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Genetic analysis and the effect upon mouse infection of nucleic acid metabolizing genes in the Lyme disease spirochete

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Genetic analysis and the effect upon mouse infection of nucleic acid metabolizing genes in the Lyme disease spirochete

by

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Abstract

The Lyme spirochete *Borrelia burgdorferi* causes the most prevalent vector-borne infection in North America. In this study, the importance of DNA metabolizing genes for the infectivity, persistence and survival to DNA damage in *B. burgdorferi* was determined. During the infection of a vertebrate host, *B. burgdorferi* undergoes antigenic variation by DNA recombination at *vlsE*, which encodes for an immunogenic surface lipoprotein required for the persistence of the spirochete. In the present study, eight gene targets were disrupted and only the RuvAB Holiday junction branch migrase subunits affected the switching at *vlsE* and the persistence of *B. burgdorferi* in mice. The disruption of these eight genes was part of a wider study aiming to identify nucleic acid metabolizing genes involved in switching at *vlsE*. Although no other genes were found to strongly affect switching, the disruption of the DEAH-box RNA helicase HrpA abolished the infectivity of *B. burgdorferi*. Since the complementation of *hrpA in trans* could not be achieved, the restoration of the wild-type gene by allelic exchange was used as an alternate strategy for complementation. The restoration of the wild-type *hrpA* did restore infectivity, confirming the importance of *hrpA*. Point mutations were also introduced by allelic exchange in motifs required for either the RNA helicase or the ATPase activity of HrpA. To avoid an intial screen of a large number of clones by sequencing, a strategy was adapted to confirm by PCR the presence of the mutation in the gene. Infection of mice with these *B. burgdorferi hrpA* mutants confirmed that the RNA helicase activity, in addition of the ATPase activity, is required for the survival of *B. burgdorferi* in

the mouse. Finally, a strategy was adapted to expediently compare the cell density of multiple cultures. This strategy was used to measure the importance of 25 nucleic acid metabolizing genes for survival of *B. burgdorferi* to DNA damage. Using this strategy, the nucleotide excision repair pathway was shown to be the sole repair pathway to be significantly involved in repair of UV-induced DNA damage in *B. burgdorferi*.

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
ATP	Adenosine triphosphate
BER	Base excision repair
BSK	Barbour-Stoenner-Kelly
CPG	*cis-syn* cyclobutane pyrimidine dimers
CSF	Cerebro-spinal fluid
dCTP	Deoxycytidine triphosphate
ddH_2O	Double-distilled water
DEA/NO	Diethylamine NONOate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphate
dsDNA	Double-stranded DNA
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EM	Erythema migrans
ESS	Endonuclease sensitivity site
gDNA	Genomic DNA
gent	Gentamicin
HCl	Hydrochloric acid
iNOS	inducible nitric oxide synthase

iTRAQ	Isobaric tags for relative and absolute quantitation
kan	Kanamycin
kDa	Kilodalton
KO	knock-out
LB	Lysogeny Broth
LMMA	N^G-L-monomethyl arginine
LPS	Lipopolysaccharide
MMC	Methyl-mismatch correction
NER	Nucleotide excision repair
Osp	Outer surface protein
P^{32}	Phosphorus-32
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDG	Pyrimidine dimer glycosylase
RML	Rocky Moutain Laboratories
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT-PCR	Reverse transcription PCR
SCID	Severe combined immune deficiency
SDS	sodium dodecyl sulfate
SSC	Saline-sodium citrate

ssDNA	Single-stranded DNA
UV	Ultraviolet

Chapter One: General Introduction

1.1 The history

In 1977, Steere *et al.* described an epidemic form of oligoarthritis in clusters of adults and children around the town of Lyme in Connecticut, USA (Steere *et al.* 1977). The disease was also characterized by a skin lesion called erythema migrans, previously associated with the bite of the sheep tick *Ixodes ricinus* in Europe (Azfelius 1921; Steere *et al.* 1977). The newly described form of arthritis was then named Lyme arthritis, later called Lyme disease, in reference to the geographical location where it was originally described (Steere *et al.* 1977). Later studies isolated a *Treponema*-like spirochete from patients with Lyme disease (Benach *et al.* 1983; Steere *et al.* 1983b) that was shown to be carried by *Ixodes* ticks (Burgdorfer 1984; Burgdorfer *et al.* 1982; Lane *et al.* 1991; Steere *et al.* 1978; Wallis *et al.* 1978). The organism was then named *Borrelia burgdorferi* in reference to Willy Burgdorfer, a medical entomologist who participated to the identification of the spirochete (Burgdorfer *et al.* 1982; Hyde and Johnson 1984; Johnson *et al.* 1984a; Johnson *et al.* 1984b). Although the organism could be cultured in the medium used for the spirochete that causes the relapsing fever, *B. hermsii*, the medium was then optimized for the culture of *B. burgdorferi* (Barbour 1984; Barbour *et al.* 1982b; Burgdorfer *et al.* 1982; Kelly 1971), which improved significantly the study of Lyme disease.

Since the original identification of *B. burgdorferi*, at least 20 species of Lyme disease spirochetes have been identified in North America, Europe and Asia and are collectively called the *B. burgdorferi* sensu lato complex (Figure 1.1)

(Baranton *et al.* 1992; Burgdorfer *et al.* 1982; Canica *et al.* 1993; Casjens *et al.* 2011; Chu *et al.* 2008; Fleche *et al.* 1997; Fukunaga *et al.* 1996; Kawabata *et al.* 1993; Marconi *et al.* 1995; Margos *et al.* 2010; Margos *et al.* 2009; Masuzawa *et al.* 2001; Postic *et al.* 1994; Postic *et al.* 2007; Postic *et al.* 1998; Richter *et al.* 2006; Richter *et al.* 2004; Rudenko *et al.* 2009a; Rudenko *et al.* 2009b; Schwan *et al.* 1993; Wang *et al.* 1997a). However, not all species are expected or have been proven to cause human Lyme disease (Rudenko *et al.* 2011) and, currently, only *B. burgdorferi* sensu stricto has been shown to cause the disease in North America (Gray *et al.* 2002; Lindgren and Jaenson 2006; Rudenko *et al.* 2011; Stanek and Reiter 2011; Stanek *et al.* 2012; Takano *et al.* 2011). For the rest of this document, *B. burgdorferi* sensu stricto will be referred to as *B. burgdorferi*. Although *B. burgdorferi* is also present in Europe, *B. afzelii* and *B. garnii* are responsible for most cases of Lyme disease in Europe and Asia (Baranton *et al.* 1992; Guner *et al.* 2003; Li *et al.* 1998; Masuzawa 2004; Ružić-Sabljić *et al.* 2001; Taragel'ová *et al.* 2008; Wang *et al.* 1997b).

1.2 The disease

Lyme disease (also known as Lyme borreliosis) can be described by phases of progression. Initially, the spirochetes enter the skin at the site of the tick bite, replicate locally (Berger *et al.* 1992; Duray and Steere 1988; Shih *et al.* 1992; Steere *et al.* 1983a) and immune cells, including macrophages, neutrophils, dendritic cells and T-lymphocytes, are recruited to the site (Duray and Steere 1988; Shih *et al.* 1992; Steere *et al.* 1983a). A blood meal lasting 48 hours or

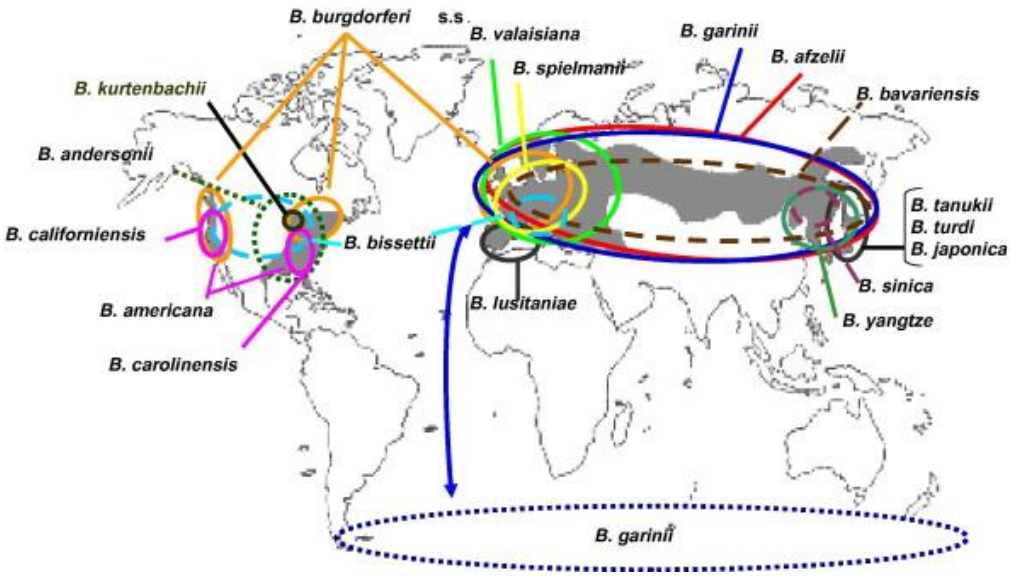

Figure 1.1. Geographic distribution of *B. burgdorferi* sensu lato.

Reproduced with permission from Margos *et al.* 2011.

more is required for a successful transmission of the pathogen by *I. scapularis* to mice (Piesman *et al.* 1987). The inoculation of *B. burgdorferi* in the skin, along with the triggered inflammatory response, results in skin lesions named erythema migrans (EM) or Bull's eye lesion within days or weeks following the inoculation (Azfelius 1921; Burgdorfer *et al.* 1982; Steere 2001; Steere *et al.* 1983a). The EM is characterized by a red central papule, often surrounded by one or multiple red rings and can be located anywhere on the body. It is present in approximately 60 to 80% of the patients infected with *B. burgdorferi* (Steere 1989) and is sometime associated with a sensation of burning or itching (Steere *et al.* 1983a). The lesion is usually cleared within weeks or months following the inoculation, even without antibiotics treatment (Mullegger *et al.* 2000; Steere *et al.* 1983a).

The second phase of Lyme disease is characterized by the haematogenous dissemination of spirochetes in tissue and organs throughout the body. Spirochetes have been cultured from the blood, lymph nodes, spleen, bone marrow, liver, synovial fluid, and CSF from some patients with symptoms of neuroborreliosis (Berger *et al.* 1992; Duray and Steere 1988; Steere 1989). Along with the EM, patients with early Lyme disease have reported a wide array of sometime less specific symptoms, including conjunctivitis, pharyngitis, myalgia, arthralgia, headaches, splenomegaly, hepatitis, fever/chills, fatigue, and stiff neck (Duray and Steere 1988; Kornmehl *et al.* 1989; Nadelman *et al.* 1996; Steere 1989; Steere *et al.* 1983a). Dissemination of *B. burgdorferi* has also been accompanied with neuropathies like meningitis, encephalopathy and facial palsy

(Reik *et al.* 1979; Steere 1989) and cardiopathies (Steere 1989; Steere *et al.* 1980).

If the infection persists for months or years, approximately 60 percent of the patients will also suffer from recurrent attacks of asymmetric oligoarthritis, mostly on large joints like the knee (Steere 1989; Steere *et al.* 1987) (Centers for Disease Control and Prevention-http://www.cdc.gov). In patients with Lyme arthritis, swelling, cartilage erosion and recruitment of immune cells to the synovial fluid is observed (Steere *et al.* 1979a) and recurrence of inflammation lasting up to 5 years has been reported (Steere 1989). It was suggested that the persistence of inflammation in the joints, even after antibiotic treatment, could be due to an autoimmune response associated with alleles of the class II histocompatibility complex, similar to what is observed in rheumatoid arthritis (Patil *et al.* 2001; Steere 1989; Steere *et al.* 1979a). Moreover, higher immunoglobulin production (IgM) and proliferation of mononuclear cells in response to *B. burgdorferi* antigens have been observed in patients with recurrences of Lyme arthritis (Sigal *et al.* 1986; Steere *et al.* 1979b).

