in *B. burgdorferi* was an allele of the *gyrB* gene conferring resistance to the coumarin antibiotics (Samuels et al. 1994a, b; Samuels 2006). A synthetic version of *gyrB* designed to minimize recombination into the native *gyrB* locus on the chromosome was used briefly as a selectable marker (Elias et al. 2003). Unfortunately, the *gyrB* mutations also affect DNA supercoiling in *B. burgdorferi* (Samuels and Garon 1993), causing pleiotropic effects such as increased expression of *groEL*, encoding a chaperonin (Alverson and Samuels 2002) and *ospC*, encoding an outer surface lipoprotein (Alverson et al. 2003; Drecktrah et al. 2013; Yang et al. 2005), which spurred the exploitation of antibiotic resistance markers from other microorganisms.

3.2 Foreign Antibiotic Resistance Genes

The efficiency of genetic manipulation of *B. burgdorferi* was enhanced when new selectable markers were developed by fusing endogenous promoters to nonnative antibiotic resistance genes (Bono et al. 2000). The strong, constitutive promoters of the flagellin and basal body rod genes *flaB* and *flgB*, respectively, were chosen to drive gene expression (Bono et al. 2000; Ge et al. 1997; Sohaskey et al. 1997). These promoters were first fused to the *aphI* gene from *Tn903*, resulting in *flaBp-aphI* and *flgBp-aphI*, which mediate resistance to kanamycin (Bono et al. 2000). Other markers were constructed soon after by the same strategy and conferred resistance to streptomycin using *aadA* from *Shigella flexneri* plasmid R100 (Frank et al. 2003) and resistance to gentamicin using *aacC1* from *Tn1696* (Elias et al.

2003). These antibiotic resistance markers function in both *E. coli* and *B. burgdorferi*, making them versatile and practical tools for genetic studies. Not all antibiotic resistance cassettes function in *B. burgdorferi*, including *cat* and *pac*, the genes encoding resistance to chloramphenicol and puromycin, respectively (Elias et al. 2003). The *cat* gene encodes a chloramphenicol acetyl transferase, but the acetylation of chloramphenicol is reversed by esterases present in the *B. burgdorferi* growth medium (Sohaskey and Barbour 1999), which might be overcome by modifying the medium. Some foreign antibiotic resistance genes driven by their own promoters function in *B. burgdorferi*, such as *erm* conferring erythromycin resistance (Sartakova et al. 2000). Others include *aph(3′)-IIIa* conferring kanamycin resistance, *aad9* conferring spectinomycin resistance, *linA′* conferring lincomycin resistance, and *aac(3)-VIa* conferring sisomycin and gentamicin resistance, but they have not been widely used (Sartakova et al. 2003). Use of the *erm* gene has been restricted by some Institutional Biosafety Committees because erythromycin can be used to treat Lyme disease patients (Terekhova et al. 2002). Spectinomycin is not efficient at selecting for transformants containing the *aadA* (and probably the *aad9*) gene because *B. burgdorferi* readily becomes resistant to this antibiotic (Criswell et al. 2006). Lincomycin has not been utilized for selection in *B. burgdorferi*, but could be adapted to expand the toolbox for genetic manipulation. While the early years entailed plodding development of methodologies for genetic manipulation, borreliologists have refined their experimental approaches and now almost exclusively employ selectable markers conferring antibiotic resistance to kanamycin, streptomycin and gentamicin.

3.3 Mutant Isolation

There are two techniques for plating and selecting transformed *B. burgdorferi*. One is semi-solid agarose plating where transformed cells are allowed to recover overnight before plating in growth medium mixed with molten agarose (Kurtti et al. 1987; Rosa and Hogan 1992; Samuels et al. 1994a). *B. burgdorferi* is mixed with the molten agarose-growth medium containing the antibiotic so the colonies form within the mixture, not on the surface. After incubation for 5 days to 2 months in a CO_2 incubator, individual colonies appear as small white clouds in the plate and are then expanded in liquid culture to be screened for the desired genetic alteration. The second method is dilution plating in which the transformed cells are diluted in 100 ml growth medium and then distributed in four 96-well plates following overnight recovery (Yang et al. 2004). Plates are incubated in a CO_2 incubator until the medium in individual wells begins to turn from red to yellow, as metabolizing *B. burgdorferi* excretes lactic acid that is indicated by phenol red in the medium. Positive wells are expanded in liquid culture and screened.

3.4 Reporter Genes

The first reporter used in *B. burgdorferi* was the *cat* gene (Sohaskey et al. 1997). Although the gene product does not confer resistance to chloramphenicol in *B. burgdorferi*, the reporter provided some of the first assays of promoter activity (Alverson et al. 2003; Sohaskey et al. 1997, 1999), just as the age of spirochete genomics was dawning (Fraser et al. 1997). More recently, the gene for β-galactosidase, *lacZ*, fused to the *ospC* promoter, was used to study the regulation of *ospC* expression in both liquid and semi-solid media (Hayes et al. 2010, 2014; Sarkar et al. 2011).

