THE BURGEONING MOLECULAR GENETICS OF THE LYME DISEASE SPIROCHAETE

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Abstract | Lyme disease is the most commonly reported vector-borne disease in North America and Europe, yet we know little about which components of the causative agent, *Borrelia burgdorferi*, are critical for infection or virulence. Molecular genetics has provided a powerful means by which to address these topics in other bacterial pathogens. Certain features of *B. burgdorferi* have hampered the development of an effective system of genetic analysis, but basic tools are now available and their application has begun to provide information about the identities and roles of key bacterial components in both the tick vector and the mammalian host. Increased genetic analysis of *B. burgdorferi* should advance our understanding of the infectious cycle and the pathogenesis of Lyme disease.

More than 20 years have passed since scientists first described Lyme disease and identified the novel tick-borne spirochaete *Borrelia burgdorferi* as the cause[1–3]. Lyme borreliosis has since been recognized as an important zoonotic disease of the Northern hemisphere, with endemic foci in those temperate regions of the United States, Europe and Asia in which infected wildlife and ticks are prevalent (BOX 1). Although much has been learned about *B. burgdorferi* since its isolation in 1982, insights into the importance of specific bacterial genes for infection of the mammalian host or tick vector have begun to emerge only recently through molecular genetic analyses.

Certain features of *B. burgdorferi* have impeded genetic investigations. Phylogenetically, spirochaetes represent a phylum of Bacteria that is distinct from other main bacterial groups[4,5] (BOX 2). Perhaps owing to this evolutionary distance, genetic tools that have been developed for other bacteria, such as selectable markers and shuttle vectors, may not work in *B. burgdorferi* without the addition of key borrelial sequences that are needed to drive gene expression or autonomous plasmid replication[6–9]. Spirochaetes also exhibit a characteristic morphology, with inner and outer membranes surrounding periplasmic flagella and a flexible cell wall[10–13]; the outer membrane of *B. burgdorferi* is easily disrupted and does not contain lipopolysaccharide (LPS)[14–16] (FIG. 1). It is not known how or if these physical characteristics limit the introduction of DNA into *B. burgdorferi*, but *Borrelia* are notoriously difficult to transform[17,18].

The unusual structure of the *B. burgdorferi* genome, however, presents a challenge for genetic studies in this organism (BOX 3). *B. burgdorferi* has a segmented genome that is composed of a small linear chromosome of approximately 900 kb and >20 different plasmids ranging in size from 5 to 56 kb[16,19]. Some plasmids are unstable during *in vitro* propagation, but are required for infectivity *in vivo*[20–23]. Heterogeneity can arise among clonal derivatives of an outgrowth population due to plasmid loss, with significant consequences for plasmid-encoded phenotypic traits, such as antigenic variation and NAD cofactor biosynthesis[24–28]. Hence, diligence must be taken to ensure that the plasmid content of genetically manipulated bacteria remains constant[25,26]. In contrast to plasmid loss, genomic rearrangements due to recombination between closely related sequences occur only rarely during *in vitro* growth[29,30]. This indicates

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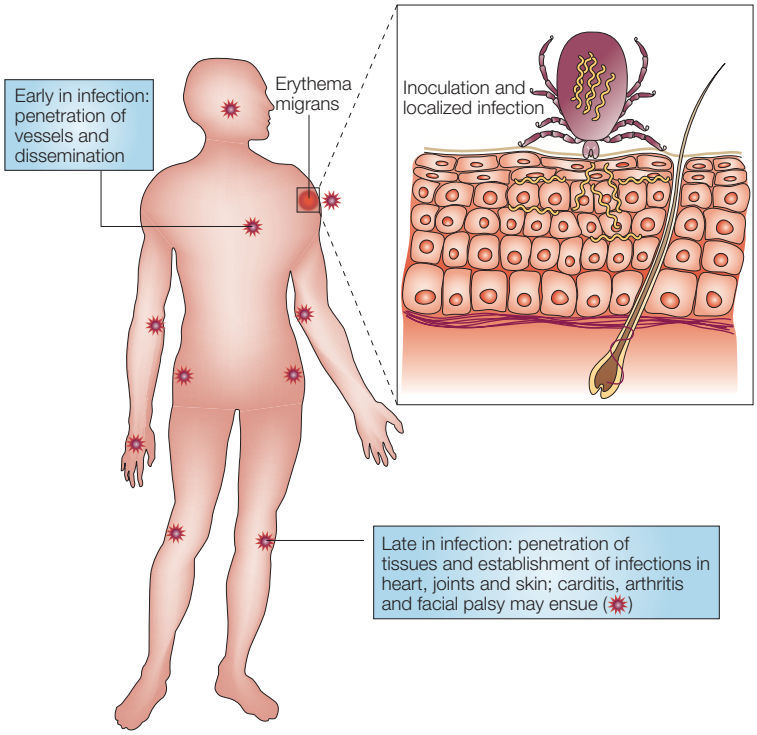
Box 1 | **Lyme disease**



The spectrum of clinical manifestations resulting from infection with *Borrelia burgdorferi* is generally termed Lyme disease in the United States and Lyme borreliosis in Europe and Asia. Tens of thousands of cases are reported annually in both North America and Central Europe, which probably makes Lyme borreliosis the most common vector-borne bacterial disease in the world[132]. Three closely related *Borrelia* genospecies — *B. burgdorferi* sensu strictu (s.s.), *Borrelia garinii* and *Borrelia afzelii* — cause Lyme disease; they are collectively referred to as *B. burgdorferi* sensu lato (s.l.)[133]. All three genospecies exist in Europe and Asia, whereas only *B. burgdorferi* s.s. has been found in the United States. Additional closely related *Borrelia* genospecies have been identified, but their potential as human pathogens has not been established[134].

Lyme disease has both acute and persistent phases, which are typically subdivided into three separate stages[135]. During stage I, or early-localized Lyme disease, manifestations include a transient inflammatory skin rash known as erythema migrans (EM), where the spirochaetes are localized in the skin. Stage I manifestations can also include a mild influenza-like illness with nonspecific symptoms. Neither EM nor influenza-like symptoms are present initially in all patients who subsequently develop stage II, or early-disseminated Lyme disease. The symptoms of the second stage of Lyme disease typically involve the organs to which the spirochaetes have disseminated, such as the joints, heart or nervous system, and include arthritis, carditis and neuropathies. Rarely, neurological symptoms, including meningitis, can be seen.
A 2–4-week course of antibiotics is generally effective therapy for the localized and early-disseminated stages of Lyme disease. A subset of patients requires longer treatment and can develop chronic symptoms that are unresponsive to antibiotics, possibly as post-infectious autoimmune sequelae[135]. If untreated, Lyme disease can progress to stage III, or late-persistent disease, with chronic arthritis, neuroborreliosis and skin disorders.

The prevalence of particular Lyme disease symptoms varies between North America and Europe, with arthritis more common in the United States and neurological and skin disorders more common in Europe[132,135]. These differences probably reflect the non-uniform geographical distribution of the three genospecies[132]. Although the connection is not absolute, different genospecies of *B. burgdorferi* s.l. are associated with distinct clinical manifestations of Lyme disease; infection by *B. burgdorferi* s.s. is frequently associated with arthritis, *B. garinii* with neurological disease and *B. afzelii* with chronic skin disorders[136]. In general, the symptoms of Lyme disease reflect the site in which the spirochaetes reside. However, a clear picture of how host and bacterial components interact, or which are responsible for the varied manifestations of Lyme disease, has not emerged.

The figure shows how spirochaetes are inoculated into the skin by the bites of infected ticks and initially establish a localized infection that can cause a painless rash known as erythema migrans. As spirochaetes migrate from the site of the tick bite, they penetrate vessels and are transiently present in the blood, in which they disseminate throughout the body. Spirochaetes subsequently exit the blood and enter various tissues and organs, where they can establish persistent infections (denoted by asterisks). The manifestations of Lyme disease vary with the stage of disease and site of infection.

that the mechanisms for homologous recombination in *B. burgdorferi* might be tightly controlled or inefficient, with potential limitations for targeted mutagenesis. Finally, although the complete sequence of the *B. burgdorferi* genome is an informative and valuable tool, it revealed no obvious virulence factors, thus providing little insight into which spirochaetal components are central to the pathogenesis of Lyme disease[16,19].

Given the difficulties associated with genetic studies in *B. burgdorferi*, one might ask if they are worth pursuing? We believe this approach is justified for several reasons. The infectious cycle and unusual genomic structure of *B. burgdorferi* present several interesting basic biological and molecular questions related to vector transmission, host adaptation and plasmid maintenance. Mutants (once generated) can be tested in an

Box 2 | **The phylum *Spirochaetes***

Members of the phylum *Spirochaetes* are easily identified by their unique coiled morphology and periplasmic flagella[4]. The phylum is composed of a single class and order, divided into three families (see figure) [137]. In addition to *Borrelia*, genetic studies have been conducted with *Leptospira*, *Brachyspira* and *Treponema*[138–143]. Listed within the GenBank taxonomy database of the National Center for Biotechnology Information there are >700 separate entries for spirochaetes. Many of these entries represent uncultured spirochaetes and indicate that our knowledge of this phylum is very limited.