Previous studies reported post-treatment or chronic Lyme disease. Most cases are associated with persistent arthritis and neuropathies even following antibiotic treatment (Asch *et al.* 1994; Dinerman and Steere 1992; Fallon *et al.* 2008; Klempner *et al.* 2001; Wormser *et al.* 2006). However, most studies failed to re-isolate *B. burgdorferi* from the treated patients and reported that prolonged antibiotic treatment is not likely to improve the symptoms (Klempner *et al.* 2001; Wormser *et al.* 2006). In the cases of post-treatment relapse of erythema

migrans, a recent study compared the serotype of the immunogenic outer membrane lipoprotein OspC in patients having two or more relapses of Lyme diseases (Lagal *et al.* 2002; Nadelman *et al.* 2012). Expression of OspC is important for the invasion of the tick salivary glands and the establishment of the infection in the mouse (Grimm *et al.* 2004; Pal *et al.* 2004b; Tilly *et al.* 2006). However, OspC is highly immunogenic and its expression must be decreased following the initial infection (Dressler *et al.* 1993; Margolis *et al.* 1994a; Xu *et al.* 2006). Nadelman and colleagues have compared the serotypes from 22 paired consecutive infections but none contained an identical OspC serotype, suggesting reinfection events instead of relapse of spirochetes from the initial infection (Nadelman *et al.* 2012).

1.3 Life cycle/Epidemiology

In North America, two geographically separated arthropod vectors can transmit *B. burgdorferi*. Whereas in the Northeastern American states and the Southeastern Canadian provinces, *B. burgdorferi* is transmitted by the *Ixodes scapularis*, Lyme disease is transmitted by *Ixodes pacificus* in Western North America (Burgdorfer 1984; Burgdorfer *et al.* 1982; Burgdorfer *et al.* 1985; Margos *et al.* 2012; Ogden *et al.* 2010). The *Ixodes* spp ticks have a two-year life cycle (Figure 1.2). The larvae take a first blood meal, usually on birds or small mammals like the white-footed mouse *Peromyscus leucopus*, by the end of the first summer/early fall (Lane *et al.* 1991). The larva then molts to the nymph stage and the presence of *B. burgdorferi* is conserved between each stage and

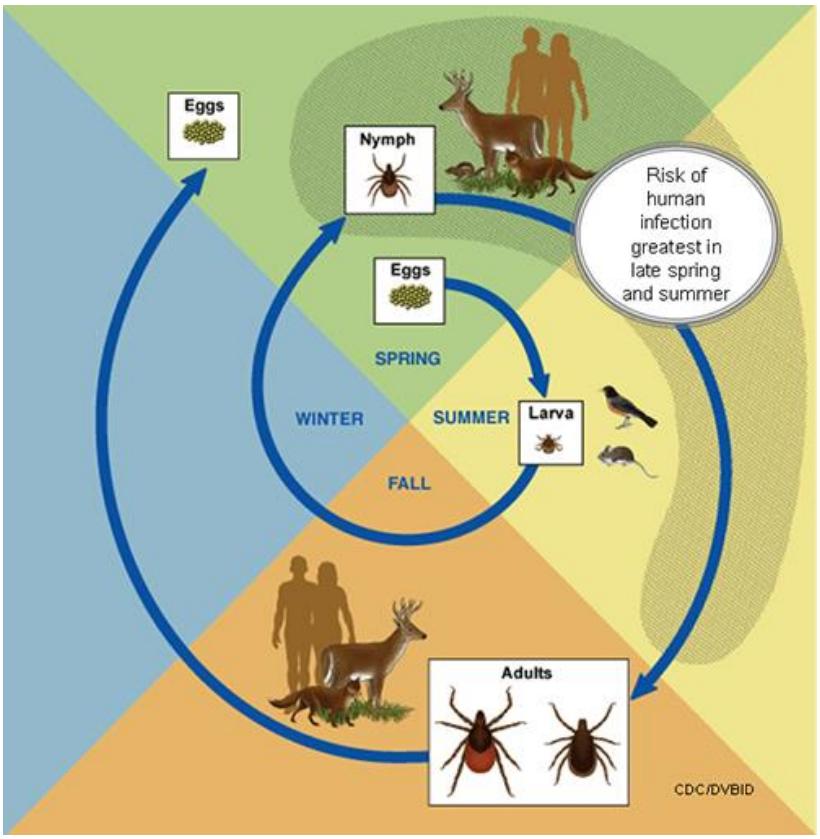

Figure 1.2. Life cycle of *I. scapularis* ticks.

Reproduced from Centers for Disease Control and Prevention (Centers for Disease Control and Prevention-http://www.cdc.gov).

the presence of *B. burgdorferi* is conserved between each stage (trans-stadially). The *Ixodes* nymph takes a second blood meal usually during the following spring/early summer on similar hosts as the larvae. The *Ixodes* nymph then molts to the adult stage. The female tick takes a third and last blood meal, usually on a bigger host like the white-tailed deer or livestock, falls off and lays eggs, usually in the following spring. Humans are considered an accidental host for *B. burgdorferi* and no transmission from an infected human host has been reported.

Although deer are not involved in the maintenance of *B. burgdorferi* (Luttrell *et al.* 1994; Telford *et al.* 1988), they appear to play an important role for the maintenance of *I. scapularis* populations. Previous studies reported a reduction of the tick population following local reduction of either the deer population or tick infestation of deer (Garnett *et al.* 2011; Rand *et al.* 2004; Wilson *et al.* 1985; Wilson *et al.* 1988). In North America, the white-footed mouse *P. leucopus* is considered to be the main reservoir for *B. burgdorferi* (Donahue *et al.* 1987; Levine *et al.* 1985). During a field study in Connecticut, Bunikis and colleagues reported that almost all *P. leucopus* collected were seropositive for antibodies against *B. burgdorferi* by the end of the summer, in time with the peak feeding time of uninfected *Ixodes* larva (Bunikis *et al.* 2004). Since there is no vertical transmission of *B. burgdorferi* (Burgdorfer *et al.* 1988; Burgdorfer *et al.* 1989; Lane and Burgdorfer 1987; Magnarelli *et al.* 1987; Schoeler and Lane 1993), each generation of larvae acquires the spirochetes through feeding on an infected host, underlining the importance of the reservoir for the disease.

Lyme disease is now the most commonly reported vector-borne disease in the United States. From 2001 to 2010, 213,515 human cases were confirmed and almost 25,000 were confirmed in 2011. However, despite multiple attempts, no vaccine against Lyme disease is currently available for use in humans (Brown *et al.* 2005; Dai *et al.* 2009; Earnhart *et al.* 2007; Earnhart and Marconi 2007; Earnhart *et al.* 2011; Marconi and Earnhart 2011; Sigal *et al.* 1998; Steere *et al.* 1998; Töpfer and Straubinger 2007). Disease onset peaks annually in the months of June and July, coinciding with the prime nymphal feeding time (Lane *et al.* 1991) and (Centers for Disease Control and Prevention - http://www.cdc.gov). Although being a reportable disease in Canada since 2010, no countrywide statistics are available. The province of Ontario reported 196 probable cases in 2009 and 2010 (from Public Health Ontario - http://www.oahpp.ca) and 57 probable human cases were reported between 2009 and 2012 in Manitoba (from Manitoba Health - https://www.gov.mb.ca/health/lyme/stats.html). Also, 20 cases of Lyme disease were confirmed in 2011 in British-Columbia, where *B. burgdorferi* is transmitted by *I. pacificus* (from BC Centre for Disease Control - http://www.bccdc.ca).

1.4 The organism

Borrelia burgdorferi is a spirochete with a flat-waved morphology (Burgdorfer *et al.* 1982; Goldstein *et al.* 1994). It has an inner and an outer membrane with a flat ribbon of 7 to 11 periplasmic flagella attached to both ends of the cell that is responsible for the waved shape of the spirochete (Charon *et al.* 2012; Charon *et*

al. 2009; Motaleb *et al.* 2000). Neither Gram-positive nor Gram-negative, unstained bacteria are observed using a dark-field microscope. The cells measure 10 to 30 μm in length and 0.33 μm in diameter, have an optimal growth temperature of 34 to 37°C in a microaerobic environment in laboratory (Barbour 1984). Sequencing of the genome revealed that *B. burgdorferi* lacks genes for amino acids synthesis, fatty acid and nucleotide biosyntheses, and the tricarboxylic acid cycle. *B. burgdorferi* uses glucose as a preferred substrate for glycolysis, but it was also shown to grow in medium containing glycerol, mannose, maltose, trehalose, chibiotose or N-acetylglucosamine instead of glucose (Hoon-Hanks *et al.* 2012; von Lackum and Stevenson 2005). It uses homofermentation for the generation of lactate, the only electron acceptor (Fraser *et al.* 1997; Johnson *et al.* 1984b).

Moreover, previous studies have shown that *B. burgdorferi* does not either accumulate or require iron for growth. Posey and Gherardini showed that culture of *B. burgdorferi* in an iron-free medium does not affect growth and that during culture in the presence of iron, less than 10 atoms per cell were present, which was the detection limit of the technology used in the experiment (Posey and Gherardini 2000). Instead of iron, *B. burgdorferi* is known to accumulate manganese and to encode a Mn transporter, which is required for infectivity and for survival in ticks (Ouyang *et al.* 2009a; Posey and Gherardini 2000). During infection, *B. burgdorferi* is exposed to the components of the immune response, including reactive oxygen species (ROS) produced by activated phagocytes (Brown *et al.* 2003; Lee *et al.* 2010; Ma *et al.* 1994; Modolell *et al.* 1994;

Ruderman *et al.* 1995). In presence of iron, the hydrogen peroxide generated by the inflammatory response is reduced to highly reactive hydroxyl radicals by the Fenton reaction, and which can react with DNA bases (Aruoma *et al.* 1989b; Cooke *et al.* 2003; Fenton 1894; Imlay *et al.* 1988). Boylan *et al.* previously showed that reactive oxygen species primarily damage *B. burgdorferi* membrane lipids but do not generate significant DNA damage, possibly because of the absence of iron (Boylan *et al.* 2008) protects the DNA from the hydroxyl radicals produced by Fenton reaction.

However, a more recent study showed that measurable levels of iron and copper are found in *B. burgdorferi* during culture (Wang *et al.* 2012). In this study, the disruption of the iron and copper binding protein BicA increased the sensitivity of the spirochete to hydrogen peroxide and was previously shown to be required for persistence in the tick and transmission from the tick to the mouse (Li *et al.* 2007b; Wang *et al.* 2012). The presence of intracellular iron suggests that in presence of oxidative stress, some level of Fenton reaction could be occurring in *B. burgdorferi* and damage in the DNA could be occurring in cells lacking BicA.

The sequencing of the genome also confirmed that *B. burgdorferi* does not encode for LPS and did not reveal any recognizable toxins or virulence factors (Fraser *et al.* 1997; Takayama *et al.* 1987).

1.5 The genome

The study of *B. burgdorferi* was greatly improved by the release in 1997 of the sequence of the almost complete genome of the strain B31, isolated in 1982 from an *Ixodes scapularis* tick collected on Shelter Island, New York (Burgdorfer *et al.* 1982; Fraser *et al.* 1997). The genome is highly segmented, with a 910,725 bp linear chromosome as well as 12 linear and nine circular plasmids (Figure 1.3). The GC content for the chromosome is 28.6%, which is significantly lower than the 50.8% found in *E. coli*. The size of the linear plasmids ranges from approximately 5 kb to 53 kb and the circular plasmids range from 9 kb to 30 kb (Fraser *et al.* 1997). The replication of the chromosome and the linear plasmids begin at the center of the molecule and progresses bidirectionally towards each end, resulting in a circular replication intermediate (Chaconas *et al.* 2001; Picardeau *et al.* 1999). Each plasmid is then separated and the ends are closed by the telomere resolvase, ResT, an essential protein encoded on the cp26 plasmid (Byram *et al.* 2004; Chaconas *et al.* 2001; Kobryn and Chaconas 2002).