The luciferase (*luc*) gene from the firefly *Photinus pyralis* has been adapted as a transcriptional reporter to assay gene expression from endogenous promoters (Blevins et al. 2007; Ouyang et al. 2010). The *luc* reporter also provided the means to follow *B. burgdorferi* infection in vivo by bioluminescence imaging of infected mice (Chan et al. 2015; Hyde et al. 2011). *B. burgdorferi* infection and dissemination of mutant strains lacking the fibronectin-binding protein BBK32 or the decorin-binding proteins DbpA and DbpB (Hyde et al. 2011) were assayed as well as the pathogenokinetics of *ospC* expression (Skare et al. 2016). Recently, a dual *luc* reporter system has been successfully implemented to study gene expression in the tick vector. The second *luc* reporter, from *Renilla reniformis*, allows for normalization to *B. burgdorferi* number, while the *P. pyralis luc* gene product can then accurately measure transcriptional activity (Adams et al. 2017a). The *luc* reporter systems developed for in vivo work provide a more convenient and lower cost system to follow gene expression in the tick-mouse model.

The convenience and versatility of GFP has made it a popular reporter for studies of *B. burgdorferi* gene expression (Babb et al. 2004; Carroll et al. 2003; Eggers et al. 2002, 2004) and served to demonstrate the functionality of an inducible gene system (Whetstine et al. 2009). The GFP reporter has the advantage of allowing the analyses of single cells, which has highlighted the heterogeneity of *B. burgdorferi* populations (Srivastava and de Silva 2008). Additionally, the yellow fluorescent protein and cyan fluorescent protein have both been used as reporters (Eggers et al. 2002). The monomeric red fluorescent protein has proved useful for studying lipoprotein secretion to the cell surface (Schulze and Zückert 2006; Schulze et al. 2010) and was a crucial component of a FACS-based protein localization screen (Kumru et al. 2010). The GFP marker could not be directly visualized in these studies, as fluorescence was compromised in the periplasm (Schulze and Zückert 2006). Constitutively expressed *gfp* has allowed for visualization of *B. burgdorferi* in live-cell imaging studies. A strain of *B. burgdorferi* containing a plasmid expressing *gfp* from the *flaB* promoter allowed for exquisite examination of the spirochete's interaction with the mouse vasculature in vivo by live-cell intravital microscopy (Moriarty et al. 2008). Stable integration of a constitutively expressed *gfp* driven by the *flaB* promoter on cp26 has served as a fluorescent tag of *B. burgdorferi* to allow in vivo studies on morphology and population dynamics in ticks (Dunham-Ems et al. 2009).

Finally, GFP has been expressed in the relapsing fever agents *B. hermsii* (Fine et al. 2011) and *B. turicatae* (Krishnavajhala et al. 2017), and used to follow the persistence of the latter in the *Ornithodoros turicatae* tick midgut and salivary glands (Krishnavajhala et al. 2017). Visualization of GFP-relapsing fever *Borrelia* in vivo should aid in describing the spirochete lifestyle in both the tick vector and mammalian hosts of these understudied *Borrelia* species.

4 Mutagenesis

Using reverse genetics to reveal the function of a gene entails generating mutations in the gene, which is often suspected of producing a particular phenotype, and isolating the resulting mutants. Although transduction has been demonstrated in *Borrelia* (Eggers et al. 2001, 2016), mutagenesis currently relies exclusively on transformation (see Sect. 2) and selection with an antibiotic resistance marker (see Sect. 3). Besides pleiotropy and polar

effects, a crucial caveat is off-target repercussions of genetic manipulation, which is particularly problematic in *B. burgdorferi* due to plasmid loss from the segmented genome (Kawabata et al. 2004; Labandeira-Rey and Skare 2001; Lawrenz et al. 2002; Purser and Norris 2000); therefore, mutant phenotypes should be confirmed by complementation or genetic reconstitution (see Sect. 6).

4.1 Gene Inactivation by Homologous Recombination

Almost every mutant in *Borrelia* has been generated by allelic exchange, requiring recombination of a synthetic DNA construct into the genome. The other mechanisms for mutagenesis, albeit not commonly utilized, are displacement of entire plasmids (Grimm et al. 2004a) and deletion of large segments of plasmids (Beaurepaire and Chaconas 2005; Bestor et al. 2010; Chaconas et al. 2001). Both *B. burgdorferi* (and other Lyme disease species) and *B. hermsii* (and other relapsing fever species) have antigenic variation systems that entail intra-genomic recombination (Nordstrand et al. 2000; Stoenner et al. 1982; Zhang et al. 1997) and a RecA homolog (Liveris et al. 2004; Putteet-Driver et al. 2004) that are likely hijacked to incorporate foreign DNA. VlsE antigenic variation in *B. burgdorferi* requires the branch migrase RuvAB (Dresser et al. 2009; Lin et al. 2009), which is also implicated in homologous recombination during genetic manipulation.