Despite their phylogenetic grouping and similar morphological characteristics, spirochaetes are quite diverse. Chromosome sizes range from 0.91–4.3 Mb, and the GC content varies from 28.6%–52.8% (REFS 16,144–147). The borreliae are the only known members of the phylum that contain linear DNA molecules. Like *Treponema pallidum* and *Treponema denticola*, *Borrelia* lack lipopolysaccharides in the outer membrane. Spirochaete species include both free-living and host-associated members, many of which are pathogens of humans and other animals. *Brachyspira hyodysenteriae* (family *Brachyspiraceae*) causes swine dysentery, *Leptospira interrogans* (family *Leptospiraceae*) causes leptospirosis in animals (including humans), whereas *Leptospira biflexa* is a free-living saprophyte. Members of the family *Spirochaetaceae* include the borreliae, *T. pallidum* (the causative agent of syphilis), *T. denticola* (associated with periodontal disease) and *Spirochaeta bajacaliforniensis*, a free-living anaerobe that is isolated from mud[148].

Spirochaetes (phylum)
- *Spirochaetes* (class)
  - *Spirochaetales* (order)
    - ***Brachyspiraceae*** (family)
      - *Brachyspira* (genus)
    - ***Leptospiraceae***
      - *Leptonema*
      - *Leptospira*
    - ***Spirochaetaceae***
      - *Borrelia*
      - *Brevinema*
      - *Cristispira*
      - *Spirochaeta*
      - *Spironema*
      - *Treponema*

experimental mouse–tick infectious cycle that closely mimics natural infections[31–35]. Few other human pathogens have an experimental animal model that accurately reproduces the components of transmission, infection and disease. Insight may also be gained into other vector-borne bacterial pathogens, such as the tick-borne agents of ehrlichiosis and tularaemia, that are even less amenable to genetic investigation, or for which the complete infectious cycle cannot be reproduced in the laboratory (TABLE 1). The unusual structure of the *Borrelia* genome also presents an opportunity to address basic molecular topics, such as possible influences of DNA topology on recombination and gene expression with linear versus circular DNA. Finally, the demonstrated strength of molecular genetic analysis of infectious diseases, particularly as applied to bacterial pathogens[36,37], makes a sound argument for attempting a similar approach with *B. burgdorferi*.

The infectious cycle

Lyme disease is considered a zoonosis because the causative agent is maintained in a natural infectious cycle that does not include humans, who only inadvertently become infected. Ticks both acquire and transmit *B. burgdorferi* sensu lato (s.l.) by feeding on a variety of small mammals that act as reservoir hosts[38–41]; birds can also be a reservoir for *Borrelia garinii* in Europe and Asia[42]. *B. burgdorferi* s.l. is transmitted by *Ixodes* ticks, mainly *Ixodes scapularis* and *Ixodes pacificus* in the United States, and *Ixodes ricinus* and *Ixodes persulcatus* in Europe and Asia[1,39,43]. Transovarial transmission of *B. burgdorferi* s.l. is infrequent and inadequate to maintain infected populations of either the tick vector or vertebrate hosts[43]. *Ixodes* ticks feed once at each of the three active stages — larval, nymphal and adult. So, uninfected larval ticks generally acquire *B. burgdorferi* s.l. by feeding on infectious vertebrates, and uninfected mammals acquire spirochaetes when fed on by infected nymphal ticks (FIG. 2).

The dynamics of *B. burgdorferi* infection in the mammalian host have not been clearly defined because very low numbers of organisms are present in immunocompetent hosts and therefore the bacteria are difficult to follow. However, it has been established in a mouse model of infection that *B. burgdorferi* initially establishes a localized infection in the skin at the site of the tick bite, transiently disseminates via the bloodstream and subsequently establishes persistent infection in various tissues, including skin, joints, heart and bladder[44]. This pattern of infection mimics what is known to occur during human infection with *B. burgdorferi* s.l. (BOX 1). Although natural rodent hosts and most laboratory mice can have persistent *B. burgdorferi* infections without signs of disease, some inbred mice develop joint and heart pathologies that resemble Lyme disease when infected with *B. burgdorferi*[35]. In addition to mice, other experimentally infected animals include rats, hamsters, gerbils, rabbits, dogs and monkeys. Significantly, infected primates can develop infection of the central nervous system (CNS) and neurological manifestations that are not common in infected rodents[45].

As small mammals are natural vertebrate hosts for Lyme disease spirochaetes and *Ixodes* ticks can be reared in the laboratory, an experimental infectious cycle was developed shortly after the spirochaete was first isolated[31,33,46]. More recently, this experimental system has been used to assess the *in vivo* phenotype of *B. burgdorferi* s.l. mutants. This infectious cycle is usually initiated by needle inoculation of mice. Subsequently, larval ticks that feed on these infected mice can acquire spirochaetes. After the moult to nymphs, the infected ticks can transmit spirochaetes to naive mice. Mice that have been infected naturally by tick bite can also be used to infect more larval ticks (FIG. 3). The artificial infection of ticks by one of several methods bypasses the need for feeding on an infected mouse[47–50]. These techniques are crucial for studying the phenotype in the tick vector of *B. burgdorferi* mutants that cannot infect or persist in the mouse. They also allow the

delivery of spirochaetes to mice by the natural route of tick bite rather than by needle inoculation, without a prerequisite infection in mice (BOX 4).

Reproduction of the entire infectious cycle in the laboratory has enabled careful analysis of the timing and mechanics of spirochaete transmission from the tick[31,51–55]. After ingestion with the bloodmeal, spirochaetes multiply in the midgut of the larval tick, where they persist through the moult to the nymphal stage. With few exceptions, the spirochaetes are restricted to the midgut and do not infect other tissues or organs in the tick. When the nymphal tick feeds, spirochaetes again replicate and some leave the midgut and migrate to the salivary glands, from where they are transmitted by saliva. Only small numbers of spirochaetes are transiently present in the salivary glands. *Ixodes* ticks feed slowly and take 3–7 days to complete a bloodmeal. Efficient transmission of *B. burgdorferi* from infected nymphs occurs after approximately 48 hours of tick attachment[31]. Immediately before and during transmission, spirochaetes undergo a marked alteration of outer membrane protein composition — the major outer surface protein (Osp) that is expressed changes from OspA to OspC, as first described by Schwan *et al.*[56] Environmental conditions that change during the bloodmeal, such as temperature, pH, nutrient and cell density, have been shown to influence transcription of the *osp* genes *in vitro* and are presumed to have roles in triggering the switch in spirochaetal surface phenotype during the tick bloodmeal[56–60].