The plasmids are expected to be present at low copy number, similar to the chromosome (Beaurepaire and Chaconas 2007; Casjens and Huang 1993; Hinnebusch and Barbour 1992). Although some plasmids can be lost without significantly affecting the infectivity of *B. burgdorferi* (Purser and Norris 2000), others are known to encode genes essential for growth in culture, for infectivity in mice or survival in ticks. Previous studies have shown that the lp25 plasmid contains the *bbe22* gene, which encodes the PncA nicotinamidase and is

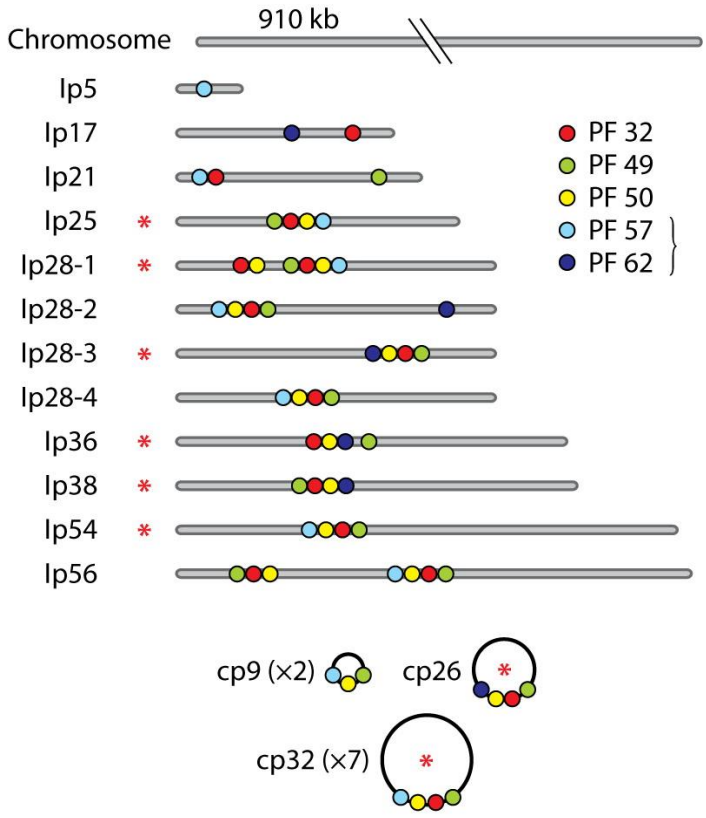

Figure 1.3. The segmented genome of *B. burgdorferi* B31.

Plasmids known to carry important metabolic functions or virulence factors are indicated with asterisks (see below). The color-coded PF32, 49, 50, 57 and 62 genes are plasmid replication/maintenance functions. PF57 and PF62 are related at the sequence level and are therefore grouped together. This figure is adapted from Casjens *et al.* 2000, Deneke and Chaconas 2008 and Chaconas and Kobryn 2010 with permission.

essential for the survival in mice, but not in culture (Labandeira-Rey and Skare 2001; Purser *et al.* 2003; Purser and Norris 2000). The same plasmid also encodes for the BptA (*bbe16*) surface protein, which is involved in persistence in the tick vector (Revel *et al.* 2005). The cp26 plasmid contains genes involved in the purine biosynthesis pathway (Margolis *et al.* 1994b), required for mouse infection (Botkin *et al.* 2006), the periplasmic membrane proteins *bbb26* and *bbb27* (Jewett *et al.* 2007a), required for growth culture, as well as the previously mentioned *resT* and *ospC* genes (Pal *et al.* 2004b; Tilly *et al.* 2007; Tilly *et al.* 2006). The lp38 plasmid encodes for the OspD outer membrane lipoprotein, which was shown to bind tick midgut, but its disruption does not affect *B. burgdorferi* survival in the tick and transmission from the tick to a naïve mouse (Li *et al.* 2007a; Stewart *et al.* 2008). The plasmid lp54 encodes the genes for OspA and OspB outer membrane lipoproteins (Bergström *et al.* 1989; Pal *et al.* 2004a; Yang *et al.* 2004), required for colonization and survival in the ticks (Bergström *et al.* 1989; Pal *et al.* 2004a; Yang *et al.* 2004), the virulence-associated decorin-binding proteins DbpA and DbpB (Guo *et al.* 1998; Shi *et al.* 2006; Shi *et al.* 2008) and the factor H-binding complement regulator-acquiring surface protein (CRASP-1), which inhibits complement deposition *in vitro* (Kenedy *et al.* 2009; Kraiczy *et al.* 2004). A second factor H-binding protein CRASP-2 is encoded on lp28-3 (Hartmann *et al.* 2006), however its disruption does not significantly affect infectivity in mice (Coleman *et al.* 2008). The plasmid lp36 encodes for the fibronectin and the glycosaminoglycans-binding protein BBK32 (Fischer *et al.* 2006; Moriarty *et al.* 2012; Norman *et al.* 2008; Probert and Johnson 1998;

Seshu *et al.* 2006) and the adenine deaminase *adeC,* involved in virulence of *B. burgdorferi* (Jewett *et al.* 2007b). The multiple cp32 plasmids encode a diversity of OspE/F-related outer membrane lipoproteins (Erp), including some that were shown to bind the complement inhibitor factor H *in vitro* (Hellwage *et al.* 2001; Hovis *et al.* 2006; Stevenson *et al.* 2002). Moreover, the lp28-1 plasmid was shown to encode for ErpT, which is expressed differentially in various mouse tissues (Fikrig *et al.* 1999). As will be discussed in the next chapter, the lp28-1 plasmid also contains the *vlsE* expression site, which undergoes sequence switching involved in antigenic variation of the surface lipoprotein VlsE, required for long-term persistence of the spirochetes in mice (Bankhead and Chaconas 2007; Lawrenz *et al.* 2004; Zhang *et al.* 1997). In contrast, other plasmids like cp9, cp32-3, lp21, lp28-2, lp28-4 and lp56 can be lost without significantly affecting the infectivity in mice (Purser and Norris 2000).

Since the sequencing of the first *B. burgdorferi* genome was published (Fraser *et al.* 1997), the sequences of 13 other strains of *B. burgdorferi sensu stricto* were published (Schutzer *et al.* 2010). Each strain contained between 13 and 21 plasmids and nine plasmids not found in the originally sequenced strain B31. The authors reported that the chromosomes are well conserved between strains but also, that there is substantial level of rearrangement in the plasmids, as it had previously been suggested (Qiu *et al.* 2004). In contrast to *B. burgdorferi* (Barbour 1988; Fraser *et al.* 1997) and to the tick-borne relapsing fever *B. hermsii* (Plasterk *et al.* 1985; Stevenson *et al.* 2000), which contain multiple linear and circular plasmids, the louse-borne relapsing fever *B.*

recurrentis, the tick-borne relapsing fever *B. parkeri,* and the *B. anserina*, which causes avian borreliosis, contain only linear plasmids (Lescot *et al.* 2008; Schwan *et al.* 2005). Moreover, another tick-borne relapsing fever, *B. duttonii*, contains linear plasmids and only a single 27,476 bp circular plasmid (Lescot *et al.* 2008).

As will be discussed in the following chapters, no orthologs of several DNA repair and recombination genes found in *E. coli* could be found in the *B. burgdorferi* genome by sequence homology (Fraser *et al.* 1997). Among others, orthologs for the SOS response regulator LexA, the MutH endonuclease involved in methyl-directed mismatch correction and the RuvC Holiday junction resolvase were not identified (Fraser *et al.* 1997).

1.6 Genetic manipulation

Genetic manipulation of *B. burgdorferi* was made possible by the development of a protocol for transformation by electroporation in 1994 by Samuels and colleagues (Samuels 1995; Samuels *et al.* 1994a). This protocol was first used to insert a mutation in the gene encoding the DNA gyrase B subunit *gyrB*, conferring resistance to coumermycin A_1 (Samuels *et al.* 1994a). Following this first breakthrough, the protocol was used to insert the complete mutated *gyrB* gene on the cp26 plasmid, leaving the wild-type copy of *gyrB* unmodified on the chromosome (Rosa *et al.* 1996). This demonstrated the use of a selectable marker for transformation, but also confirmed that homologous recombination is possible in *B. burgdorferi*. Subsequently, other selectable markers were amplified

from different bacteria, either with their native promoter or fused to the *B. burgdorferi PflgB* or *PflaB* constitutive promoters that drive expression of flagella biosynthesis. The selectable markers include the antibiotic resistance cassettes for gentamicin (Elias *et al.* 2003; Elias *et al.* 2002), kanamycin (Bono *et al.* 2000), erythromycin (Sartakova *et al.* 2000), and streptomycin/spectinomycin (Frank *et al.* 2003), which made possible the selection for multiple recombination events. The strategies used for gene disruption, insertion of point mutation and complementation in *B. burgdorferi* will be discussed in the following chapters.

A significant limitation to genetic manipulation of *B. burgdorferi* remains the low transformation efficiency and the slow growth of the bacteria. Following electroporation, the bacteria requires an 18 to 24 hour recovery incubation and, with a 6 to 8 hour doubling time, the plates must be incubated for up to two weeks before clones can be recovered. Moreover, homologous recombination events resulting in the insertion of the selectable marker are estimated between 10^{-5} to 10^{-8} events per viable cell. Up to 50 micrograms of DNA is used to increase the number of transformants recovered. Early studies reporting transformation of *B. burgdorferi* used the high-passage, non-infectious B31-A strain (Bono *et al.* 2000; Rosa *et al.* 1996; Samuels *et al.* 1994a). However, transformation efficiency is significantly decreased when a low-passage, infectious strain is used. The presence of lp25 and/or lp56 was then shown to correlate with the low transformation efficiency (Lawrenz *et al.* 2002). However, as mentioned previously, the *bbe22* gene on lp25 is essential for infectivity in mice and so the transformants need to retain lp25 for infection studies (Purser *et*

al. 2003). Analysis of the sequenced genome showed that *bbe02*, another gene on lp25 shares sequence similarity with other bacterial restriction-modification systems (Lawrenz *et al.* 2002). The same study also identified *bbq67*, a second putative restriction-modification gene, located on lp56 (Lawrenz *et al.* 2002). Subsequent disruption of *bbe02*, in presence or absence of lp56, confirmed its importance in limiting transformation of *B. burgdorferi* (Kawabata *et al.* 2004). Although the loss of lp56 does not affect infectivity in mice, it has been shown to decrease transmission of the spirochete from the mice to the tick vector (Jacobs *et al.* 2006) and so, its loss should be avoided. In 2008, Chen and colleagues reported a 30 to 50-fold increase in transformation efficiency of infectious *B. burgdorferi* strain B31 following an *in vitro* treatment of the DNA with the CpG methyltransferase M.SssI (Chen *et al.* 2008). The increase in the transformation efficiency without the inactivation of the restriction-modification systems is a significant improvement. Alternatively, transformation efficiency can be increased by transforming a strain lacking lp25, but which expresses BBE22 from a shuttle plasmid (Purser *et al.* 2003). This complementation *in trans* of PncA has been shown to be sufficient to restore the infectivity of *B. burgdorferi* in mice (Purser *et al.* 2003).

1.7 Mouse infection system

Early research on the pathogenesis of *B. burgdorferi* was done using the important natural reservoir for the spirochete in North America, the white-footed mouse *P. leucopus* (Donahue *et al.* 1987; Levine *et al.* 1985). Although this

infection model represents the natural host of the spirochete, even persistently infected animals do not present symptoms to a level similar to the human disease: for example, neuroborreliosis, arthritis and carditis (Moody *et al.* 1990).

All strains of laboratory mice tested are susceptible to infection by *B. burgdorferi* and tissue dissemination of the spirochetes (Barthold *et al.* 1990; Barthold *et al.* 2010). However, neuroborreliosis is not observed in mice and the development of pathologies varies greatly between various strains. The most commonly used strains of wild-type mice are C3H/HeN, BALB/c, C57BL/6 and DBA/2 because of their difference in disease manifestation but not infection. The C3H/HeN mice can develop significant arthritis and carditis, while the C57BL/6 and DBA/2 mice are described as resistant to Lyme disease because little to no arthritis and mild carditis are observed (Armstrong *et al.* 1992; Barthold *et al.* 1990; Ma *et al.* 1998). However, the difference in pathologies between the strains does not correlate with the bacterial burden in the organ affected, since the spirochetes disseminated to the organs of BDA/2 and C57BL/6 mice at a level similar to C3H/HeN mice (Barthold *et al.* 1990; Brown *et al.* 2003; Brown and Reiner 1998). The BALB/c mice present an intermediate susceptibility to the disease (Barthold *et al.* 1990; Ma *et al.* 1998). Whereas increase in the bacterial dose used for the infection can result in level of disease similar to C3H/HeN mice, this is not the case in C57BL/6 mice (Ma *et al.* 1998).