One curious phenomenon that was observed during allelic exchange (Brisson et al. 2012; Knight et al. 2000) and also documented during VlsE variation (Coutte et al. 2009) is the presence of mosaic recombination products, which implies that the crossover hops back and forth between the donor and recipient DNA strands, so the recombination machinery skips short stretches of sequence. This intermittent recombination generates further diversity during antigenic variation (Brisson et al. 2012; Coutte et al. 2009), but the caveat for genetic manipulation is that not all the expected changes may be present, so mutant and flanking sequences should be experimentally confirmed.

4.2 Site-Directed Mutagenesis

There have been only a handful of studies in which the sequence of a gene or regulatory element in its endogenous genomic location was specifically and subtly altered (as opposed to grossly deleting or inserting a gene) in *B. burgdorferi*. In the original report of transformation, a single codon of the *gyrB* gene was mutated to confer resistance to the antibiotic coumermycin A_1 (Samuels et al. 1994a; Samuels 2006). Several other genes have since been surgically manipulated in situ.

The C-terminal domain of the A subunit of DNA gyrase is uniquely synthesized as an independent DNA-binding protein termed Gac in *B. burgdorferi* (Knight and Samuels 1999). In order to disrupt *gac* expression without affecting the essential *gyrA* gene in which *gac* is embedded, the ribosome-binding site, the start codon and the second methionine codon of *gac* were mutated, which abrogated Gac production, although the mutant phenotype was disappointingly suppressed (Knight et al. 2000).

Rrp2 is an enhancer-binding protein and two-component system response regulator (Fraser et al. 1997; Samuels 2011). Exhaustive efforts failed to generate a null *rrp2* mutant, suggesting that Rrp2 had an essential function. Yang et al. (2003) changed a single residue in

the putative activation domain, which eliminated expression of *rpoS* and the RpoS regulon, establishing the Rrp2-RpoN-RpoS pathway paradigm for gene regulation during the enzootic cycle (Burtnick et al. 2007; Caimano et al. 2007; Hübner et al. 2001; Samuels 2011), but retained the essential, and still unidentified, function of Rrp2.

OspC is a transmission factor required to establish *B. burgdorferi* infection in the vertebrate host (Dunham-Ems et al. 2012; Fingerle et al. 2007; Gilbert et al. 2007; Gilmore and Piesman 2000; Grimm et al. 2004b; Pal et al. 2004; Stewart et al. 2006; Tilly et al. 2006), but its molecular function has not been fully elucidated. Marconi and colleagues have mutated both a presumptive ligand-binding site (Earnhart et al. 2010) and a cysteine residue involved in quaternary structure (Earnhart et al. 2011): infectivity was attenuated when the binding site was mutated, although the specific ligand has not been defined, and oligomerization was prevented by eradicating the disulfide bond.

The regulation of *ospC* expression has also been probed by site-directed mutagenesis. OspC levels increase dramatically during transmission from the tick to the vertebrate (Fingerle et al. 2002; Gilmore and Piesman 2000; Leuba-Garcia et al. 1998; Montgomery et al. 1996; Ohnishi et al. 2001; Rathinavelu and de Silva 2001; Schwan et al. 1995; Schwan and Piesman 2000), but OspC synthesis is turned off after the vertebrate infection is established in order for the spirochete to evade adaptive immunity (Crother et al. 2004; Liang et al. 2002a, b; 2004; Tilly et al. 2006). We (Drecktrah et al. 2013) introduced site-directed mutations in situ into an inverted repeat that constitutes part of an *ospC* operator (Xu et al. 2007) and demonstrated that impairing the intra-complementarity of the inverted repeat reduced induction of *ospC* expression in response to environmental factors in vitro.

Site-directed mutations have also been incorporated in *trans* into the genome by transforming a mutated gene or regulatory sequence on a shuttle vector into a null background or fused to a reporter, respectively. Targets of these plasmid-borne dissections include the *ospC* operator/promoter (Eggers et al. 2004; Yang et al. 2005), the *ospE* and *ospF* promoters (Eggers et al. 2006), *ospA* (Schulze and Zückert 2006), *ospC* (Kumru et al. 2011a, b), *p13* (Kumru et al. 2011a), *bb0646* (Shaw et al. 2012), and *bbd18* (Hayes et al. 2014).

4.3 Promoter Fusion

Borrelia promoters have been fused to selectable markers (see Sect. 3.2), reporters (see Sect. 3.4), and transposons (see Sect. 7) as well as genes of interest to effect constitutive, high-level expression, often as an alternative approach when attempts to disrupt the gene fail. One of the first applications was the fusion of the *flaB* promoter to the *ospC* ORF, which corroborated that constitutive synthesis of OspC during vertebrate infection prevented *B. burgdorferi* from avoiding the immune response (Xu et al. 2006). In a more recent study, Tilly et al. (2013) swapped the promoters and ORFs for three outer membrane lipoprotein genes, including *ospC* and *vlsE*, revealing that OspC and VlsE have similar in vivo functions.