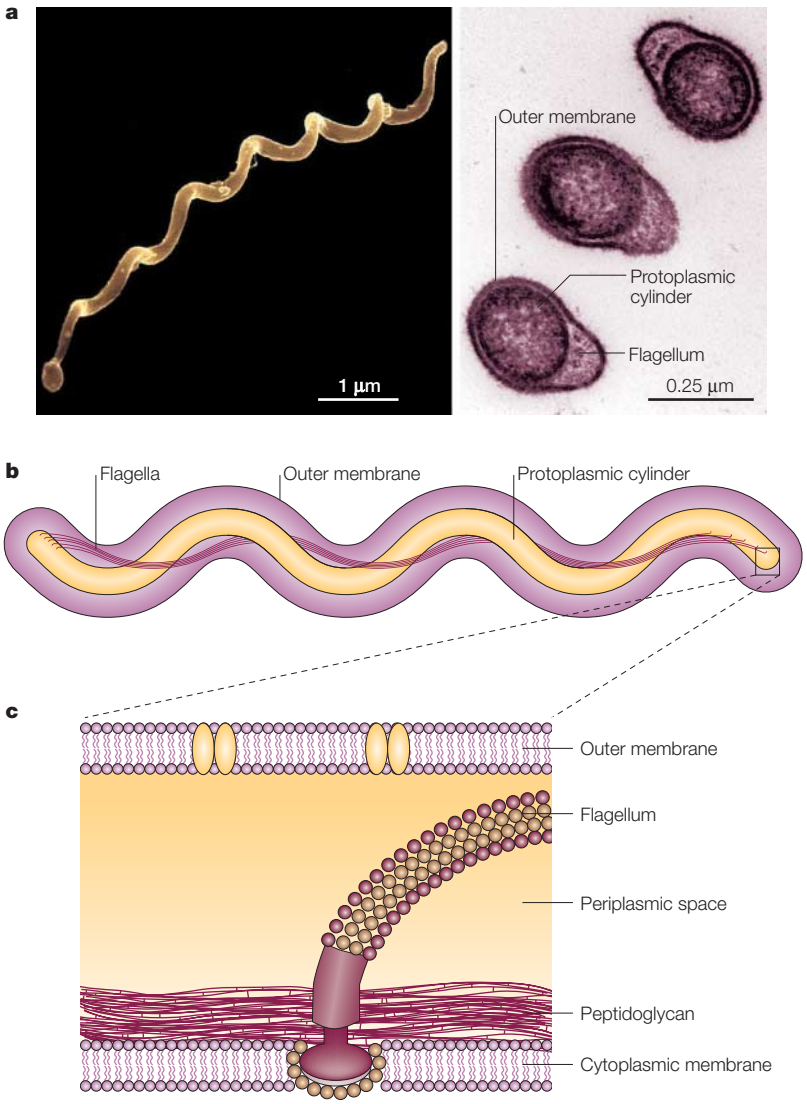


Figure 1 | **Structure and morphology of *Borrelia burgdorferi*. a** | Scanning (left) and transmission (right) electron micrographs of *Borrelia burgdorferi*. The helical shape of *Borrelia* (visible in the scanning electron micrograph) is imparted by the periplasmic flagella, which can be seen in the cross-sectional view of the spirochaete in the transmission electron micrograph. Micrographs provided by David Dorward and Elizabeth Fischer, Rocky Mountain Laboratories, NIAID, NIH. Left-hand panel is reproduced with permission from *Nature* 11 Dec 1997 (cover image) © Macmillan Magazines Ltd. **b** | Diagram of the spirochaete. Flagellar insertion points are located near the termini of the spirochaete. Bundles of flagella wind around the flexible, rod-shaped protoplasmic cylinder of *Borrelia* and overlap in the middle. The outer membrane constrains the flagellar bundles within the periplasm. **c** | Detailed diagram of flagella. Each flagellum is inserted into the cytoplasmic membrane and extends through the cell wall into the periplasm. Flagella are multi-component, complex structures. Spirochaetal motility results from coordinated rotation of the flagella. Part **c** is modified with permission from REF. 90 © (2002) Annual Reviews.

Characterization of the protein composition of spirochaetes in the vertebrate host has been difficult owing to the paucity of organisms in this setting. Information has been inferred from analysis of the bacterial proteins that are recognized by the host immune response[61], as well as by quantitative PCR analysis of transcripts of particular genes[62]. Direct microarray analysis of *in vivo* gene expression is not feasible owing to the small quantity of spirochaetal RNA that can be isolated from infected mammals; even the use of arrays for qualitative determination of *B. burgdorferi* gene expression in vertebrate hosts is difficult and prone to misinterpretation[63]. Akins and colleagues developed an *in vivo* culture system to directly analyse spirochaetes in the mammalian host: *B. burgdorferi* are grown in culture medium within a dialysis membrane chamber (DMC) implanted in the peritoneal cavity of a rat[64]. Spirochaetes grow to sufficient numbers in DMCs to allow direct analysis of their protein and RNA composition[65]. Although not identical to a natural infection, the patterns of gene expression and protein synthesis of *B. burgdorferi* s.l. grown in DMCs are characteristic of mammalian host-adapted organisms, such as down-regulation of OspA expression and induction of OspC expression[65,66]. Earlier work by Jonsson and co-workers demonstrated that *B. burgdorferi* could be isolated from subcutaneous implants in mice up to 14 weeks after inoculation[67]. However, spirochaetes were not confined to the implanted chamber and did not replicate to sufficient levels to allow direct analysis of proteins in host-adapted bacteria withdrawn from the chamber. Recent reports by Crother *et al.* represent a significant achievement in which spirochaetal proteins were directly extracted from the tissues of infected mice and rabbits and visualized on immunoblots of two-dimensional gels[68,69]. A limited amount of material is obtained by this approach, but it is a powerful tool with which to directly identify proteins that are synthesized by spirochaetes in the mammalian host.

Box 3 | **The *Borrelia burgdorferi* genome**

The complete genome sequence and plasmid content have been defined for only one strain of *B. burgdorferi* s.s., the original tick isolate and type strain B31 (REFS 16,19). Efforts are currently underway to sequence the complete genomes of several additional *B. burgdorferi* isolates[149]. As described, the B31 genome is composed of a small linear chromosome of approximately 900 kb, and 12 linear and nine circular plasmids that total 610 kb. Additional small circular plasmids were subsequently described in strain B31 (REF. 150). Other *B. burgdorferi* s.l. strains contain a linear chromosome and numerous linear and circular plasmids, but their plasmid content and relationship to B31 plasmids have not been precisely determined[151,152]. Recent studies indicate that horizontal transfer of chromosomal sequences and plasmids occurs among spirochaetes within the same geographical and ecological niche, in contrast to earlier reports that found little evidence for DNA exchange with sequence comparisons of geographically disparate isolates[149,153,154]. An unusual component of the *Borrelia* genome is a family of closely related, but distinct, 32-kb circular plasmids (cp32s)[19,155,156]. As many as nine separate cp32 plasmids can coexist within the same bacterium[153]. A few variable loci exist amidst a background of almost identical genes, and these loci define the unique nature of individual cp32s. The cp32 plasmids represent prophage genomes and might provide a mechanism for horizontal DNA transfer[75,157].

Coding sequences comprise approximately 90% of the *B. burgdorferi* chromosome, and most of the 769 chromosomal genes are homologous to genes of known function[16]. By contrast, the plasmids have a higher content of non-coding sequence and pseudogenes, and <10% of the 419 plasmid genes have predicted functions[19]. Although plasmids of pathogenic bacteria often carry genes that are important for infection of their hosts, none of the plasmid genes of *B. burgdorferi* have similarity to known bacterial virulence genes. This fostered the hypothesis that *B. burgdorferi* plasmids encode functions that are specific to the spirochaete infectious cycle, and that the *B. burgdorferi* host–pathogen interactions are dissimilar from those of other well-studied bacterial pathogens[19]. Subsequent analyses by multiple investigators demonstrated that a large proportion of plasmid genes are differentially expressed in response to environmental cues that distinguish various stages of the infectious cycle[60,65,66,129–131].

B. burgdorferi plasmids vary in stability, with some frequently lost after only a few generations of *in vitro* growth and others stably maintained with continuous passage [20,21,26,78,158]. The consequences of plasmid loss for growth of *B. burgdorferi in vivo* also vary; some plasmids encode functions that are critical for survival in a natural host, whereas others are lost without impact on the infectious cycle[22,23,25,83]. As growth *in vitro* is an inseparable component of genetic studies, great care must be taken to assure that spontaneous plasmid loss does not occur during genetic manipulations. In addition, plasmid-encoded restriction–modification systems limit transformation with certain constructs, providing an unwanted positive selection for variants lacking particular plasmids[82]. Hence, demonstration that the plasmid contents of mutants remain identical to that of the wild-type bacterium with which they are being compared has become an essential component of genetic studies in *B. burgdorferi*.

Genetic tools

Some of the following genetic tools are shown schematically in FIG. 4.

Selectable markers. Genetic studies in *B. burgdorferi* did not get underway until more than 10 years after the initial isolation of the spirochaete. Samuels and co-workers published the first description of transformation of *B. burgdorferi* in 1994 (REF. 17). They used resistance to coumermycin, which was conferred by the *gyrB* gene of a spontaneous *B. burgdorferi* mutant, as a genetic marker to select for introduced DNA. This seminal study both demonstrated the feasibility of genetic analysis in *B. burgdorferi* and established electroporation as a method to transform these spirochaetes. Although important advances in the genetic system of *B. burgdorferi* have been made in the ensuing 10 years, the basic method of transformation by electroporation and selection in solid media remains largely as first described. Coumermycin resistance conferred by the mutated *gyrB* gene, however, was found to be an inefficient genetic marker for gene inactivation owing to the high frequency with which incoming DNA containing this marker recombined at the chromosomal *gyrB* locus rather than undergoing allelic exchange at the targeted site[18,70]. Elias *et al.* later circumvented this limitation

Table 1 | **Characteristics and genetic tools of some vector-borne bacterial pathogens**

Pathogen	Vector	Disease	Genome sequence	*In vitro* culture	Genetic techniques	Infection studies
B. burgdorferi	*Ixodes* ticks	Lyme disease	Yes	Yes	M, Tr, Tn	E
Some borreliae	*Ornithodoros* ticks	Relapsing fever	No	Yes	—	E
Yersinia pestis	Fleas	Plague	Yes	Yes	M, Tr, Tn	E
Francisella tularensis	*Ixodes* ticks	Tularaemia	No	Yes	M, Tr, Tn	A
Rickettsiae	Louse, ticks	Typhus, spotted fever	Yes	No (intracellular)	M, Tr, Tn	E
Bartonella quintana	Louse	Trench fever	Yes	Yes	M, Tr	E
Anaplasma and *Ehrlichia*	*Ixodes* ticks	Ehrlichiosis	No	No (intracellular)	—	E

A, animal model, but no experimental vector-borne transmission; E, established vector–mammal experimental infectious cycle; M, selectable marker; Tn, transposon mutagenesis; Tr, transformation system.