The differences observed between the strains of mice appear to be related, to some extent, to the innate immune response to the infection. This is supported by different observations. Using SCID mice, which lack a functional

adaptive immune response (Blunt *et al.* 1995), does not eliminate the differences between strains of mice (Barthold *et al.* 1992). However, the infection of C3H/HeN mice lacking the chemokine receptor CXCR2 ($CXCR2^{-/-}$) resulted in a significant decrease in joint swelling as well as a decrease in neutrophil recruitment (Brown *et al.* 2003). In this study, the CXCL1 and MCP-1 chemokines were expressed at a significantly higher level in the knee joint of C3H/HeN mice compared to the C57BL/6 mice. CXCL1 is only known to bind to CXCR2 and MCP-1 binds CCR2. Although the disruption of CCR2 did not affect the level of arthritis, the absence of CXCR2 significantly affected the disease (Brown *et al.* 2003). In a recent study, the disruption of *B. burgdorferi bba57* abolished transmission from tick to mouse and delayed the dissemination of the spirochetes to bladder, skin and heart when needle infection was used (Yang *et al.* 2013). Whereas the bacterial burden of the mutant strain reached a level similar to the wild-type strain at three weeks post-infection, the number of bacteria in the joint remained significantly lower for the mutant than the wild type up to 4 weeks post-infection. In this study, increasing the amount of bacteria used for the infection did result in a bacterial burden in the joint similar to wild type from week 2 to week 4 post-infection. Although the number of spirochetes in the tissue was similar, ankle swelling remained significantly lower. Changes in the expression profile of chemokines in the tissue showed that among others, the expression of the CXCR2 ligands CXCL1 and CXCL5 was significantly decreased (Yang *et al.* 2013). As opposed to early Lyme arthritis, which is characterized by a neutrophil-dominated environment, the macrophages

represent the majority of the cells recruited (Barthold *et al.* 1993; Ruderman *et al.* 1995). Another study reported that the expression of the *B. burgdorferi* basic membrane proteins (Bmp) A and B is significantly higher in the joint than in the skin, bladder and heart of C3H/HeN mice (Pal *et al.* 2008). The disruption of BmpA and B abrogated the dissemination of the spirochetes to the joint tissue and the associated swelling of the ankle (Pal *et al.* 2008).

The kinetics of dissemination of *B. burgdorferi* to organs was established by PCR and by culture of organs from C3H/HeN mice infected with either the *B. burgdorferi* N40 strain (Barthold *et al.* 1991) or the B31 strain clone 5A4 (Coutte *et al.* 2009). *B. burgdorferi* N40 could be cultured from the spleen and the blood from days 2 and 3 post-infection, respectively and spirochetes were cultured from the kidney from day 4 and from the ear at day 10. However, no samples were collected between days 4 and 7 and days 7 and 10 post-infection. In this study, inflammation at the joint was observed from day 3 and from day 7 in the heart (Barthold *et al.* 1991). In the second study, samples were collected on days 4, 7, 10 and 14. Although no cultures from the heart, bladder, joint and skin were positive on day 4, spirochetes were grown from all 4 organs collected on day 7 (Coutte *et al.* 2009).

1.8 Antigenic variation

Prior to the discovery of *B. burgdorferi*, antigenic variation in *Borrelia* was already being studied in the tick-borne relapsing fever spirochete, *B. hermsii* (Meleney 1928). As opposed to *B. burgdorferi sensu lato*, which is transmitted by a hard-

bodied, slow feeding *Ixodes* sp. ticks (Burgdorfer *et al.* 1982), tick-borne relapsing fever spirochetes are transmitted by soft-bodied, fast-feeding *Ornithodoros* sp. ticks (Barbour and Hayes 1986; Barbour *et al.* 1982a; Dutton and Todd 1905). *B. hermsii* is responsible for most cases in North America and is transmitted by the *O. hermsi* tick (Dworkin *et al.* 2002). The bacteria express a surface exposed lipoprotein called variable major protein (Vmp) (Barbour *et al.* 1982a; Barstad *et al.* 1985). The gene encoding this antigen undergoes gene conversion (Burman *et al.* 1990; Plasterk *et al.* 1985; Restrepo *et al.* 1994). A silent *vmp* gene is unidirectionally recombined in the single sub-telomeric expression site, replacing the previously expressed allele. The newly expressed Vmp variant needs to differ enough from the previous gene not to be recognized by the antibodies produced against the previously expressed protein (Burman *et al.* 1990; Plasterk *et al.* 1985). This second population then grows in the absence of immune pressure from the antibody response, giving rise to a new peak of spirochetemia, until the host immune system responds to the new protein (Burman *et al.* 1990; Plasterk *et al.* 1985; Stoenner *et al.* 1982). Each population expressing a Vmp that is not recognized by the immune serum against the other Vmp is called a serogroup, based on the allele number that they are expressing (eg. clones of the serogroup 7 are all expressing Vmp7) (Stoenner *et al.* 1982).

The *B. hermsii* genome contains genes for two subgroup of Vmp. Based on the protein size, the variable small proteins (Vsp, approximately 20 KDa) and the variable large proteins (Vlp, approximately 36 KDa). Sequence analysis identified 59 *vsp*/*vlp* alleles organized in clusters on different plasmids (Barbour

et al. 1983; Dai *et al.* 2006; Restrepo *et al.* 1992). The recombination of a silent allele into the expression site involves an upstream homology sequence (UHS) of about 50 nt ranging from the transcriptional start site to inside the signal peptide coding region. It also involves a downstream homology sequences (DHS) of approximately 200 nt, dispersed across the different silent locus and the expression site (Dai *et al.* 2006).

In 1997, Zhang and colleagues described in *B. burgdorferi* the identification of *vmp*-like sequence cassettes (*vls*) (Zhang *et al.* 1997) (Figure 1.4). Moreover, the high or low infectivity phenotype of different strains correlates with the presence or absence of *vls*, which is located at the right-end of the plasmid lp28-1, known to be required for persistence in mice (Purser and Norris 2000; Zhang *et al.* 1997). The sequencing of a portion of the plasmid confirmed the presence of a *vls* expression site, named *vlsE*, located close to the right telomere. The expression site is composed of a central 570-bp cassette (*vls1*), flanked by two unique sequences. Upstream from *vlsE*, 15 silent cassettes similar to *vls1* (between 90 and 96% of sequence similarity with *vls1*) are identified. The cassettes are located towards the center of the plasmid, in the opposite orientation than *vlsE*. All the cassettes, including *vls1*, are flanked by 17 bp direct repeats. The alignment of the *vls* cassettes showed that the sequence variations between the cassettes are clustered in six variable regions, interspaced by six invariable regions. The *vlsE* ORF was shown to encode a surface-exposed lipoprotein called VlsE that is expressed in culture. Infection studies in mice showed that spirochetes recovered after four weeks of infection

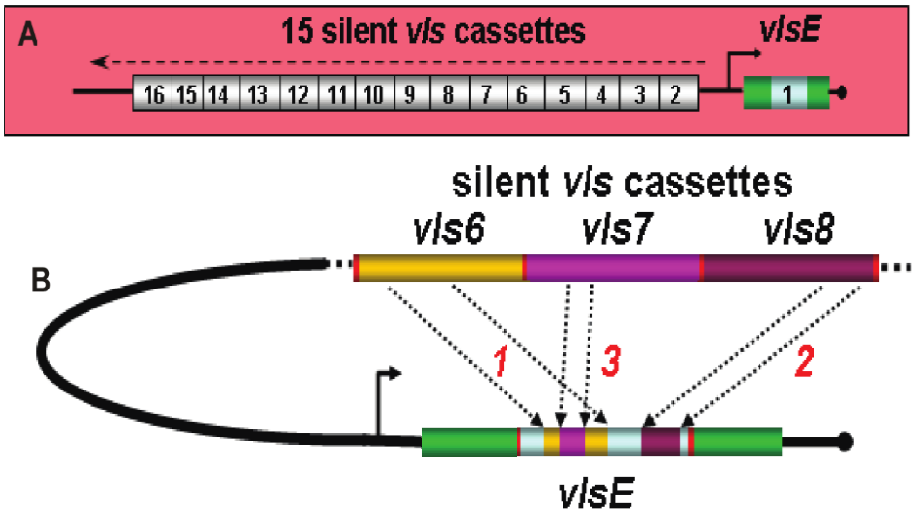

Figure 1.4. Organisation of the *vlsE* locus.

A) Representation of *B. burgdorferi vlsE* locus preceded by 15 *vls* silent cassettes in the opposite orientation from the expression site. The *vlsE* ORF is composed of a variable central cassette that is flanked by conserved regions. **B)** Illustration of a series of three sequential gene conversion events, generating sequence variation at *vlsE*. In this example, variable length of sequence from *vls* 6, 7 and 8 silent cassettes are sequentially recombined in the expression site. The red bars represent the 17 bp direct repeats. Reproduced with permission from Norris 2006.

have non-parental sequence at *vlsE*. The sequencing of the expressed *vls* cassettes after passage in mice confirmed that all the clones analyzed from the same organ contained different sequences. However, unlike the complete gene conversion observed in *B. hermsii*, where a complete or almost complete ORF is recombined in the expression site and each relapse represents a unique serotype, each *vlsE* sequence contained a mosaic of sequences from different silent cassettes and different in each clone. Although sera from infected mice recognized the parental strain, which expresses *vls1*, the same sera had decreased reactivity with *vlsE* from clones recovered from the mice. Similarly, an antibody against *vls1* has strong reactivity with *vlsE* from the parental strain but little reactivity against *B. burgdorferi* proteins after the passage in mice (Zhang *et al.* 1997). This suggested that sequence variation at *vlsE* results in a decreased recognition by host antibodies, but recombination of sequence appears to occur in a different manner than in *B. hermsii*. Following studies confirmed that switching of sequence at *vlsE* results from continuous random segmental gene conversion (Zhang and Norris 1998a). Whereas switching of *vmp* sequence in *B. hermsii* is continuous and occurs in culture at an estimated rate of 10^{-4} to 10^{-3} switch per cell per generation (Stoenner *et al.* 1982), switching at *vlsE* in *B. burgdorferi* occurs only during mouse infection (Zhang and Norris 1998b). The molecular mechanisms involved in switching remain unknown in both organisms. However, the next chapter focuses on identifying genes required for switching at *vlsE* in *B. burgdorferi*.

1.9 Gene regulation

The complex life cycle of *B. burgdorferi* suggests that the bacteria must differentially control the expression of various genes to adapt to each host-specific environment. During its life cycle, *B. burgdorferi* persists in the tick midgut, until it takes another blood meal. The spirochetes then migrate to the salivary gland to invade the vertebrate host and then disseminate and persist in multiple organs until it is acquired from the blood by another tick (Burgdorfer 1984; Burgdorfer *et al.* 1988; Donahue *et al.* 1987; Piesman *et al.* 1987). This complex cycle suggests that *B. burgdorferi* must persist using different carbohydrates, interact with various tissues, grow at different temperatures and pH, and respond to the passage from one environment to another. In 1995, Schwan and colleagues showed that the OspA and OspC surface proteins are differentially expressed in the tick and in the mouse (Schwan *et al.* 1995). In the unfed tick midgut or when *B. burgdorferi* is cultured at 24°C, only OspA is detected and when spirochetes are recovered from the tick midgut engorged following a blood meal, both proteins are detected. However, spirochetes cultivated at 37°C express OspC, and serum from infected mice react with OspC, but not OspA, suggesting that only OspC is expressed in the mouse (Schwan *et al.* 1995). Moreover, expression of OspA decreases the infectivity in mice (Strother *et al.* 2007). Following this initial study, OspA was shown to bind to the tick midgut receptor TROSPA (Pal *et al.* 2004a). Expression of OspC is required for the migration of *B. burgdorferi* from the midgut and binds to the tick salivary gland protein SALP15 to the salivary gland and for the early infection in the

mouse (Anguita *et al.* 2002; Ramamoorthi *et al.* 2005). Moreover, OspC was shown to bind to human plasminogen (Lagal *et al.* 2006; Onder *et al.* 2012). However, the importance of the OspC-plasminogen interaction for infection remains to be defined.