4.4 Cre-*lox* and Telomere-Mediated Recombination

Perhaps the most conspicuous genetic method missing from the *Borrelia* armamentarium is markerless mutagenesis, which is somewhat surprising because Bestor et al. (2010) have developed an elegant Cre-*lox* recombination system that functions in *B. burgdorferi*. Two *loxP* sites were inserted several kilobase pairs apart into one of the linear plasmids and the Cre recombinase was introduced on a shuttle vector, which resulted in the deletion of a substantial swath of genes (Bestor et al. 2010). Chaconas et al. (2001) fashioned a similar system utilizing a replicated telomere that is inserted into a linear plasmid and triggers a telomere resolvase-mediated deletion between the inserted telomere and the end of the linear plasmid opposite the origin of replication (Beaurepaire and Chaconas 2005). Genetic borreliology stoically awaits a tractable system to make markerless mutations.

5 Inducible Gene Expression

The ability to artificially regulate gene expression in *B. burgdorferi* has been immensely valuable for probing the function of essential genes and for assaying the kinetic requirements of gene expression in vivo (Samuels 2011). Failure to generate a null mutant after exhaustive attempts suggests that the targeted gene is essential (Bandy et al. 2014; Chu et al. 2016; Dunn et al. 2015; Lenhart et al. 2012; Liang et al. 2010). In these instances, a conditional mutant, generated by replacing the endogenous promoter with an inducible promoter, allows for gradual reduction of gene expression, which is not possible with a null mutation. Essentiality is demonstrated if growth ceases in the absence of gene expression. In addition, the phenotype associated with decreased expression may provide clues to the function of the gene. Inducible systems can also be harnessed to experimentally overcome a growth phenotype even if the gene is not essential. For example, changes in morphology, metabolism or gene expression may be challenging or laborious to assess in a null mutant, but may be expedited using a conditional mutant where expression is induced for outgrowth and then stopped by removing the inducer.

5.1 *lac* System

A couple of strategies for inducible gene expression that have been workhorses in other bacteria have been adapted for *B. burgdorferi*. The first was based on the *lac* operon of *E. coli* where the repressor LacI binds to operators to repress gene expression (Jacob and Monod 1961). Repression is relieved by an inducer, such as isopropyl β-D-1-thiogalactopyranoside (IPTG), which binds LacI and causes an allosteric change that inhibits binding to the operators. In *B. burgdorferi*, the strong *flgB* promoter (Ge et al. 1997) fused to *lacI* was inserted in *bbe02* on lp25, resulting in high levels of LacI production (Gilbert et al. 2007). The inducible *flac* promoter was fashioned by integrating an optimized operator, *lacO*$_{id}$ (Oehler et al. 1994), into the *flgB* promoter; the hybrid *flacp* is then fused to any gene (Gilbert et al. 2007). In vitro, IPTG induced OspC production in a *flacp-ospC* strain (Gilbert et al. 2007) and RpoS production in a *flacp-rpoS* strain (Caimano et al. 2007; Gilbert et al. 2007). Furthermore, using a mouse model in which IPTG was administered in the drinking water, OspC was required immediately upon injection of *B. burgdorferi* into the mouse as well as during the early stages of murine infection (Gilbert et al. 2007). *flacp* has also been fused to *bosR*, encoding a transcriptional regulator (Hyde et al. 2010), *bamA* and *tamB*,

encoding the outer membrane protein transport machinery (Iqbal et al. 2016; Lenhart and Akins 2010), and *ftsH*, encoding a membrane protein protease (Chu et al. 2016).

Another inducible system based on the *lac* operon consists of the *flaB* promoter fused to *lacI* and the hybrid T5/*lac* promoter *pQE30* from plasmid QE30 (Blevins et al. 2007). IPTG induced expression of several genes fused to *pQE30*, including *bptA*, encoding an outer surface lipoprotein required for tick persistence (Blevins et al. 2007), *rrp2* (Groshong et al. 2012; Ouyang et al. 2014a), *csrA*, encoding an RNA-binding protein (Ouyang et al. 2014b), *dhhP*, encoding a cyclic-di-AMP phosphodiesterase (Ye et al. 2014), and *resT*, encoding the telomere resolvase (Bandy et al. 2014).

5.2 *tet* System

In addition to the *lac*-based gene regulation in *B. burgdorferi*, there have been two other inducible systems incorporating tetracycline resistance regulation from the *E. coli* transposon *Tn*10 (Cabello et al. 2006; Whetstine et al. 2009). The TetR repressor binds to the *tet* operator and the tetracycline analog anhydrotetracycline induces gene expression. In one version, the *flaB* promoter was fused to *tetR* and the *tet* operator was combined with the *B. burgdorferi ospA* promoter forming the hybrid P_{ost} (Whetstine et al. 2009), which was fused to *gfp* (Whetstine et al. 2009) as well as *bpaB* and *ebfC*, which encode small DNA-binding proteins (Jutras et al. 2012a, b). In the other *tet* system in *B. burgdorferi*, *tetR* was also driven by the *flaB* promoter and expression of *gfp* or *bmpA*, encoding an outer membrane protein, was controlled by fusing *tet* operators to the *bmpA* promoter (Cabello et al. 2006).