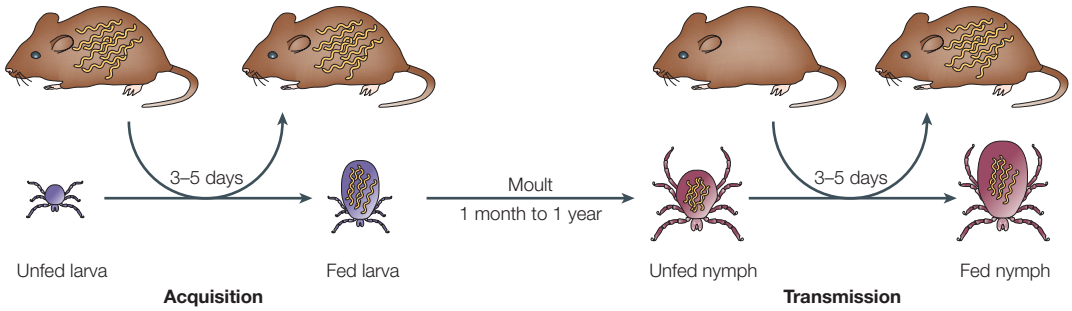


Figure 2 | **The natural infectious cycle of *Borrelia burgdorferi*.** Uninfected larval ticks acquire *B. burgdorferi* by feeding on infected small wild mammals, primarily rodents. Spirochaetes multiply and persist in the midguts of infected ticks through the moult to the nymphal stage. When infected nymphal ticks feed, the spirochaetes migrate from the midguts to the salivary glands, from where they can be transmitted to a naive mammalian host.

with a synthetic *gyrB* gene that confered resistance to coumermycin[71]. The nucleotide sequence of this synthetic gene was changed in the wobble position of the codons to maximize the difference from the *B. burgdorferi gyrB* gene, while encoding a protein of identical amino acid sequence to the coumermycin-resistant allele. When used as a selectable marker in transformations, this technique eliminated recombination with the chromosomal *gyrB* gene. However, possible effects on gene expression by alteration of DNA supercoiling complicate the use of coumermycin-resistant *gyrB* as a selectable marker.

As an alternative to coumermycin resistance conferred by a mutated *gyrB* gene, Sartakova *et al.* demonstrated the utility of foreign antibiotic-resistance genes expressed from their native promoters[72,73]. In particular, the *ermC* gene from *Staphylococcus aureus* has been used as a selectable marker in *B. burgdorferi* (TABLE 2). Bono *et al.* took a slightly different approach and fused a foreign antibiotic-resistance gene, *kan*, to a strong *Borrelia* promoter[8]. The *kan* gene was inadequately expressed from its native promoter in *B. burgdorferi*, but conferred resistance to kanamycin in both *B. burgdorferi* and *Escherichia coli* when transcribed from a *Borrelia* promoter[7,8]. The *kan* cassette also illustrated an effective strategy for the development of additional selectable markers for *B. burgdorferi* that confer resistance to other antibiotics, including gentamicin and streptomycin[71,74]. Some other common selectable markers, such as those conferring resistance to ampicillin and tetracycline, cannot be used in *B. burgdorferi* because these antibiotics are clinically useful in the treatment of Lyme disease.

Strains. Most genetic studies have used the type strain B31 of *B. burgdorferi* sensu stricto (s.s.) owing to the advantage offered by the availability of the genome sequence (TABLE 2). However, transformation of other *B. burgdorferi* s.s. strains is feasible, and mutations or foreign DNA have also been introduced into strains N40 (REF. 9), Ca-11.2A[75], 297 and HB19 (TABLE 2). So far, transformation of strains from the genospecies *B. garinii* or *Borrelia afzelii* has not been reported, but there are no obvious obstacles to genetic studies with these spirochaetes. Over the years, individual laboratories have chosen to work with different *B. burgdorferi* strains for a variety of reasons. In general, the strain chosen for gene inactivations reflects the one with which a particular laboratory is most familiar and has conducted experiments related to the gene of interest. No rigorous comparisons have been made regarding the relative merits of different *B. burgdorferi* strains for genetic investigations.

Shuttle vectors. Several shuttle vectors have been developed for *B. burgdorferi*. The first description of autonomous replication of introduced DNA in *B. burgdorferi* was with a broad host-range vector from *Lactococcus lactis*[72]. This represented an important development in the genetic analysis of *B. burgdorferi*, but its subsequent use as a shuttle vector has not been widespread because it is relatively unstable. Additional shuttle vectors have been developed that combine replication regions of *E. coli* vectors with sequences from endogenous linear and circular *B. burgdorferi* plasmids[9,76–78]. These *Borrelia* sequences contain open reading frames (ORFs) that have paralogues on *B. burgdorferi* plasmids[16,19]. The precise roles of these paralogous genes are unknown, but they encode plasmid maintenance functions and confer incompatibility with the *B. burgdorferi* plasmid from which they are derived. At least one of these shuttle vectors has been shown to be relatively stable and provides an important tool for complementation in *B. burgdorferi*[9].

A gene encoding green fluorescent protein (GFP) was introduced on the broad host-range shuttle vector and shown to cause spirochaetes to fluoresce when expressed from a strong *Borrelia* promoter[72]. GFP has since been used as a reporter gene on other shuttle vectors in *B. burgdorferi* to measure promoter activity and monitor gene expression in response to environmental signals[77,79]. Prior to the development of stable replicons for *B. burgdorferi*, a reporter construct containing the *cat* gene, which encodes chloramphenicol acetyl transferase, was used in transient assays in *B. burgdorferi*[6]. This *cat* reporter has also been used more recently in conjunction with a shuttle vector to measure the effects of supercoiling on the expression of *osp* genes[80]. So far, constructs containing other reporter genes, such as *lacZ*, have not been developed or used to monitor gene expression in *B. burgdorferi* owing to technical limitations related to the growth media and codon usage of *Borrelia*, but they remain viable options.

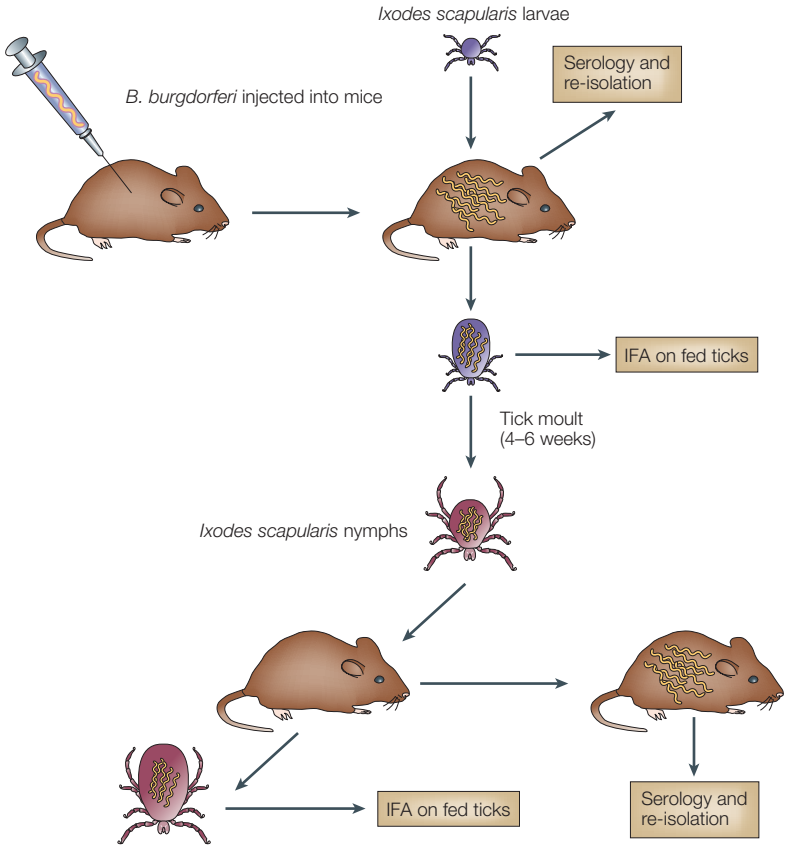


Figure 3 | **The experimental infectious cycle of *Borrelia burgdorferi*.** Approximately 4 weeks after mice have been inoculated with *B. burgdorferi* by injection, uninfected larval ticks are allowed to feed on infected mice. Infection in mice is assessed by seroconversion to *B. burgdorferi* antigens and re-isolation of organisms. The acquisition of *B. burgdorferi* by feeding ticks is determined on a subset of larval ticks by an immunofluorescence assay (IFA) of tick midguts with antisera that recognize *B. burgdorferi*. Remaining larval ticks moult to nymphs and subsequently feed on naive mice. The percentage of infected nymphs is determined by the IFA. The transmission of *B. burgdorferi* to mice by feeding nymphal ticks is assessed by serology and re-isolation.