The sequencing of the *B. burgdorferi* genome identified two two-component systems; one is Hpk1-Rrp1. The histidine kinase Hpk1 (BB0420) and the regulatory response protein Rrp1 (BB0419) are co-transcribed, constitutively expressed in culture at 25°C and 37°C and their expression is significantly up-regulated during tick feeding (Rogers *et al.* 2009). Rrp1 was shown to have phosphorylation-dependent diguanylate cyclase activity required for the synthesis of the cyclic-bis(3′→5′) dimeric GMP (c-di-GMP) secondary messenger (Ryjenkov *et al.* 2005) and the co-transcription of *hpk1* with *rrp1* suggests that Hpk1 may be the kinase that phosphorylates Rrp1 (Ryjenkov *et al.* 2005). The deletion of Rrp1 resulted in a growth defect at 25°C and 37°C, but significantly more severe at 25°C (Rogers *et al.* 2009). Microarray analysis showed changes in the expression of 140 genes after growth at 33°C, including 131 with lower level of expression in the mutant (Rogers *et al.* 2009). However, the parental strain used in this study was a non-infectious strain and when a different, infectious strain was used, in two other studies, Rrp1 was shown to modulate a smaller array of genes (Caimano *et al.* 2011; He *et al.* 2011). Despite such differences between the studies, all three groups reported that the expression of genes involved in the transport and metabolim of glycerol (Caimano *et al.* 2011; He *et al.* 2011). The disruption of *rrp1* in an infectious parental strain was also

used to show that Rrp1 is essential for the survival of *B. burgdorferi* in the ticks, but dispensable for infectivity in mice (Caimano *et al.* 2011; He *et al.* 2011; Kostick *et al.* 2011). In addition to Hpk1 and Rrp1, *bb0363* was identified as encoding a c-di-GMP phosphodiesterase, which hydrolyzes c-di-GMP *in vitro* (Sultan *et al.* 2010). Disruption of *bb0363* affected the motility of *B. burgdorferi* and a complete loss of infectivity in mice. However, the disruption of *bb0363* did not appear to affect survival in ticks (Sultan *et al.* 2010).

The second two-component system found in *B. burgdorferi* is the Hk2/Rrp2. Disruption of Rrp2 could not be achieved, suggesting an essential role for the response regulator in culture. However, the insertion of a mutation in Rrp2 ATP-binding domain was shown to abolish *B. burgdorferi* infectivity in mice but had no effect on the survival in ticks (Boardman *et al.* 2008), suggesting that the ATPase activity of Rrp2 has an additional function during mice infection, that is not essential for the growth of *B. burgdorferi* in culture. Moreover, microarray analysis showed that at least 144 genes are differentially expressed in the mutant, including 125 genes with an increased expression, compared to the wild-type parental strain (Boardman *et al.* 2008). Among these genes with a higher expression in the mutant strain is the alternative sigma factor RpoS (σ^s) (Boardman *et al.* 2008; Ouyang *et al.* 2008). The expression of RpoS was previously shown to increase following a temperature increase and a pH decrease in late logarithmic to stationary phase culture (Caimano *et al.* 2007). In this system, the expression of RpoS was shown to be induced by the binding of the second alternative sigma factor, RpoN (σ^{54}) (Hübner *et al.* 2001). The RpoN

factor binds to a specific promoter -24/-12 site upstream of *rpoS* (Burtnick *et al.* 2007) and its activity depends on the Rrp2 (Yang *et al.* 2003). However, as opposed to *rrp2*, the disruption of *hk2* had no impact on *B. burgdorferi* infectivity (Burtnick *et al.* 2007). The phosphorylation of Rrp2, required for its activity, was later shown to occur using acetyl phosphate as the phosphate donor (Burtnick *et al.* 2007; Xu *et al.* 2010).

Another regulator of gene expression, including of *rpoS*, was originally predicted to be a Fur/PerR ortholog, sharing 50.7% sequence homology with *Bacillus subtilis* PerR (Boylan *et al.* 2003; Fraser *et al.* 1997). In *B. subtilis*, the oxidation of the PerR metalloprotein induces its release from the DNA and the expression of the genes it represses (Herbig and Helmann 2001). However, the *B. burgdorferi* Fur/PerR putative ortholog binds more strongly to the promoter region of *bicA*, a *napA*/*dps* ortholog, in presence of *t*-butyl peroxide (Boylan *et al.* 2003). It was subsequently shown to be required for the expression of *bicA* in response to oxidative stress. In *E. coli*, *dps* is expressed in response to oxidative stress and protects the DNA from damage by binding iron (Martinez and Kolter 1997; Zhao *et al.* 2002). The *B. burgdorferi* Fur/PerR putative ortholog was then named borrelial oxidative stress regulator (BosR) (Boylan *et al.* 2003). However, the role of BosR in the response to oxidative stress in *B. burgdorferi* remains unsure (Boylan *et al.* 2006; Hyde *et al.* 2006; Katona *et al.* 2004; Ouyang *et al.* 2011; Ouyang *et al.* 2009b; Samuels and Radolf 2009; Seshu *et al.* 2004). Binding activity of BosR requires Zn^{2+} but is independent of Mn^{2+} and Fe^{2+}. Following this study, the expression of BosR was shown to be up-regulated in the

mouse (Medrano *et al.* 2007) and is required for infection in the mouse but not in the tick vector (Hyde *et al.* 2009; Ouyang *et al.* 2009b). Moreover, BosR was shown to bind to the promoter of *rpoS* (Ouyang *et al.* 2011) and induces its expression (Hyde *et al.* 2009; Ouyang *et al.* 2009b). The disruption of *bb0647* (*bosR*) was shown to affect the expression of 88 genes (Hyde *et al.* 2006), including 50 genes that are not regulated by *rpoS* (Ouyang *et al.* 2009b), demonstrating that BosR regulates the expression of genes independently of *rpoS*.

At lower cell density, a longer *rpoS* mRNA transcript is synthesized, extending upstream from the -24/-12 RpoN binding site (Lybecker and Samuels 2007; Samuels 2011). In this situation, the small RNA $DsrA_{Bb}$ was shown to be required for the expression of RpoS at 37°C. The binding of $DsrA_{Bb}$ to the extended 5' sequence of the transcript is suggested to allow translation by blocking the formation of a predicted stem-loop structure that would block access to the ribosome-binding site on the transcript (Archambault *et al.* 2013; Lybecker and Samuels 2007). In this case, the expression of RpoS would be controlled by the RpoD (σ^{70}) sigma factor. However, the expression of $DsrA_{Bb}$ is not influenced by the temperature or the cell density and the disruption of $DsrA_{Bb}$ did not affect the expression of RpoS induced by pH or at high cell density. Moreover, the disruption of $DsrA_{Bb}$ did not affect infectivity in mice by needle injection (Lybecker and Samuels 2007).

Recently, the *Borrelia* host adaptation regulator (BadR) has been shown to bind to the promoter of *rpoS in vitro* and the disruption of BadR increases the

expression of RpoS. The expression of BadR is also required for survival of *B. burgdorferi* in wild-type and SCID mice (Miller *et al.* 2013).

1.10 DNA repair

Little is currently known on how damage in DNA is repaired in *B. burgdorferi*. As mentioned previously, *B. burgdorferi* does not accumulate or require iron for growth *in vitro* and no proteins are known to be iron dependent (Posey and Gherardini 2000). This suggests that DNA and protein damage resulting from the Fenton reaction between Fe^{2+} and the oxygen species would be predicted to be minimal in *B. burgdorferi* (Boylan *et al.* 2008; Gunther *et al.* 1995; Posey and Gherardini 2000). A previous study by Boylan and colleagues showed that reactive oxygen species are toxic for *B. burgdorferi* primarily through damage in membrane polyunsaturated lipids, a primary target for oxygen radicals (Boylan *et al.* 2008). No enzymes for fatty acids biosynthesis were found by sequencing (Fraser *et al.* 1997) and the analysis of the composition of the membrane confirmed that *B. burgdorferi* incorporates lipids from the environment, including polyunsaturated lipids, which are sensitive to oxidative damage (Beermann *et al.* 2000; Boylan *et al.* 2008; Esterbauer *et al.* 1992; Hossain *et al.* 2001; Requena *et al.* 1996). The disruption of BosR was shown to result in a slight increase in sensitivity to hydrogen peroxide (Hyde *et al.* 2009) and the *B. burgdorferi* superoxide dismutase A (SodA) is required for infectivity in mice (Esteve-Gassent *et al.* 2009), suggesting some level of sensitivity to oxidative damage in *B. burgdorferi*.

In another study, Bourret *et al.* determined the cellular targets and the sensitivity of *B. burgdorferi* to reactive nitrogen species (RNS). The main targets of RNS in *B. burgdorferi* are proteins that contain free and zinc-bound cysteine thiols. However, the disruption of the nucleotide excision repair proteins UvrB and UvrC did result in a significant increase in sensitivity to RNS, but not to reactive oxygen species, demonstrating that *B. burgdorferi* DNA is damaged by RNS and the nucleotide excision pathway is required for the survival.

The DNA repair pathways present in *B. burgdorferi* and their importance will be further described in the Chapter 4.

1.11 Research projects

Three research projects will be developed in the next chapters.

1. Role of *B. burgdorferi* nucleic acid metabolizing genes in switching at *vlsE*.
2. Role of the *B. burgdorferi* DEAH-box RNA helicase *hrpA* upon murine infection.
3. Importance of *B. burgdorferi* nucleic acid metabolizing genes for survival to DNA damage.

Chapter Two: Role of *B. burgdorferi* nucleic acid metabolizing genes in switching at *vlsE*[*].

2.1 Introduction

For long-term infection of a vertebrate host, *B. burgdorferi* requires the expression of a 35-kDa surface lipoprotein, named VlsE, which undergoes constant antigenic variation during the infection (Zhang *et al.* 1997). This protein is encoded at a single *vlsE* expression site, located at the right end of the linear plasmid lp28-1. This *vlsE* site contains a central variable cassette flanked with conserved sequence regions and just downstream from *vlsE* are 15 *vls* silent cassettes oriented in opposite direction from *vlsE*, towards the center of the plasmid (Zhang *et al.* 1997) (see Figure 1.4). These *vls* silent cassettes and *vlsE* are flanked by 17-bp direct repeats (Zhang *et al.* 1997).

During vertebrate host infection, a variable length of sequence from the silent cassettes is unidirectionally recombined into the *vlsE* variable region through gene conversion events, ultimately resulting in a VlsE protein made of a mosaic of sequences from various cassettes (Zhang and Norris 1998a, b). Previous studies showed that although *B. burgdorferi* lacking *vlsE* is unable to

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[*] All the experiments and figures in this chapter were done by Pierre-Olivier Hardy, with the exception of the experiments involving the *ruvA* mutant and the figures 2.2 and 2.6, which were done by Ashley R. Dresser. The results for *ruvB*, *mutS1*, *nth* and *ruvA* mutants were published in Dresser AR, Hardy P-O, Chaconas G (2009) Investigation of the genes involved in antigenic switching at the *vlsE* locus in *Borrelia burgdorferi*: An essential role for the RuvAB branch migrase. PLoS Pathog 5(12): e1000680. doi:10.1371/journal.ppat.1000680. The table 2.9 was published in Walia R, Chaconas G (2013) Suggested role for G4 DNA in recombinational switching at the antigenic variation locus of the Lyme disease spirochete. PLoS ONE 8(2): e57792. doi:10.1371/journal.pone.0057792.

persist in wild-type mice (Labandeira-Rey *et al.* 2003; Labandeira-Rey and Skare 2001; Purser *et al.* 2003; Purser and Norris 2000), long-term persistence and dissemination of the spirochetes is not affected in the absence of the acquired immune response in severe combined immune deficiency (SCID) mice (Labandeira-Rey *et al.* 2003; Purser *et al.* 2003). However, the complementation of *vlsE* in the absence of the silent cassettes locus is not sufficient to restore persistence of *B. burgdorferi* in immune competent mice (Bankhead and Chaconas 2007; Lawrenz *et al.* 2004). Moreover, Bankhead and Chaconas (2007) (Bankhead and Chaconas 2007) deleted the left-end of lp28-1 by inserting a replicated telomere in the plasmid, leaving only *vlsE*, the 15 *vls* silent cassettes, the genes required for the autonomous replication of the plasmid and three potential ORF of unknown coding function. This strain had an infectivity level similar to wild-type *B. burgdorferi*. These results confirmed that the antigenic variation of VlsE involves the *vls* silent cassettes and is required for *B. burgdorferi* to evade clearance by the host adaptive immune response (Bankhead and Chaconas 2007).