Thus, a few variations on inducible gene regulation have been developed for *B. burgdorferi*, although the *lacI* systems are more widely used and, notably, function in vivo in an animal model (Chu et al. 2016; Gilbert et al. 2007).

6 Complementation

The fulfillment of molecular Koch's postulates assigning a function to a gene product in a microbe requires complementation of an isogenic mutant (Falkow 1988). In bacteria, including *B. burgdorferi*, synthetically constructed mutants are typically complemented either in *cis* by genetically restoring the wild-type allele to the native locus or in *trans* by introducing the functional gene to a heterologous locus, often on a plasmid vector. Each approach has strengths and weaknesses.

6.1 Obstacles

The barriers to *B. burgdorferi* transformation, especially the restriction-modification systems (the *bbe02* and *bbq56* gene products) and loss of genomic elements, some of which are essential for host infection, are particularly pertinent to complementation. In addition to the aforementioned tactics of *bbe02* disruption and DNA methylation, various methods facilitate transformation of infectious mutant strains for complementation. A *bbe02* mutant has been deployed as the parental strain, in both wild-type and mutant *bbq56* backgrounds (Chan et al. 2015; Gilbert et al. 2007; Kasumba et al. 2015; Rego et al. 2011). This modification serves double duty by increasing transformation efficiency and furnishing a selection for

lp25, which is readily lost during in vitro cultivation, but essential for host infection (Grimm et al. 2004a; Labandeira-Rey and Skare 2001; Purser and Norris 2000; Purser et al. 2003; Revel et al. 2005; Strother and de Silva 2005). Another method employed to ease complementation utilizes the *pncA* gene from lp25, which is necessary and sufficient to maintain mouse infectivity, carried on a shuttle vector, along with the complementing gene, and transformed into a strain that lacks lp25 (and lp56) (Seshu et al. 2006; Xu et al. 2005). This approach allows for vertebrate host studies, but not tick experiments as undefined genes from lp25, besides *pncA*, are necessary for persistence in the tick (Grimm et al. 2005; Revel et al. 2005; Strother and de Silva 2005). More recently, a suicide plasmid that recombines both the complementing gene and a selectable marker into the *bbe02* locus on lp25 has been established that is competent for in vivo experiments in the tick-mouse model (Kasumba et al. 2015). Thus, what was once an impediment to genetic manipulation and animal studies, a restriction-modification system on a plasmid unstable in vitro but required in vivo, has been exploited to study the role of gene products in B. burgdorferi.

6.2 *Trans*-complementation and Shuttle Vectors

B. burgdorferi sequences essential for plasmid partitioning and replication have been incorporated to construct several shuttle vectors that originally made complementation experiments possible. pBSV2 was the first, and likely remains the most popular, shuttle vector; it contains a 3.3-kb segment from the endogenous plasmid cp9 and is capable of autonomous replication in both *E. coli* and *B. burgdorferi* (Stewart et al. 2001). This region contains three open reading frames of a paralogous gene family (PGF) that is not homologous to any known replication or partitioning genes, but is found in many of the *B. burgdorferi* circular and linear plasmids (Casjens et al. 2000; Chaconas and Norris 2013; Zückert and Meyer 1996). In all, there are five different members in the PGF, with different combinations present on *B. burgdorferi* plasmids (Beaurepaire and Chaconas 2005; Chaconas and Norris 2013; Eggers et al. 2002; Zückert and Meyer 1996). pBSV2 (Stewart et al. 2001), and its derivatives containing alternative selectable markers (Elias et al. 2003; Frank et al. 2003), as well as the pCE320 series derived from cp32 (Eggers et al. 2002) are stably maintained both in vitro and in vivo. A number of shuttle vectors have been constructed using the PGFs from lp25, lp28-1, lp17, a cp32, and cp26 (Beaurepaire and Chaconas 2005; Byram et al. 2004; Eggers et al. 2002; Stewart et al. 2003; Tilly et al. 2012). Interestingly, all of these shuttle vectors are incompatible with their native plasmid from which the partitioning region has been cloned, as evidenced by transformation displacing the endogenous plasmid (Chaconas and Norris 2013), except cp26, which contains other essential genetic elements (Bandy et al. 2014; Byram et al. 2004).

Broad-host-range plasmids also have served as shuttle vectors to complement *B. burgdorferi* mutants, and to ectopically express genes. These plasmids can be replicated and maintained in a number of different bacterial hosts. The plasmid pJRS525 and related pGK12, both from *Lactococcus lactis*, have been used to complement *B. burgdorferi* mutants and express *gfp*, respectively (Hübner et al. 2001; Sartakova et al. 2000). pGK12 is less stable, without selection, and present at lower copy number than the more widely used pBSV2, potentially practical properties for plasmid curing and matching gene dosage during complementation (Sartakova et al. 2000).