Transposon mutagenesis. An exciting recent development has been random tagged mutagenesis of *B. burgdorferi* by insertion of a transposon derived from the *mariner* element[81]. Prior to this, a genetic method for random tagged mutagenesis of *B. burgdorferi* was not available. Initial results indicate that the insertion of the transposon is both random and saturating in *B. burgdorferi*, as required for effective genetic screens[81]. Transposon libraries provide a means to identify putative virulence genes and to characterize the roles of other genes. The repertoire of standard genetic tools available for *B. burgdorferi*, however, is not complete. Although a method now exists for genome-wide mutagenesis, localized saturating mutagenesis of a plasmid or chromosomal region is still not possible. Among other things, a tightly controlled inducible promoter, a conditionally replicating plasmid and an effective counter-selectable marker still need to be developed. Consequently, it is not currently possible to demonstrate the essential nature of specific genes in *B. burgdorferi* by creating conditional lethal mutants. However, the genetic system of *B. burgdorferi* continues to grow and the rate of progress suggests that these techniques will soon be developed.

Transformation of *B. burgdorferi*

The most obvious feature of genetic transformation by electroporation of *B. burgdorferi* is that it occurs at low frequency and efficiency[18]. In other words, few bacteria in the electroporated population become transformed and it requires a large quantity of DNA to achieve even that. This aspect of the genetic system of *B. burgdorferi* was apparent in the first transformations of non-infectious spirochaetes by Samuels *et al.* and still persists[17]. Putative restriction–modification systems encoded on plasmids prevent transformation with some shuttle vectors[82], but do not seem to influence allelic exchange events[83–85]. These phenomena are incompletely understood and apparent inconsistencies reflect a limited knowledge of the mechanisms involved. The transformation of infectious clones is even more difficult; this continues to impede investigations of gene function *in vivo* because the generation of mutants in an infectious background is limiting. Low transformation efficiency generally does not present a problem for targeted gene inactivation in non-infectious *B. burgdorferi* because it is relatively easy to generate large quantities of plasmid DNA that contains allelic exchange constructs. However, it does prevent the introduction of a complex DNA library into *B. burgdorferi* because few transformants are obtained with even a single species of incoming DNA. The exception to this limitation is transformation of certain non-infectious *B. burgdorferi* clones that lack putative restriction systems; in this case, some shuttle vectors and constructs yield thousands of transformants and present the opportunity for creation of a library of *B. burgdorferi* transformants[81,82].

Although transformation of *B. burgdorferi* by electroporation yields few transformants, a better method has not been identified. An alternative chemical method of transformation has been shown to work with *B. burgdorferi*, but it does not generate more transformants and so offers few benefits compared with electroporation[18]. Efforts to introduce DNA by conjugation with *E. coli* have been unsuccessful (K.T., unpublished observations), and generalized transducing phage for *B. burgdorferi* have not been identified. Higher levels of transformation in *B. burgdorferi* will probably be achieved as a consequence of increased knowledge of the factors that influence the fate of introduced DNA, such as identification and characterization of restriction–modification systems, rather than by the development of an entirely new transformation method.

Another feature of *B. burgdorferi* that affects genetic studies is slow growth. The number of spirochaetes doubles about every 5 hours under optimal *in vitro* conditions and colonies take 1–2 weeks to appear. So a successful transformation experiment, from the initial electroporation to the point at which a mutant has been isolated and confirmed to have the correct molecular structure and retain all plasmids, takes approximately 3–4 weeks to complete. Finally, *B. burgdorferi* is an obligate parasite throughout its natural life cycle and has limited metabolic capabilities. As a result, it can be propagated *in vitro* only in a complex, undefined growth medium known as BSK[86,87].

Box 4 | **Artificial infection of ticks**

The standard method of experimentally infecting ticks (other than by feeding on an infected mouse) is technically challenging and limited by the number of ticks that can be infected. It involves placing a small capillary tube filled with a solution of spirochaetes over the mouthparts of individual ticks[48]. This is feasible with nymphs but not with the smaller larval ticks. A variation on this technique developed by Narasimhan *et al.* uses microinjection of individual nymphal ticks through the rectal aperture[50]. Although still technically demanding, this latter method has the advantage of delivering a known quantity of spirochaetes. Recently, a much simpler technique for artificially infecting ticks was developed by Policastro and Schwan[49]; it merely involves immersing batches of ticks in a culture of spirochaetes. In addition to the ease with which it can be done, this immersion method can also provide large numbers of infected larval ticks, the stage at which the vector typically acquires spirochaetes in nature. The ability of *Borrelia burgdorferi* mutants to establish a midgut infection, persist through the moult and be transmitted in the nymphal bloodmeal can be accurately assessed with ticks artificially infected by this method. A method developed by Burkot *et al.* can also yield infected larval ticks[47]. This method is technically challenging, however, because it requires removing the skin of a mouse to which ticks have attached and then placing it in contact with a culture of spirochaetes to allow ticks to continue feeding and to ingest spirochaetes.

The undefined nature of some essential BSK components, such as rabbit serum and a crude preparation of bovine serum albumin, limits the ability to identify auxotrophic mutants. BSK medium is cumbersome to make and use, and although it is commercially available, batch-to-batch variability and occasional supply shortages have resulted in many investigators choosing to prepare BSK themselves. In summary, conducting genetic studies with *B. burgdorferi* requires more patience and attention to technical detail than is usually required in prokaryotic systems, but there are no insurmountable hurdles such as those presented by uncultivatable, intracellular or otherwise genetically intractable bacteria.

Phenotypic analysis of *B. burgdorferi* mutants

As mentioned previously, non-infectious *B. burgdorferi* are considerably easier to transform than infectious bacteria for poorly understood reasons. This is perhaps due to traits carried by plasmids that have been lost from non-infectious strains, such as restriction–modification systems that could degrade incoming DNA or altered surface properties that might allow more efficient introduction of DNA. Alternatively, attenuated bacteria might exert less stringent control over homologous recombination than infectious *B. burgdorferi*. As a consequence of this characteristic, whatever the basis, most genetic manipulations of *B. burgdorferi* over the past 10 years have been conducted with non-infectious bacteria. Inherent to such studies, the phenotypes of any resulting mutants cannot be assessed in the tick vector or mammalian host, so no direct information about the contributions of particular genes to infectivity, transmission or disease pathogenesis is obtained. Despite this limitation, insights into the importance of several spirochaetal components have been gained by careful analysis of the *in vitro* phenotypes of non-infectious mutants.

An exciting recent development in Lyme disease research has been the ability to conduct genetic studies with infectious *B. burgdorferi* and assess the phenotypes of resulting mutant bacteria in the experimental mouse–tick infectious cycle. The first published reports of genetic manipulation of infectious *B. burgdorferi* appeared in 2001 and overcame a technical barrier that had been in place since 1994 (REFS 9,25,88). This development resulted from the careful optimization of existing methods to allow the recovery of extremely rare transformants from infectious *B. burgdorferi*. However, transformants resulting from these initial experiments were either not tested for their ability to infect mice, or were non-infectious as an indirect consequence of the experiment[9,25,88]. These studies laid an important foundation for subsequent work by demonstrating the feasibility of transforming infectious *B. burgdorferi*, the requirement for a well-characterized wild-type clone and the diligence that must be maintained throughout an experiment to ensure that the plasmid contents of both wild-type and mutant bacteria remain constant[25]. In addition, complementation of mutations was recognized as a crucial component of genetic investigations in *B. burgdorferi*[25,88]. TABLE 2 contains a comprehensive summary of the *B. burgdorferi* mutants that have been constructed so far. Here, we will highlight just a few of these studies.