However, the genes required for the recombination events at *vlsE* remain unknown. Among the other bacteria known to encode proteins undergoing antigenic variation through sequence recombination, only the obligate human pathogen *Neisseria gonorrhoeae* has been significantly studied (Vink *et al.* 2011). Pathogenic strains of *N. gonorrhoeae* express a Type IV pilus, primarily composed of PilE subunits (Kellogg *et al.* 1968; Kellogg *et al.* 1963; Meyer *et al.* 1982; Punsalang and Sawyer 1973; Scheuerpflug *et al.* 1999; Swanson *et al.*

1971). Similar to *vlsE* in *B. burgdorferi*, the *N. gonorrhoeae pilE* gene undergoes segmental gene conversion resulting in antigenic variation (Haas and Meyer 1986; Segal *et al.* 1986). Variable lengths of sequence from a silent gene copy, located in various *pilS* loci, are recombined into the *pilE* expression site replacing the previously expressed sequence (Haas and Meyer 1986; Haas *et al.* 1992). Previous studies showed that disruption of *N. gonorrhoeae recA* (Koomey *et al.* 1987), *rep* (Kline and Seifert 2005b), *rdgC* (Mehr *et al.* 2000), *recJ* (Sechman *et al.* 2005; Skaar *et al.* 2002), *recR*, *recG*, *ruvA*, *recO*, *recQ* (Sechman *et al.* 2005), *ruvB*, *ruvC* (Sechman *et al.* 2006) or *mutS* (Criss *et al.* 2010) affected pilin antigenic variation. Moreover, the integration host factor (IHF) and its binding to the *pilE* promoter is required for the transcription of PilE (Fyfe and Davies 1998; Hill *et al.* 1997). Compared to *N. gonorrohoeae*, no ortholog of *recX*, *recO*, *recQ*, *recR*, *rdgC* and *ruvC* are found in the sequenced genome of *B. burgdorferi* (Fraser *et al.* 1997). Moreover, Liveris *et al.* showed that disruption of *B. burgdorferi recA* does not decrease switching at *vlsE*, but appeared to impair allelic exchange, suggesting that the recombination at *vlsE* would involve a RecA-independent response that is either induced, or repressed, only in the host. The present study was part of a wider project aiming to identify genes required for switching at *vlsE* in *B. burgdorferi*. Our data demonstrate that the disruption of the branch migrase subunits *ruvA* and *ruvB* almost completely abolished *vlsE* switching in *B. burgdorferi*. Moreover, similar results were obtained in parallel by Lin *et al.* using a transposon mutagenesis strategy (Lin *et al.* 2009).

2.2 Materials and Methods

2.2.1 Strains and primers used and growth conditions

All the primers used in this study are listed in Table 2.1, Table 2.2 and Table 2.3 and were synthesized by the University of Calgary Core DNA services (Calgary, AB, CA). Plasmids and *E. coli* strains used in this study are listed in Table 2.4 and *B. burgdorferi* strains in Table 2.5.

2.2.2 Growth and transformation of *E. coli*

All plasmid amplification and storage was done in *E. coli* DH5α transformed as described. To prepare chemically competent *E. coli* cells, a culture was grown until it reached an optical density of 0.55. The cells were then placed on ice for 10 minutes and centrifuged for 10 minutes at 2,500 x g at 4°C. After resuspending the pellet in TB buffer (10 mM PIPES pH 6.7, 55 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCl), the cells were centrifuged for 10 minutes at 2,500 x g at 4°C. The pellet was resuspended in TB buffer with 7.5% DMSO, aliquotted and froze at -80°C until they were used for transformation. To transform chemically competent *E. coli*, ligation products were incubated 30 minutes with chemically competent *E. coli* DH5α (prepared using common protocol) on ice, 45 seconds at 42°C and 5 minutes on ice before adding one ml of Lysogeny Broth (LB - prepared in house using common protocol). Cultures were incubated one hour at 37°C with shaking at 250 rpm and 150 µl were plated on LB agar plates containing the antibiotic required for selection. Plates were incubated overnight at 37°C. Selected colonies were grown in 5 ml LB for 12 to 16 hours at 37°C with shaking at 250 rpm with the appropriate antibiotic for selection. DNA from each

Table 2.1. Primers used in this study.

Primers used for knockout plasmid construction			
Genotype	**Target**	**Inverse**[1]	**Knockout**
mutS1	B1067-GGGATCTTGAGAAATACTCTCCTAAAG	B1069-NheI-gcgctagctagctagGGCGCATATAGGCTCTTTTGT	B1140-CCAGGCTAGATCATGTTGAATTTT
	B1068-CAAGATCGTTGCCCTGCTTTTC	B1070-NheI-gcgctagctagctagGTCTCTTTCCCATTGCAGTTTTGC	B1141-TAGTGCTCAACAACAGGATGCC
ruvA	B1359-ATAATCTTCCATGGCAAT	B1376-NheI-catttcctagctagcCTTGAATCCACTGGTCTT	B1419-CTCGCTATTAATCAAAAGC
	B1360-TCCATGTATAGTCTTTATGTGA	B1377-NheI-catttcctagctagcATAACTCAACTTTGTCTGACAA	B1420-AAATGAATTAAAACTTTTTGG
ruvB	B1200-CGAAAATAGTATAAGCTTTTTAAGCTC	B1202-NheI-gcgctagctagctagGCTCAAGAGGGACTCCTCGT	B1249-CATAGGCTCAGACCAATAATAGAAG
	B1201-CTTTGGTTTTCATTAAGACCATCC	B1203-NheI-gcgctagctagctagCACTCTTCTCATCAAGACCTGTT	B1250-TTTCTCTTTATTATCTCAACAAGCTC
nth	B1486-GGCCAAATTTTCTCCTAAAAATT	B1488-NheI-ctagctagctagCCCGCAATTATTGTAGATACTCAT	B1501-CTCCATACCTTTTTGAAAGGTACG
	B1487-GCATTGATTCATGGATGGATT	B1489-NheI-ctagctagctagCCAAATTATCTGTTGTTCTTGCA	B1502-GATCCAAGAATAACATTTGCCG
exoA	B1478-GGGTAGGCAATTTAGCGTATCT	B1480-NheI-ctagctagctagGCTAGAGAGAGAAATATGGGGTG	B1503-CTATCTCTGTAAGGCTGCTTGGAG
	B1479-CTTGCATTGTTATATGCCTTGG	B1481-NheI-ctagctagctagGGCAACTGTTCTCTTAAGGCTT	B1504-GATCCTTATTAAATATCCTAAATGTATCCAC
uvrC	B1470-GCGAATACGAAGCATTGCTT	B1472-NheI-ctagctagctagGGGAATGCCTTTTAAAGAAAACTA	B1995-GGAAAATTAGTTGAAAGAGATGC
	B1471-GGCAATATATCTTTATAGGTTCCAAT	B1473-NheI-ctagctagctagTTTCTCCTGGATGAACATGG	B1996-CCATAGAAGCTACTGTTTCTTGAC
uvrD	B1178-GGGATACTAATGATGTTGTTAAATTTG	B1834-NheI-gcgctagctagctagGAGGAGCTGGGTAAAAAACTTTTTG	B1874-CAACTCAAAATAGTTCTAATAAAAGG
	B1833-CTCAGGGATAAAGATAATGTCATAAG	B1835-NheI-gcgctagctagctagCCTCATTTGCAATGCCCAC	B1875-AAATAATAAAAGAGACTCTTTGGC
dnaK1	B1825-GTGTTCATGAAAAAATGGATAGG	B1827-NheI-gcgctagctagctagGCTTTAAATCAAGATGAAGTTGTAGC	B1870-GGGCAAAGTCGACTTGG
	B1826-GGAGAAAACAAACAGATCTCATTAG	B1828-NheI-gcgctagctagctagCGAAAGAACAGTATAGGTGTCGC	B1871-CTAAAATTGTAGCAGAAGGAAAGG
polA	B1685r-TATTTAGCTATTGTTGGAGACAGGTC	B1686-NheI-gcgctagctagctagGAAATGGAAGAAAATGGAATTTACC	B1688-CAGCATCTTCGGATGAATAGC
	B1684f-CAAGCTCAATTTGAGAATAATCAGC	B1687-NheI-gcgctagctagctagGATATGTATTTAGCCTTTTTAAGGC	B1689-CTATATTCCAATCGAAGCCAAAGG
dnaK2	B1829-GCAAAAAACCAAATGGTTACAAACC	B1831-NheI-gcgctagctagctagGGAGAAACTAAAGACATGGTACTCC	B1872-CGGAGACACACATCTTGGTG
	B1830-CGCTTGAAATTTTTTCAGAATATTC	B1832-NheI-gcgctagctagctagGGTTGACTTTACTTCAAAAACACC	B1873-GAATGCCACCCTGAATAGC
parC	B1458-GGTAATACAATGAAATATCATCCCC	B1460-NheI-ctagctagctagTGTTGAAAATTTGCTTAAAATTCC	B1510-CAAGCGAAGTGATTGAAAAGTT
	B1459-AACATAATAAAAATTGTCAAGCCTG	B1461-NheI-ctagctagctagCCCTAGGCAAAGATAGCTCTATGG	B1511-GTGCCTTAAAACCTCTGACAATAT
parE	B1462-TGATGAGTTTATTATGGGTTACGG	B1464-NheI-ctagctagctagATCCCTAAGCTTAAGGATTGCA	B1512-ATGATGGAGGTACCCATCAGAC
	B1463-GGAGAAATTTCACCAAGGCC	B1465-NheI-ctagctagctagCTCCCCATAATTATTTGTATGGG	B1513-CTGAGTCGTTCATTGTCAACTACT
ligA	B1482-TTTTGCTTTCTTCCAAACTAGC	B1484-NheI-ctagctagctagGATGGTGTTGTTCTTAAGGTTAGTG	B1508-GATGGCGTTCTTGAAAAAGCTCTTACTAGAGG
	B1483-GTCGGAAAAACCCTTCAATATCC	B1485-NheI-ctagctagctagCGATAGAACATCCATCAATCTTT	B1509-CAAGGGATTGACTTTAAAACCC
smg	B1474f-GCCGCCCTTTTAATGTCAAT	B1476-NheI-ctagctagctagGCCCTTGTAGTAAATAGCAAAAGG	B1499-CATCAGACAGACCTGATACCAATC
	B1475r-CGTATATGATCTTGATAAACAACTTGA	B1477-NheI-ctagctagctagAGGAGCTTTAATTACAGCTGAGC	B1500-CTTGGGCTGTTGTTGGTTCTAG
ssb	B1466f-ATCGTTGGCCATTTGTTTTT	B1468-NheI-ctagctagctagTCTAAGAACAGCCATGCCAC	B1514-GAAGAATGAAGAAGAATGATGAATGG
	B1467r-GCGAATTAGAAGAAGATTAGGGTT	B1469-NheI-ctagctagctagGCAAGATTCAGATGTTAATAGTTTGA	B1515-GTATTAGAACCTGAACTAAACATTTGC
Primers used for gentamicin resistance cassette cloning and transformant screens			
B348-CGCAGCAGCAACGATGTTAC			
B349-CTTGCACGTAGATCACATAAGC			
B1281-ATCGTCTATGCTTAAGCTCTT			
B415-CATTTCCTAGCTAGCGGCGAATGGCGCGGCCGCCCTAGG			
B416-CATTTCCTAGCTAGCACGCGTAAGCCGATCTCGGCTTG			
B820-CATTTCCTAGCTAGCCCTAGGTAATACCCGAGCTTCAA			

[1] Small letters identifiy sequence added for the indicated restriction sites.