6.3 Genetic Reconstitution

The other way to complement a mutant in *B. burgdorferi* is genetic reconstitution. This strategy involves allelic exchange at the original mutated locus to restore the wild-type gene (Hübner et al. 2001; Tilly et al. 2001). The restriction-modification systems do not pose a hurdle with this method as linear, non-autonomously replicating DNA substrates are used, but loss of native plasmids during this second transformation remains a concern. The advantage of genetic reconstitution is that returning the wild-type gene to its native locus restores the copy number, regulation, supercoiling state, and any small noncoding RNAs that may have been disrupted in the mutant. Disadvantages of genetic reconstitution include additional manipulation of the targeted loci, which may cause polar effects, thereby confounding comparison of the mutant and complemented strain phenotypes. Genetic reconstitution in a separate noncoding region of the genome may alleviate some of these concerns, but we now know that *B. burgdorferi* has an extensive, and incompletely characterized, sRNA transcriptome that must be considered when altering any region of the genome that is considered "non-coding" (Adams et al. 2017b; Arnold et al. 2016; Lybecker and Samuels 2017; Popitsch et al. 2017).

6.4 Caveats and Considerations

Occasionally, despite a plethora of painstaking attempts, complementing a *B. burgdorferi* mutant is simply not successful (Stewart et al. 2008). The reason is rarely clear, but some mutants have severe growth phenotypes, including retarded replication, elongated morphology and "clumping," which adversely affect the preparation of competent cells or efficient electrotransformation and recovery. In the case where a mutant is unable to be complemented, the last best course to assign a function to a mutated gene is to generate multiple independent clones from separate transformations. If all the clones have the same phenotype, then this is stronger evidence than relying on the phenotype of a single mutant. This line of reasoning is obviously not as robust as complementation of the mutant, but decreases the chance that an unrelated second-site mutation in the genome is responsible for the phenotype, as that change is unlikely to occur in two independent mutants, particularly if two different selectable markers are used. There are several analyses that should be undertaken if complementation does not restore the wild-type phenotype (Bugrysheva et al. 2005). Of course, the plasmid profile should be determined following all genetic manipulations, and transformants lacking an intact genome should not be further studied. Second, an alternative complementation construction (in *trans* or genetic reconstitution) should be attempted as both methods have their limitations. Third, the recombinant DNA in the mutant should be recovered from the complemented strain and sequenced to determine if any changes have occurred during genetic manipulation. Inadvertent mutations could be introduced during subcloning in *E. coli* or in *B. burgdorferi*. If the sequence is correct, then the level of gene expression should be compared between the complemented strain and the isogenic wild type by qRT-PCR or Northern blot analyses. Northern blot analysis of the mRNA has the advantage of detecting differential transcript processing between the two strains, which may affect stability or translation. A different complementation strategy would be prudent if the levels or kinetics of expression are significantly different between the complemented and wild-type strains. Finally, if all else fails, then polar effects from the mutation, second-site mutations and disruption of small noncoding RNAs should be

considered. If the expression of adjacent genes has been altered in either the mutant or complemented strain, then another strategy such as site-directed mutagenesis may be warranted. Recently, *B. burgdorferi* has been found to have an extensive collection of noncoding sRNAs, nearly all of unknown function (Adams et al. 2017b; Arnold et al. 2016; Lybecker and Samuels 2017; Popitsch et al. 2017). These include antisense, intergenic, and intragenic (within a gene on the coding strand) RNAs. Mutant construction and complementation may alter these previously unappreciated sRNAs, resulting in failed complementation or confounding data interpretation. These genetic elements should be considered when designing both mutant and complementation strategies. Finally, the effect of extraneous second-site mutations on complementation may finally be possible to address as the cost of sequencing entire bacterial genomes continues to decrease. This may be especially applicable to the small genome (*1.5 Mb) of *B. burgdorferi* (Fraser et al. 1997). While there remain numerous challenges for genetically manipulating *B. burgdorferi*, particularly during complementation, there is now truly light at the end of the tunnel.

7 Transposon Mutagenesis and High-Throughput Genetics

Transposon mutagenesis is undoubtedly an exceedingly powerful genetic manipulation tool (Lin et al. 2014; Morozova et al. 2005; Stewart et al. 2004; Stewart and Rosa 2008). The most popular transposon system employed in *B. burgdorferi* entails a hyperactive allele of *Himar1*, a *mariner*-family transposable element from *Haematobia irritans*, fused to a strong, constitutive promoter and carried on a suicide vector; also included are the inverted terminal repeats that serve as binding sites for the transposon and flank a ColE1 origin as well as a selectable marker (Stewart et al. 2004; Stewart and Rosa 2008). The genome-wide mutagenesis technology has led to the generation of a signature-tagged mutant (STM) library in an infectious background (Botkin et al. 2006; Lin et al. 2012), the application of transposon sequencing (Tn-seq) (Troy et al. 2013, 2016), and the identification of scores of genes involved in diverse aspects of spirochete physiology as well as disease pathogenesis (Botkin et al. 2006; Khajanchi et al. 2016; Lin et al. 2009, 2012, 2015; Liu et al. 2009; Morozova et al. 2005; Ramsey et al. 2017; Stewart et al. 2004; Troy et al. 2013, 2016). A notable caveat is that STM and Tn-seq libraries may include mutants that either carry second-site mutations or are missing genomic elements; again, the segmented nature of the genome is a challenge for high-throughput methodologies in *Borrelia*. Prudence dictates that clean mutants be constructed before a function is ascribed to a gene. For example, the tour de force implementation of in vivo expression technology, another high-throughput genetic screen, inadvertently identified a red herring due to the loss of a plasmid (Ellis et al. 2014), although the system has proved insightful in defining an infectivity-associated sRNA transcriptome in the spirochete (Adams et al. 2017b).