Motility

The ability of *B. burgdorferi* to move through highly viscous media is thought to be critical to the ability of this spirochaete to penetrate tissues and disseminate within the mammal or tick[89]. In support of this hypothesis, approximately 6% of the *B. burgdorferi* chromosome encodes proteins that are involved in motility and chemotaxis. So far, motility mutants have not been generated in infectious *B. burgdorferi*. However, Charon and colleagues have acquired substantial information about the structure and function of the motility organelles — the periplasmic flagella — from genetic studies with non-infectious *B. burgdorferi*[90]. Flagella are inserted near the ends of the spirochaete and overlap in the centre of the cell (FIG. 1). Translational motility of the spirochaete requires that flagellar motors at either end of the cell rotate in opposite directions. Inactivation of the *flaB* gene, which encodes the major flagellar filament protein, resulted in non-motile, rod-shaped spirochaetes that lacked periplasmic flagella, whereas complementation of the *flaB* mutation restored helical shape and motility along with flagella[91,92]. This demonstrated that flagella impart both shape and motility to *B. burgdorferi*, in contrast to other bacteria (and some spirochaetes) in which shape is determined by the peptidoglycan layer of the cell wall. In addition, FlaB influenced the amount of another flagellar filament protein at a post-transcriptional level[93]. This result is consistent with previous observations that *B. burgdorferi* lacks the typical transcriptional cascade control of motility gene expression by an alternative sigma factor that is common in other flagellated bacteria[94–97]. Finally, analysis of a rare *B. burgdorferi* double mutant that is deficient in chemotaxis indicated that structural asymmetry exists between the flagellar motors

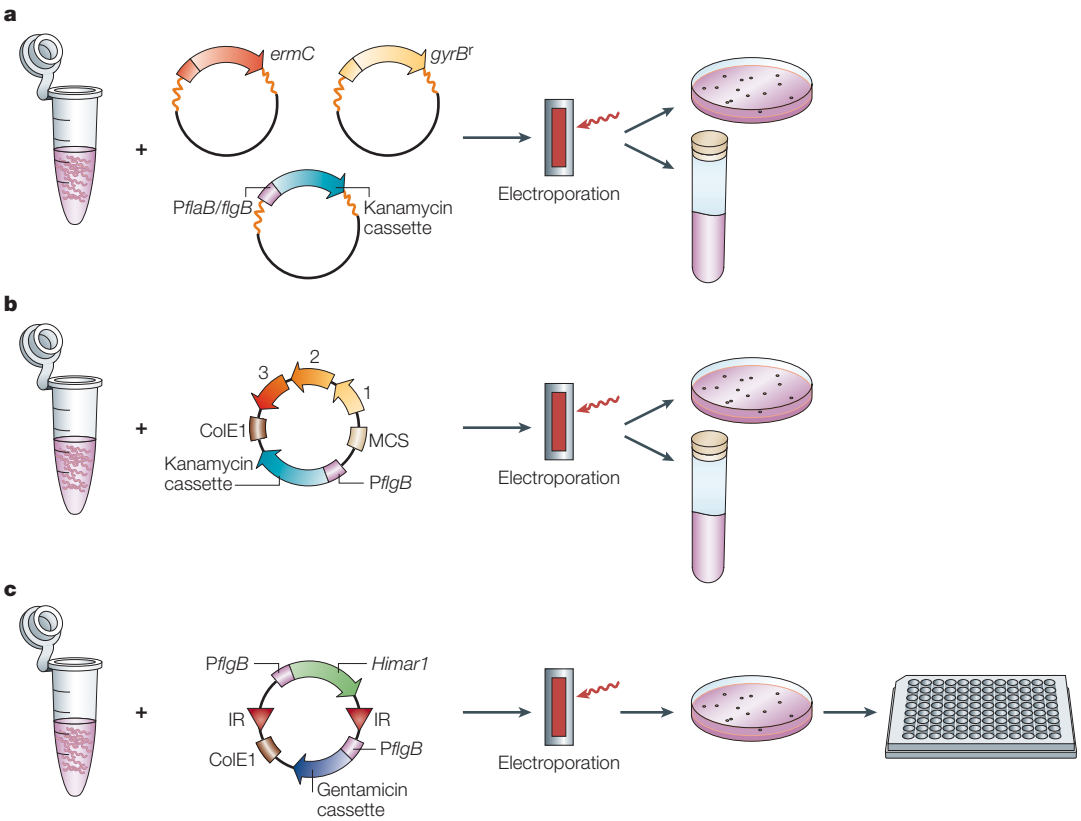


Figure 4 | **Genetic tools for *Borrelia burgdorferi*. a** | Allelic exchange. Following electroporation with gene inactivation constructs, spirochaetes are allowed to recover for 24 hours before imposing antibiotic selection in solid and liquid media. *B. burgdorferi* mutants exhibit antibiotic resistance that is conferred by selectable markers inserted into a targeted site by allelic exchange with gene inactivation constructs. Representative examples of markers are shown. *ermC*, foreign gene expressed from its native promoter conferring resistance to erythromycin; *gyrB*^{r}, mutant form of *B. burgdorferi gyrB* gene conferring resistance to coumermycin; kanamycin cassette, foreign gene expressed from a constitutive *Borrelia* promoter conferring resistance to kanamycin. **b** | Shuttle vectors. Genes for plasmid replication and maintenance in *Borrelia* (open reading frames 1, 2 and 3), a multiple cloning site (MCS), a selectable marker (such as the kanamycin cassette) and an *Escherichia coli* plasmid origin of replication (ColE1) are present. Following electroporation, transformants are selected in liquid or solid media on the basis of antibiotic resistance carried by the shuttle vector. **c** | Transposon mutagenesis. A member of the *mariner* family of transposons was adapted for random tagged mutagenesis in *Borrelia burgdorferi*[74]. A suicide vector was used for transient expression of the *Himar1* transposase from a strong *Borrelia* promoter, P*flgB*. Also present on the suicide vector are a selectable marker conferring resistance to gentamicin in *Borrelia* and a ColE1 origin of replication, flanked by inverted repeats (IR) that define the mobilizable element. Following electroporation, transient expression of *Himar1* mobilizes the transposon, which is randomly inserted at a single site in the *Borrelia* genome. A library of stable transformants containing random transposon insertions is selected with gentamicin. Individual mutants are isolated as colonies in solid media and then picked to 96-well plates for subsequent analyses. The ColE1 origin allows rescue of the element from digested genomic DNA for easy identification of the insertion site.

at either end of the cell[98]. Such flagellar asymmetry distinguishes *B. burgdorferi* from most other bacteria. Additional studies are required to elucidate the role of spirochaete motility *in vivo*, but much has been learned about flagellar structure and function through these analyses of non-infectious motility mutants.

Plasmid replication and telomere resolution

B. burgdorferi has a small linear chromosome and more than 20 distinct linear and circular plasmids[16,19]. This is the largest number of plasmids of any characterized bacterial genome. Together, the plasmids comprise more than one-third of the DNA in the cell and an increasing amount of data indicates that many *B. burgdorferi* plasmids encode essential functions that are required by the spirochaete in its natural infectious cycle[20–23]. Genetic studies have identified the minimal element that is required for plasmid replication in *B. burgdorferi* and shown that plasmid maintenance functions are conserved between linear and circular replicons[9,76,77,99]. The linear DNA molecules of *Borrelia* have covalently closed hairpin ends, which are referred to as telomeres[100]; the addition of telomeres to a circular replicon in *B. burgdorferi* is sufficient to convert it into a linear plasmid[99]. A single enzyme has been shown to resolve replicated telomeres with a site-specific DNA breakage and reunion reaction that regenerates covalently closed hairpin ends[99,101,102]. The only copy of the gene encoding the telomere resolvase, *resT*, is present on a ubiquitous 26-kb circular plasmid, cp26, of *B. burgdorferi*[102,103]. Telomere resolution is presumably required for replication of the linear plasmids and the

Table 2 | **Mutants constructed in *Borrelia burgdorferi***

Gene	Putative function	Marker	Replicon	Background*	References
Targeted insertions					
gyrB	DNA gyrase	couR	Chromosome	B31(N)	17
gac	DNA binding	couR‡	Chromosome	B31(N)	159
rpoS	Transcription	couR, ermR	Chromosome	B31, 297(N)	71,88,160
flaB	Flagellin	kanR	Chromosome	B31(N)	91
rpoN	Transcription	ermR	Chromosome	297(N)	88
p13	Porin	kanR	Chromosome	B31(N)	161
*oppA*II	Peptide binding	kanR	Chromosome	B31(i)	25
*cheA*1 & 2	Chemotaxis	kanR, ermR	Chromosome	B31(N)	98
luxS	Quorum sensing	ermR	Chromosome	297(I)	113
p66	Integrin binding	kanR	Chromosome	HB19(N)	162
rrp2	Response regulator	ermR, strR‡	Chromosome	297(i)	117
ctpA	Peptidase	kanR	Chromosome	B31(N)	163
oppAIV/guaB	Intergenic	couR	cp26	B31(N)	70
ospC	Unknown	couR, kanR, strR	cp26	B31(N,I), 297(i),	84,111,164
*oppA*IV	Peptide binding	couR	cp26	B31(N)	105
guaB	IMP dehydrogenase	couR	cp26	B31(N)	165
chbC	Chitobiose transport	couR, gmR	cp26	B31(N, i, I)	25,83,110
BBB29	Glucose transport	kanR	cp26	B31(N)	78
resT§	Telomere resolution	gmR	cp26	B31(N)	78
BBE05/06	Intergenic	gmR	lp25	B31(I)	85
BBF29	Pseudogene	gmR	lp28-1	B31(I)	85
*oppA*V	Peptide binding	kanR	lp54	B31(N)	8
ospA	Midgut adhesin	strR	lp54	297(I)	119
Random transposon insertions					
BB0102	Unknown	gmR	Chromosome	B31(N)	81
BB0257 *ftsK*	Cell division	gmR	Chromosome	B31(N)	81
BB0323	LysM motif	gmR	Chromosome	B31(N)	81
BB0347	Fibronectin binding	gmR	Chromosome	B31(N)	81
BB0414	Methyltransferase	gmR	Chromosome	B31(N)	81
BB0562	Unknown	gmR	Chromosome	B31(N)	81
BB0608	Dipeptidase	gmR	Chromosome	B31(N)	81
BB0827	Helicase	gmR	Chromosome	B31(N)	81
BB0830 *sbcC*	Exonuclease	gmR	Chromosome	B31(N)	81
BBB18 *guaA*	GMP synthetase	gmR	cp26	B31(N)	81
BBR41	Pseudogene	gmR	cp32-4	B31(N)	81
BBO36	Unknown	gmR	cp32-7	B31(N)	81
BBN16	Pseudogene	gmR	cp32-9	B31(N)	81
BBN26	Unknown	gmR	cp32-9	B31(N)	81
BBD14/15	Intergenic	gmR	lp17	B31(N)	81