Table 2.2. Primers used for individual plasmid screening [1]

Plasmid	Forward (5' → 3')	Reverse (5' → 3')
lp21	B421-TGTGGTTGCTAAAACCCAAGCGT	B422-TTGTTTCTAATTGCTCTGAATTGCATCC
lp28-2	B423-CCCTCATCAAGTTTTTCCATGTGTTTTT	B424-AGGTGGCCTTTCCGAGCTTGTACCTTAC
lp28-3	B425-AACACTATCTTAAATGTCCCCCACAA	B426-GTGGAAGAGTGGTTATGGTCAATTTT
lp25	B427-AGAATTATGTCGGTGGCGTTGT	B428-ATTAAAGCCGCCTTTTCCTTGGT
lp28-1	B429-CGGGGATCCAGCCAAGTTGCTGATAAGGACGACCC	B430-ACGGCAGTTCCAACAGAACCTGTACTATCT
lp56	B431-ACTATTAAGACGAGCAATAAAAAGTCCA	B432-GACGAAGCAAAGAAGGATTTGGATCACC
cp26	B433-ATAGCCCATTCCAGACATTAAACCGCCT	B434-AGTTCCCCAAATAACAGCAATCTGCGA
lp17	B435-ACTGCAATCTGCCCAAGCTACATAATCT	B436-AAGGTAAGGACGGTTGTCTACATGGATT
cp32-9	B437-TATCAAAAAAGTGCTGTTTTATAG	B438-TAATCTCAAATATTCTTCTTTATG
lp38	B439-AGCAGGCAGAACAAAACATGCAAAAACTG	B440-TCCAAGCTATTTCTACGGCCTCTTTAGC
cp32-3	B441-GCAAGTTCCCACGATAACACACCCGTAT	B442-TTTTCATATCCCCTCCTAGCTTTATTGCC
lp36	B443-TTCTTATCCCTGACTTTCACTTTTGAGG	B444-TCCTTTACTTCTATGTTTTTACTTTCCTTGGT
cp9	B445-GGACTGGTATTTACTCCGGCTGATAGAGC	B446-CCTTAATGATGAGGCCGATGAAGTTGC
cp32-8	B447-GAAGATTTAAACAAAAAAATTGCG	B448-GTAATCACTTCTTTTTTACCATCG
lp54	B449-GCAAAATGTTAGCAGCCTTGACGAGAAA	B450-TAGATCGTACTTGCCGTCTTTGTTTTTT
cp32-1	B451-ACGATAGGGTAATATCAAAAAAGG	B452-AGTTCATCTAATAAAAATCCCGTG
cp32-2/7	B453-GGAATGTATTAATTGATAATTCAG	B454-GCGAACTAAATAGTGCCTTATGGG
cp32-6	B455-GACTTTACATAGTATAAATGCTTTTGG	B456-TCTCGTTTTATAAAATAAGTAGG
lp28-4	B457-TCACCTCAGCTAATCTATTTATCGACAC	B458-AAGCGCGGAGTTTTCGGCTG

The primers were first published in Purser, J. E. and S. J. Norris (2000) (Purser and Norris 2000).

Table 2.3. Primers used for the plasmid screen by multiplex PCR[1]

Plasmid targets	Sequence[1] 5' → 3'	Amplicon size (bp)	Concentration in 5X mix (µM)
Linear plasmids			
lp21	B2056 - ATGATCATATTTTTATTATCCCCGCAC	400	1.5
	B2057 - TGAATCTTGCCCTTGACATAACTACC		1.5
lp28-3	B2058 - CTAAGACAGGCCCCGTATGAGG	375	1.5
	B2059 - GCTAATTGTCGCCACAGTGCTATG		1.5
lp38	B2060 - CATCGGCTTTGGTTGCTACATTACC	350	3
	B2061 - GGTTGATTGATGGCTTTCTACGC		3
lp28-1	B2062 - ACACCGCACTAACATCGGGTTC	325	1
	B2063 - GATACACCTCCTAGTTTGGGTCCTC		1
lp25	B2064 - GAATGTATTTTGTATCCAAACCGGAG	300	3
	B2065 - TTAGCTTTTCTGGTTTGAACGCC		3
lp36	B2066 - GTGTTTATGTAGTGTTCTTTTAGCGACC	275	0.5
	B2067 - GTATTTTGCTACTACCGCCTTTTGTC		0.5
lp17	B2068 - TGTTGGGAAAACTACCTTAAGCGG	250	0.5
	B2069 - TTGCACATCTCTTCTAAAAGTTCCACTC		0.5
lp56	B2070 - AAGTTCCTCCAAATCTTAGTCCCG	225	2
	B2071 - TCATGATGGGCATTGGGGT		2
lp54	B2072 - ACGATTGGGACAGCGTTTTAGG	200	0.5
	B2073 - AACTGAAAAAAGAATTGTGGGAAGAGC		0.5
lp28-2	B2074 - GGTCGTGTTTCGTCATCTCCAC	175	0.5
	B2075 - GCGTATTGGATAGTCTGCGAGG		0.5
lp28-4	B2076 - ATGTGGGATGCTATAATCGTGCC	150	0.5
	B2077 - TTGGTGGTGGCGCAGTGC		0.5
lp5	B2078 - CACTAGATCACATGAATCAAGAATTGTTC	125	2
	B2079 - GGTTTTATAATTAACTTCGTGTTGAGTCG		2

Plasmid targets	Sequence[1] 5' → 3'	Amplicon size (bp)	Concentration in 5X mix (µM)
Circular plasmids			
cp32-8	B2093 - TGAATTTATACCCCATCAAGAGTTGAG	375	3
	B2094 - TCCATATTGAATTCATTATTGCCCG		3
cp32-1	B2095 - CATTAAGATTGATGCCGTGGAA	350	3
	B2096 - CTGGGCCTAGAATCGCTGC		3
cp26	B2097 - GAAGTGGAAGATTGTCAGAAAGAGCC	325	0.5
	B2098 - CTTCCAGAGTCTGTTCCGCCTG		0.5
cp32-4	B2099 - CTAGCGATATGACCAATGAAGTTATAAC	300	2
	B2100 - TTTTGTGCCTGGGCTCAAAC		2
cp32-6	B2101 - TAAAGGATAAGATAGAGAAGTCGGATCC	276	1.5
	B2102 - CAACTTTCCCTTGATTTTTAGCCC		1.5
cp32-9	B2103 - TTCACATGGAAGAAATTTTGCATAC	250	1.5
	B2104 - AGCGTCTGATTGCTCATAAGCC		1.5
cp9	B2105 - GATGAACTTGCCGGGGATTG	226	1.5
	B2106 - CGTATCAAAGGAGTCTTTATACCCAGTG		1.5
cp32-7	B2107 - AACAACAAGACTTTCTGCGAACATC	200	3
	B2108 - GAAAGCTTCTGTAAGTTCCCCTTTAAG		3
cp32-3	B2109 - ATTGAGGAAGAACTTGAAAAGCTAGC	170	3
	B2110 - ATATCCCCTCCTAGCTTTATTGCC		3

The primers were first published in Bunikis, I. *et al.* (2011) (Bunikis *et al.* 2011).

Table 2.4. Plasmids and *E. coli* strains used in this study.

Gene target	Locus	Gene description	Plasmid	*E. coli* strain number (GCE)	Polarity of *gent* cassette	T7 terminator	Gene disruption	Merodiploid
mutS1	BB0797	mismatch repair protein	pPOH2	1604	forward	–	+	–
ruvB	BB0022	Holliday junction helicase	pPOH6	1618	forward	–	+	–
nth	BB0745	endonuclease III	pPOH92	3219	forward	+	+	–
exoA	BB0534	exodeoxyribonuclease III	pPOH32-3	1696	forward	–	+	+
uvrC	BB0457	excinuclease ABC, subunit C	pPOH81-2	3209	reverse	+	+	+
uvrD	BB0344	DNA helicase II	pPOH55-2	2146	forward	+	+	+
			pPOH55-1	2145	reverse	+	+	+
dnaK1	BB0264	heat shock protein 70	pPOH51-2	2138	forward	–	+	–
			pPOH51-1	2137	reverse	–	–	–
polA	BB0548	DNA polymerase I	pPOH47-1	2125	forward	–	–	+
			pPOH47-2	2126	reverse	–	–	+
			pPOH47-3	2127	reverse	+	–	+
dnaK2	BB0518	heat shock protein 70	pPOH53-1	2141	reverse	+	–	+
			pPOH53-2	2142	forward	+	–	–
parC	BB0035	DNA topoisomerase IV	pPOH30-2	1689	forward	+	–	+
			pPOH97	3232	reverse	–	–	+
parE	BB0036	DNA topoisomerase IV	pPOH100	3235	reverse	+	–	+
			pPOH98	3233	forward	–	–	–
smg	BB0297	DNA processing	pPOH31-2	1692	reverse	–	–	–
			pPOH96-1	3224	reverse	+	–	+
ligA	BB0552	DNA ligase	pPOH33-2	1698	forward	+	–	+
ssb	BB0114	Single-stranded DNA-binding protein	pPOH99	3234	forward	+	–	–
			pPOH94-2	3222	forward	–	–	–

Table 2.5. *B. burgdorferi* strains used in this study.

Genotype	Target locus	Description	Strain (GCB)	Missing plasmids	Reference
wt		*B. burgdorferi* strain B31 clone 5A4	920		(Purser and Norris 2000)
knockout strains					
uvrC	*bb_0457*	Excinuclease ABC, subunit C	537	cp9	This study
			538	cp9	
uvrD	*bb_0344*	DNA helicase	541	lp38, lp28-4	
			542	lp38	
dnaK1	*bb_0264*	Heat shock protein – Chaperone	539		
			540		
exoA	*bb_0534*	Exonuclease III	531		
			534		
ruvB	*bb_0022*	Holliday junction helicase	513		(Dresser *et al.* 2009)
			514	lp28-4, cp9	
mutS1	*bb_0797*	mismatch repair protein	502		
			504		
nth	*bb_0745*	endonuclease III	525		
			526		
*ruvA**	*bb_0023*	Holliday junction helicase	1174		
			1175		

* Experiments were done by Ashley R. Dresser.

clone was recovered using the GeneJET Plasmid Miniprep kit (Fermentas, Burlington, ON, CA) according to the manufacturer's instructions. To prepare DNA for transformation in *B. burgdorferi*, 5 ml of LB with the appropriate antibiotic was inoculated with a single colony and incubated 8 hours at 37°C with shaking (250 rpm). The grown culture was diluted in 50 ml LB with antibiotic and incubated 12 to 16 hours at 37°C with shaking (250 rpm). DNA was extracted using the Qiafilter Midiprep kit (Qiagen, Toronto, ON, CA) following the instructions from the manufacturer. Recovered DNA was precipitated (0.1 M sodium acetate pH 5.2 and 2 volume of ice-cold ethanol) during 45 minutes at -80°C and centrifuged 25 minutes at 16,110 x g at 4°C in an Eppendorf 5415R centrifuge (Mississauga, ON). The supernatant was discarded and the dried DNA was resuspended in ddH_2O at a final concentration of 5 µg/ml. DNA samples were kept at -20°C.

2.2.3 Growth and transformation of *B. burgdorferi*

B. burgdorferi was cultivated in BSK-II medium prepared in-house (Barbour 1984) and supplemented with 6% rabbit serum (Cedarlane Laboratories, Burlington, ON, CA). Cultures were incubated at 35°C with 1.5% CO_2 environment. For samples recovered from mice or cultured in the presence of blood, an antibiotic cocktail (0.2% DMSO, 20 µg/ml phosphomycin, 50 µg/ml rifampicin and 2.5 µg/ml amphotericin B) (Sigma-Aldrich, Oakville, ON, CA) was added to the culture media. Cell densities were determined by direct counting on a dark-field microscope, using a Petroff-Hausser Counting Chamber (VWR, Edmonton, AB, CA).

The protocol to prepare and transform electrocompetent *B. burgdorferi* was based on previous studies (Bono *et al.* 2000; Rosa *et al.* 1996; Samuels *et al.* 1994a). For each strain to be prepared, a 60 to 200 ml culture was incubated until it reached a cell density between 7 x 10^7 and 1 x 10^8 spirochetes per ml. The culture was incubated 15 to 30 minutes on ice and centrifuged 15 minutes at 6,000 x g at 4°C in a Beckman-Coulter Avanti J-20 XP centrifuge (Mississauga, ON, CA). Supernatant fluid was discarded, pelleted cells were resuspended on ice in 30 ml of ice-cold $dPBS^{2+}$ (4.3 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, 1.4 mM KH_2PO_4, 1 M $CaCl_2$, 4 M $MgCl_2 \cdot 6H_2O$, pH 7.4) and centrifuged 12 minutes at 6,000 x g at 4°C. Supernatant fluid was discarded, cells were resuspended a second time on ice in 20 ml of ice-cold EPS (0.27 M sucrose, 15% glycerol) and centrifuged 12 minutes at 6,000 x g at 4°C. Pelleted cells were resuspended on ice in 1.8 ml of ice-cold EPS, transferred in a 2.0 ml plastic tube and centrifuged 12 minutes at 6,000 x g at 4°C in an Eppendorf 5415R centrifuge (Mississauga, ON, CA). Supernatant fluid was removed and cells were resuspended on ice in 100 to 300 µl EPS. To measure the optical density at 600 nm, 35 µl of competent cells were diluted in 750 µl of EPS. The optical density of the diluted sample was measured and the final volume of competent cells was adjusted to reach an optical density of about 0.36 at 600 nm. Aliquots of 50 µl were kept at -80°C.