8 Counterselection

Counterselectable markers (CSM) select for the loss of a DNA sequence (Reyrat et al. 1998) and can provide another avenue to generate markerless mutations. CSM generally confer susceptibility to a substance that is lethal to an organism. This scenario requires that the parental strain in the absence of the CSM is resistant to the substance used for negative selection, which limits employing counterselection.

Counterselection has been demonstrated using the *rpsL* gene, encoding the S12 ribosomal protein, in the spirochetes *Leptospira biflexa* (Picardeau et al. 2001) and *B. burgdorferi* (Drecktrah et al. 2010). A mutant *rpsL* gene located on the chromosome confers resistance to the antibiotic streptomycin (Criswell et al. 2006). The presence of a second, wild-type allele of *rpsL* in *trans* renders the merodiploid *B. burgdorferi* susceptible to streptomycin; although the mechanism has not been fully defined, susceptibility is dominant because translational errors by the sensitive ribosomes (Karimi and Ehrenberg 1994, 1996) will poison the cell. The addition of streptomycin was shown to select for the loss of the plasmid carrying the wild-type *rpsL* gene that confers susceptibility, thus providing the first functioning CSM in *Borrelia* (Drecktrah et al. 2010). Additionally, mutations to the *parC* gene, encoding a subunit of topoisomerase IV, confer resistance to fluoroquinolones in *B. burgdorferi* (Galbraith et al. 2005), providing another potential CSM.

9 Surrogate Genetics

Many barriers to genetic manipulation of *B. burgdorferi*, including low transformation efficiency, loss of endogenous plasmids during transformation, low GC content, slow growth, and expensive, complex media, have made surrogate genetics using *E. coli* an attractive and productive method to study spirochete gene function. Numerous *B. burgdorferi* genes have been heterologously expressed in *E. coli* mutants to complement the phenotype in order to define the gene products: *grpE* and *dnaJ* (Tilly et al. 1993), *guaA* (Margolis et al. 1994), *lon* (Cloud et al. 1997; Coleman et al. 2009), *gac* (Knight and Samuels 1999), *oppA* (Lin et al. 2001), *pncA* (Purser et al. 2003), *recA* (Liveris et al. 2004; Putteet-Driver et al. 2004), *rpoS* (Eggers et al. 2004), *rel$_{Bbu}$* (Bugrysheva et al. 2005), and *hfq* (Lybecker et al. 2010). Conversely, the *E. coli hfq* gene has been heterologously expressed in a *B. burgdorferi hfq* mutant and partially complemented the phenotype, providing evidence that the spirochete has an Hfq-like RNA chaperone (Lybecker et al. 2010). Several endogenous *B. burgdorferi* promoters are capable of driving gene expression in *E. coli*. In fact, determining the consensus sequences for the sigma factors RpoS and RpoN was facilitated by studies in *E. coli* (Alverson et al. 2003; Burtnick et al. 2007; Caimano et al. 2004; Eggers et al. 2004, 2006). In addition, key experiments characterizing the promoter for the operon encoding the decorin-binding proteins (Ouyang et al. 2010) and describing the transcriptional regulator BosR (Boylan et al. 2003) were performed in *E. coli*.

Curiously, *B. burgdorferi* itself has served as a surrogate to heterologously express genes from less genetically tractable spirochetes, such as *Treponema pallidum*, the etiological agent of syphilis. *T. pallidum* lacks systems for both genetic manipulation and in vitro culture. The *T. pallidum* gene encoding the putative lipoprotein TP0435 was expressed in *B. burgdorferi* and shown to mediate adherence to host cells (Chan et al. 2016). *tp0435* expression was driven by its endogenous promoter, demonstrating that a *T. pallidum* promoter functions in *B. burgdorferi* (Chan et al. 2016). Using a similar system, the *T. pallidum* gene *tp0751*, encoding pallilysin, was also expressed in *B. burgdorferi* to complement an adhesin-attenuated mutant and restore interactions with the mouse vasculature in vivo (Kao et al. 2017). The promise of these surrogate systems to study surface-exposed adhesins provides a significant step toward the goal of developing *T. pallidum* vaccine candidates. *B. burgdorferi*, which has its own experimental hurdles, can,

surprisingly, serve as a valuable tool to facilitate studies in other genetic-less members of the spirochete phylum.