*The letters in parentheses following the strain designations denote the following: N, non-infectious bacteria transformed; I, infectious bacteria transformed and wild-type background confirmed in resulting mutant; i, infectious bacteria transformed, but wild-type background of resulting mutant not confirmed. ‡Point mutation linked to selectable marker. §Only survives as mero-diploid with wild-type copy of gene. couR, coumermycin resistance; ermR, erythromycin resistance; gmR, gentamicin resistance; GMP, guanosine monophosphate; IMP, inosine monophosphate; kanR, kanamycin resistance; strR, streptomycin resistance.

linear chromosome. Consistent with this hypothesis, Byram *et al.* recently demonstrated that the *resT* gene is essential and, as a consequence, the cp26 plasmid cannot be lost from viable spirochaetes[78]. In addition to *resT*, cp26 carries several genes with presumed physiological functions[16,104,105] and the *ospC* gene[106,107], the transcription of which is induced during transmission of the spirochaete from vector to host[56,108]. The presence of cp26 in all *B. burgdorferi* isolates, its stability during *in vitro* passage and the essential nature of the *resT* gene that it carries have led to questions about the definitions of the terms 'chromosome' and 'plasmid' and how they apply to the complex segmented genome of *B. burgdorferi*[78,109].

Essential gene on an unstable plasmid

There is a well-documented association between plasmid loss during *in vitro* propagation of *B. burgdorferi* and reduced infectivity in mice[20–23]. Two linear plasmids, lp25 and lp28-1, are relatively unstable during growth in culture, but are required for persistent infection of the mammalian host[22,23]. The phenotype of *B. burgdorferi* mutants lacking lp25 is particularly striking due to their inability to infect even immunodeficient mice or grow in DMC implants, suggesting a physiological defect[22,28]. In the first published account of genetically manipulated *B. burgdorferi* that were subsequently shown to be infectious for mice, Purser *et al.* demonstrated that transformation of *B. burgdorferi* with a shuttle vector containing the *pncA* gene of lp25 replaced the requirement for lp25 *in vivo*[22,28]. The *pncA* gene product encodes a nicotinamidase and mutation of an amino acid in the active site eliminated both enzymatic activity and infectivity. This indicated that *pncA* has a physiological function (likely to be the biosynthesis of NAD) that is required *in vivo*, but dispensable for *in vitro* growth.

The instability of lp25 during *in vitro* propagation of *B. burgdorferi* continues to plague investigators because the loss of this plasmid during the course of an experiment renders mutants non-infectious, regardless of the nature of the mutation that was introduced. As described above, this inadvertent loss of lp25 can be 'patched' by reintroduction of *pncA* on a shuttle vector[22,28]. If the shuttle vector is also used to complement the introduced mutation, the presence of *pncA* ensures that the shuttle vector is stably retained by transformants after they are injected into mice. Grimm *et al.* recently demonstrated that it is also possible to reintroduce the entire lp25 plasmid of *B. burgdorferi*, consequently restoring infectivity[85]. This was done by insertion of a selectable marker into lp25 in wild-type *B. burgdorferi*, followed by transformation of this marked plasmid into spirochaetes that lack lp25. A similar approach was taken to restore another plasmid, lp28-1, that is frequently lost during culture but which is required for persistent infection of mice[85]. These studies demonstrated the feasibility of experimentally manipulating the plasmid content of *B. burgdorferi* through selective displacement and restoration of individual plasmids.

Non-essential gene on a stable plasmid

The *pncA* gene is one of the few ORFs on lp25 that have homology to genes of known function, whereas putative functions are ascribed to at least 10 genes on cp26, including telomere resolution by the *resT* gene product[16,19,28,102,103]. As described above, *resT* is required for bacterial growth, thus conferring ubiquity and stability to cp26 and raising doubts about the definition of cp26 as a plasmid[78]. Several other genes on cp26 are presumed to provide important physiological or biological functions at some stage of the spirochaete's infectious cycle[16]. These include the genes for OspC[106,107], components of oligopeptide[105] and sugar transport mechanisms[110], and purine biosynthesis[104]. Mutations have been made in many of these genes, however, indicating that, unlike *resT*, they are not required for *in vitro* growth (TABLE 2). So far, only two cp26 loci have been tested for importance for growth in the mouse or tick — *ospC* (described below)[84,111] and *chb*[83]. The proteins encoded by the *chb* locus function as a transporter for chitobiose[83,110]. Chitobiose is the dimer subunit of chitin, a component of the tick cuticle that is not found in mammals. Chitobiose can be converted into *N*-acetylglucosamine (GlcNAc), which is an essential component for cell wall biosynthesis. Tilly *et al.* demonstrated that inactivation of the *chbC* gene in *B. burgdorferi* resulted in the inability of spirochaetes to use chitobiose as a source of GlcNAc[110]. Surprisingly, *chbC* mutants exhibited no growth defect in either the tick vector or the mouse, and were efficiently transmitted between them[83]. So, *B. burgdorferi* is not dependent on this cp26-encoded transporter at any stage of its natural infectious cycle, even in the tick where chitobiose should be present. Potentially, the *chb* locus confers a selective advantage to *B. burgdorferi in vivo* under conditions that have not been accurately reproduced or measured in the experimental infectious cycle. The *chbC* gene is adjacent to *resT* on cp26; this tight linkage indicates that *B. burgdorferi* might retain the ability to utilize chitobiose in particular circumstances by proximity of the *chbC* locus to a gene that is essential for bacterial replication under all conditions. Similarly, linkage to the essential *pncA* gene on lp25 might allow non-essential genes to be retained for availability in a particular setting in which they confer a selective advantage. The unusual genomic organization of *B. burgdorferi* poses interesting questions about the evolution of genomes, and how and why a highly segmented genome such as that of *B. burgdorferi* might have arisen.

Quorum sensing in *B. burgdorferi*?

Analysis of the genome sequence of *B. burgdorferi* identified a homologue of the *luxS* gene on the chromosome, sparking interest in the potential role of quorum sensing during the spirochaete's infectious cycle[16]. LuxS can be involved in the synthesis of a small signalling molecule, autoinducer-2 (AI-2), that is used in quorum sensing by some bacteria. Initial studies indicated that the *luxS* gene of *B. burgdorferi* complemented a *luxS* deficiency in *E. coli*, and the supernatant from these bacteria altered the pattern of protein synthesis in *B. burgdorferi*, indicating that Lyme disease spirochaetes might use an AI-2-dependent mechanism of quorum sensing during their life cycle[112]. However, no AI-2 activity could be detected in cell-free supernatants from dense *B. burgdorferi* cultures, indicating that LuxS-mediated quorum sensing may only be operative *in vivo*[112]. In the natural infectious cycle, spirochaetes probably only achieve sufficiently high cell density for quorum sensing in the tick midgut. So, it was perhaps not surprising when another group of researchers subsequently demonstrated that a *luxS* mutant of *B. burgdorferi* was capable of infecting mice by needle inoculation[113]. However, recent experiments from the

same laboratory demonstrate that *luxS*-deficient *B. burgdorferi* can also colonize ticks and be transmitted by tick bites[114]. Wild-type and *luxS*-mutant *B. burgdorferi* caused similar levels of inflammation in heart and joints of infected mice. Although these results question the hypothesis that *B. burgdorferi* uses AI-2-for quorum sensing, they do not eliminate the possibility that such a system exists. If present, however, it is either not essential for host adaptation or redundant with another mechanism for quorum sensing.

Regulation of *osp* gene transcription

The OspA protein is abundant on the surface of *B. burgdorferi* residing in the midguts of unfed ticks; however, as the ticks feed, OspA is downregulated and replaced with OspC [56,108,115,116]. This switch in spirochaetal surface proteins is hypothesized to be required for migration of *B. burgdorferi* from the midgut to the salivary glands of the tick, and/or adaptation to the mammalian host after transmission[56,116]. Environmental conditions such as temperature, pH, nutrients and cell density, which change between unfed and feeding ticks, have been shown to affect the synthesis of OspA and OspC by spirochaetes *in vitro*[56–60]. Hubner and colleagues used a genetic approach to investigate the regulatory mechanisms by which *B. burgdorferi* modulates *osp* gene transcription in response to environmental signals[88]. They found that the alternative sigma factor RpoS regulates induction of *ospC* transcription in response to temperature. Transcription of RpoS in turn was regulated by another alternative sigma factor, RpoN. A recent study from the same research group suggests that a two-component signal-transduction system of *B. burgdorferi* senses the environmental cues and activates RpoN[117]. This was elegantly demonstrated by site-directed mutagenesis of an important amino acid in the activation domain of the putative response regulator that eliminated transcription of both *rpoS* and *ospC*[117]. Attempts to completely inactivate the response regulator gene were unsuccessful, indicating that functions other than RpoN activation were essential. Data from other groups suggest that changes in DNA supercoiling[80] and dissolved oxygen[118] can alter *osp* gene transcription in *B. burgdorferi*, potentially through this signal-transduction pathway.

These studies provide important insight into how *B. burgdorferi* might sense and adapt to external environmental changes to successfully complete a complex life cycle. The putative significance of this regulatory network *in vivo* could not be determined, however, even though these experiments were initiated with infectious *B. burgdorferi*. Although complementation of the *rpoN* and *rpoS* mutations restored induction of *ospC* gene transcription, these complemented mutants could not infect mice, indicating that they had other unidentified defects that resulted in loss of infectivity[88]. This inadvertent outcome illustrates the importance of complementation in genetic studies in *B. burgdorferi*, particularly with regard to the interpretation of mutant phenotypes in the natural infectious cycle.

The roles of OspA/B and OspC in mice and ticks

Several research groups recently investigated the roles of OspA/B and OspC on *B. burgdorferi* in the experimental infectious cycle[84,111,119]. Grimm *et al.* found that *B. burgdorferi ospC* mutants were unable to colonize mice and that this defect was independent of acquired immunity[84]. Complementation with a wild-type copy of *ospC* restored infectivity, demonstrating that the mutation was responsible for the defect. In contrast to their inability to infect mice, *ospC* mutant spirochaetes colonized the midguts of artificially infected larval ticks, persisted through the moult and migrated to the salivary glands during the nymphal bloodmeal. Naive mice that are fed on by these infected nymphal ticks did not become infected, indicating that *B. burgdorferi* require OspC for mouse infectivity, whether introduced by needle inoculation or tick bite. These results indicate that the switch from OspA to OspC on spirochaetes in the tick midgut during the bloodmeal is not required for migration to the salivary glands, but is in preparation for transmission to the mammalian host[84]. This would be an adaptive response in anticipation of the next host environment.

Pal and co-workers reached a somewhat different conclusion regarding the role of OspC in *B. burgdorferi*[111]. These investigators also inactivated the *ospC* gene in an infectious clone and complemented the mutation. The phenotypes of the resulting *ospC* mutant and complemented *B. burgdorferi* in mice were not reported, so no conclusions were drawn regarding the requirement for OspC in the mammalian host. However, a different result than that of Grimm and colleagues was obtained for the phenotype of the *ospC* mutant in ticks. Pal *et al.* found that both mutant and complemented spirochaetes multiplied within the midguts of artificially infected ticks, but that spirochaetes lacking *ospC* were unable to invade the salivary glands[111]. These investigators concluded that the switch from OspA to OspC is necessary for *B. burgdorferi* to migrate from the salivary glands, and suggested a role for OspC as a salivary gland adhesin. This would be an adaptive response that is relevant within the tick, not in preparation for transmission to the mammalian host.

The basis for the disparity in the phenotypes of *ospC*-mutant *B. burgdorferi* in these two experiments is not known. However, they differ in several technical details. The infectious clones were derived from different strains, the *ospC* mutations were structurally dissimilar and ticks were artificially infected with mutant bacteria by different methods and at different stages. Additional studies will be required to determine why conflicting results were obtained and which conclusion more accurately reflects the role of OspC for *B. burgdorferi* in ticks.

Until more data are available it is perhaps premature to speculate on the putative function of OspC in either the tick vector or the mammalian host. However, data from both studies indicate that OspC is not required by *B. burgdorferi* for colonization of the tick midgut. This result is not surprising as spirochaetes do not normally express OspC during the initial stage of tick infection[56]. The finding by Grimm and colleagues that OspC is absolutely required for colonization of the mammalian host[84] is somewhat unanticipated because previous data suggested

that OspC expression by spirochaetes was downregulated in the mammal shortly after transmission from the tick[120]. The inability of spirochaetes lacking OspC to infect immunodeficient SCID mice, or to establish even localized infections in the skin or joints of immunocompetent mice, further demonstrates the importance of OspC as a virulence factor in mammals[84]. Structural data for OspC indicate that it is a dimeric, largely α-helical protein with a potential binding pocket for a small ligand[121,122]. Although there is no sequence homology, the structure of OspC is similar to that of the extracellular domain of an aspartate receptor[121,122]. However, the structural determinants of OspC that fulfil its undefined yet critical function in the mammalian host have not been identified.

There is a single published genetic investigation of the role of OspA in infectious *B. burgdorferi*[119]. The *ospA* gene is present on lp54, a linear plasmid that is conserved among isolates but which is not essential for growth *in vitro*[123–125]. Yang *et al.* recently demonstrated that inactivation of *ospA* did not impair the ability of the spirochaete to infect mice or cause disease[119]. Again, this was not an unanticipated finding because spirochaetes do not typically make OspA in the mammalian host[116]. However, *ospA*-mutant *B. burgdorferi* did not efficiently colonize ticks[119], which is consistent with earlier studies that suggested a role for OspA as a midgut adhesin[126]. The structure of OspA indicates that it is composed primarily of β-sheets and is very different from OspC, except for the presence of a potential small binding pocket[122,127,128]. Hence, although the precise functions of OspA and OspC currently remain undefined, genetic studies have defined opposing requirements for these major surface components of *B. burgdorferi* in the tick vector and mammalian host, as reflected by their reciprocal patterns of expression in these environments. Hopefully, future genetic studies will provide information about what these Osp proteins actually do that make them essential at different stages of the infectious cycle.

Summary

The genetic system of *B. burgdorferi* has developed significantly since transformation of this pathogen was first achieved 10 years ago[17]. It is now possible to perform targeted gene inactivation in infectious clones, complement mutations in *cis* or *trans*, and compare isogenic wild-type, mutant and complemented clones in an experimental mouse–tick infectious cycle that accurately reproduces the natural life cycle. The availability of the *B. burgdorferi* s.s. genome sequence led to the construction of microarrays with which to monitor global gene expression[66,129]. Genomic and proteomic analyses represent important tools for the identification of genes that are differentially expressed at various stages of the infectious cycle, which in turn are good candidates for future genetic studies[60,65,130,131]. Microarrays also represent a valuable tool with which to compare gene expression in mutant and wild-type bacteria and identify regulatory networks or pleiotropic effects of a mutation.

The development of genetics in *B. burgdorferi* results from the efforts and contributions of numerous laboratories. Many improvements still need to be made for genetic manipulation of *B. burgdorferi* to be efficient and routine. Of high priority are finding ways to stabilize plasmid content, enhance transformation frequency and streamline the plating technique. The future direction of genetic studies in *Borrelia* includes the development of screens to identify important but unrecognized genes that provide critical functions at various stages of the infectious cycle. The recent development of transposon mutagenesis in *B. burgdorferi* makes this feasible. There are also many characterized genes of *B. burgdorferi* for which their roles as potential virulence factors remain to be tested in the infectious cycle. Finally, genetic studies will be instrumental in distinguishing between spirochaetal components that are required for infectivity and those that directly contribute to disease pathogenesis.

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Competing interests statement

The authors declare no competing financial interests.

Online links

DATABASES

The following terms in this article are linked online to:

Entrez: http://www.ncbi.nlm.nih.gov/Entrez/
Borrelia burgdorferi | *Borrelia garinii* | cp26 | *Escherichia coli* | *flaB* | *gyrB* | *Lactococcus lactis* | lp25 | lp28-1 | *luxS* | *pncA* | *Staphylococcus aureus*

Infectious Disease Information:
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Lyme disease

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