10 Conclusions

The post-genomic era has ushered in powerful genetic approaches that, combined with robust animal models, have elevated spirochetology from its relegated plight as a microbiological curiosity into the limelight. The disciplines of *Borrelia* microbiology and Lyme disease pathogenesis have come to depend on genetic manipulation, and the study of relapsing fever spirochetes is not too far behind. Mutagenesis in these recalcitrant bacteria is still not for the faint-hearted, but most molecular laboratories are proficient at reverse genetics, although ignoring several considerations and caveats remains perilous. Certain genetic tricks, notably markerless mutations, are still absent from the borreliologist's quiver, but this just requires some attention and a bit of technical elbow grease. The state-of-the-art technologies for inducible gene expression in vivo and intravital microscopy are destined to reveal incredible insights into how these wily microbes interact with their hosts and are maintained in nature.

Acknowledgments

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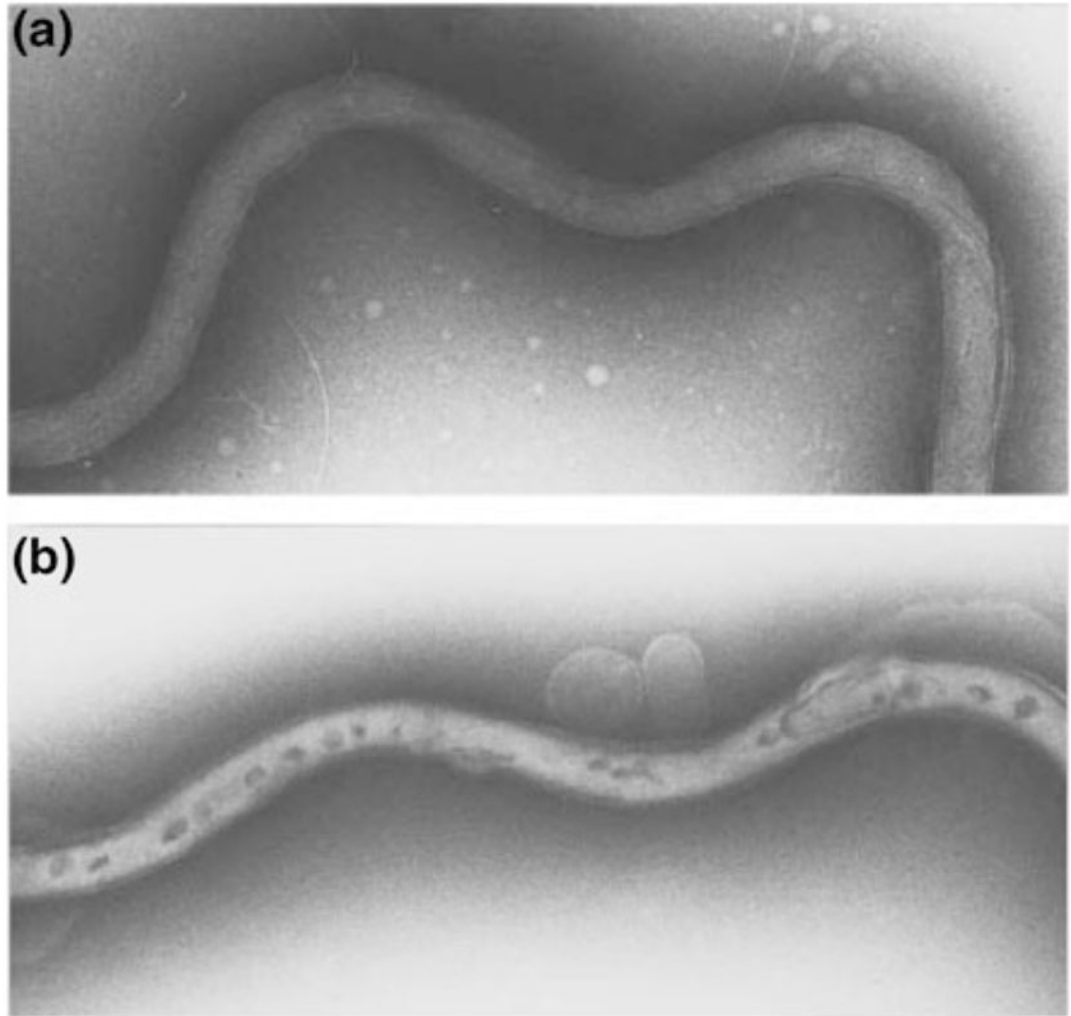
Fig. 1.

B. burgdorferi before (**a**) and *after* (**b**) electroporation as visualized by negative staining and transmission electron microscopy. Note the darkly stained regions that are thought to be transient pores generated by the electric pulse that serve as a passageway for DNA to enter the spirochete. Reprinted with permission from Samuels and Garon (1997)