To transform electrocompetent *B. burgdorferi*, competent cells were incubated on ice for 15 minutes with 50 µg of DNA and electroporated in a 2 mm gap cuvette at 200 Ω, 25 µF and 2.5 kV. Immediately following electroporation,

cells were diluted in 10 ml of pre-warmed culture medium and incubated 20 to 24 hours at 35°C for recovery. Following incubation, the culture was further diluted in 100 ml of culture medium containing the appropriate antibiotic for selection and plated on 96 well plates (250 µl/well). Plates were incubated at 35°C for 8 to 12 days, until wells containing possible clones start changing color, passing from red to yellow, which indicated growth. Each possible clone was diluted 1:100 in 5 ml of culture media containing the appropriate antibiotic and incubated at 35°C until the culture became yellow. Clones were analyzed by PCR and sequencing using *B. burgdorferi* genomic DNA (gDNA).

2.2.4 Extraction of *B. burgdorferi* genomic DNA

The *B. burgdorferi* culture was centrifuged for 15 minutes at 6,000 x g at 4°C and the supernatant fluid was discarded. Cells were resuspended in one ml of 1x PBS (Invitrogen, Burlington, ON, CA) and transferred to a 1.5 ml microcentrifuge tube. Cells were centrifuge 5 minutes at 16,110 x g at 4°C in a Eppendorf 5415R centrifuge (Mississauga, ON, CA) and the supernatant fluid was discarded. Pelleted cells were resuspended in 170 µl of buffer (100 mM Tris-HCl pH 8.5, 10 mM EDTA, 30 mM NaCl). Once the pellet was well resuspended, 8.5 µl of 10% SDS and 12 µg of RNaseA (New England BioLabs, Pickering, ON, CA) was added and the sample was incubated 30 minutes at room temperature. Following incubation, 15 µg of proteinase K (New England BioLabs, Pickering, ON, CA) was added and the sample was incubated either four hours or overnight at 37°C. DNA was then extracted with 200 µl of phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma-Aldrich, Oakville, ON, CA). The sample was incubated

20 minutes at room temperature with inversion and centrifuged one minute at 16,110 x g in a Eppendorf Microcentrifuge 5415D (Mississauga, ON, CA). The upper phase was then transferred in fresh phenol:chloroform:isoamyl alcohol and incubated 20 minutes with inversion. Following a second one minute centrifugation, the upper phase was transferred to a new 1.5 ml microcentrifuge tube containing one ml of isopropanol (Sigma-Aldrich, Oakville, ON, CA), mixed by inversion and centrifuged for 20 minutes at 16,110 x g. Supernatant fluid was discarded, and the pellet washed with 600 µl of 70% ethanol and centrifuged 10 minutes at 16,110 x g. The supernatant fluid was discarded and the dried pellet was resuspended in TE buffer (10 mM Tris-HCl pH 7.4, 1 mM EDTA), incubated for one hour at 50°C, followed by one hour at 37°C and overnight at 4°C. All genomic DNA samples were stored at 4°C.

2.2.5 Southern blot

For Southern blotting, approximately 600 ng of *B. burgdorferi* genomic DNA was digested with 20 units of HindIII (New England BioLabs, Pickering, ON, Canada) for 3 hours at 37°C. The DNA was then separated by electrophoresis in a 10 cm long, 1% agarose gel for 90 minutes at 80 V in 1x RML TAE buffer (0.4 M Tris, 1.14% glacial acetic acid, 0.01 M Na_4EDTA). DNA was stained for 30 minutes with ethidium bromide (0.5 µg/ml), visualized under UV light using a FluorChem 8900 imager and AlphaEaseFC software (Alpha Innotech). After documentation, the gel was incubated for 45 minutes in depurination solution (1% HCl), followed by two 15 minutes incubations in denaturation solution (1.5 M NaCl, 0.5 M NaOH) and a last 15 minutes incubation in renaturation solution (1 M

Tris-HCl pH 7.4, 1.5 M NaCl). Between each incubation, the gel was washed for 2 minutes with distilled H_2O. The DNA was then transferred to nucleic acid transfer nylon membranes (Hybond-N+, Amersham, GE Healthcare Life Sciences, Baie d'Urfe, QC, CA) overnight. For the transfer assembly, the gel was covered with the nylon membrane, two pieces of Whatman 3MM paper, paper towels and a weight was added on the top. The gel, the membrane and the Whatman papers were soaked in 6x SSC (3 M NaCl, 5.7 M sodium citrate, pH 7.0) prior to the assembly. Alternatively, when a duplicate of the membrane was required, the gel was placed between two similar transfer settings. After overnight transfer, the DNA was crosslinked using UV Stratalinker 1800 (Stratagene, La Jolla, CA, USA), following instructions from the manufacturer.

The probe for the *PflgB*-driven gentamicin resistance cassette was first PCR amplified from the *B. burgdorferi* shuttle plasmid pBSV2G (Stewart *et al.* 2001) using B348 and B349 primers. The probe for the portion of the gene that was deleted in the KO strain was PCR amplified from *B. burgdorferi* gDNA using the primers designed to confirm the gene disruption by PCR. Each 100 µl PCR mix contained either 80 ng of *B. burgdorferi* genomic DNA or 5 ng of pBSV2G DNA, 2 units of Phusion high-fidelity DNA polymerase (New England Biolabs, Pickering, ON, Canada), 1x Phusion HF reaction buffer, 250 µM dNTPs and 0.25 µM each primer. The PCR program used was an initial denaturation of 2 minutes at 94°C, then 25 cycles of 30 seconds at 94°C, 30 seconds at 50°C, 1 minute at 50°C and at the last cycle, a final elongation step of 7 minutes at 68°C. The PCR product was purified by electrophoresis in a 1.2% agarose gel with 1x RML TAE

buffer and extracted from the gel using Qiaquick Gel Extraction kit (Qiagen, Toronto, ON, CA), following instructions from the manufacturer. A second PCR similar to the first one was done using 1 µl of purified PCR product from the first reaction as DNA template. This second PCR product was purified using Qiaquick PCR Purification kit (Qiagen, Toronto, ON, CA). To label the probe, 5 µl of purified PCR product was first added to 16 µl of ddH_2O, incubated 5 minutes at 100°C and then labelled with 50 µCi of [α-^{32}]dCTP (PerkinElmer, Woodbridge, ON, CA) using the Random Primers DNA Labeling System (Invitrogen, Burlington, ON, CA) according to the instructions from the manufacturer.

The membrane was incubated two and a half hours with rotation at 55°C in 10 ml of BLOTTO (6x SSC, 0.1% SDS, 0.5% non-fat dry milk, 1 mM NaPPi). The probe was added with fresh BLOTTO and incubated with the membrane overnight at 55°C with rotation. The membrane was then washed three time with 2x SSC, 0.1% SDS, followed by three washes with 0.2x SSC, 0.1% SDS. All washes were done at 55°C for 20 minutes. The membrane was then dried on Whatman 3MM paper, covered with Saran wrap and exposed to phosphor screen (PerkinElmer, Woodbridge, ON, CA) in a film cassette for various amount of time. An image was then acquired using Packard Cyclone Phosphoimager and analyzed using the OptiQuant software (PerkinElmer, Woodbridge, ON, CA).

2.2.6 Determining the plasmid profile of *B. burgdorferi*

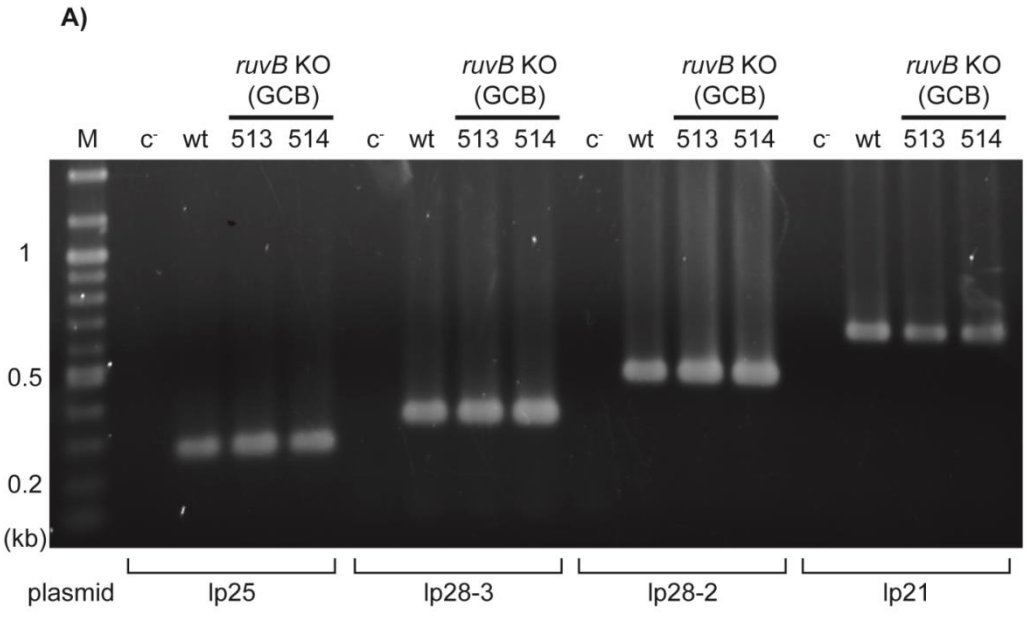
The plasmid content for each clone was established either by independent PCR for each plasmid, as described previously (Purser and Norris 2000; Tourand *et al.* 2006) or by multiplex PCR with minor modifications to the method recently

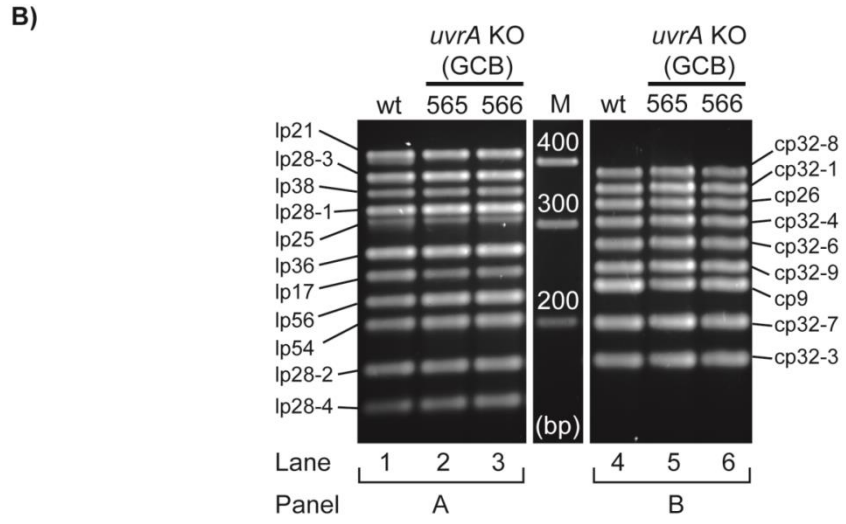
described (Bunikis *et al.* 2011) (see Figure 2.1). For the first PCR strategy, a separate PCR is prepared for each plasmid tested. The primers used are listed in Table 2.2. Each 20 µl PCR mix contained 80 ng of template *B. burgdorferi* gDNA, 1x ThermoPol buffer, 0.5 unit Taq DNA polymerase (New England BioLabs, Pickering, ON, CA), 200 µM dNTPs and 0.25 µM of each primer. The PCR program used was an initial 2 minute denaturation at 94°C, 25 cycles of 94°C for 30 seconds, 50°C for 30 seconds and 68°C for 1 minute, followed by a final 7 minutes extension at 68°C. The PCR products were analyzed by electrophoresis in a 1.2% agarose gel with 90 minutes migration at 80 V in 1x RML TAE buffer. The DNA was stained 30 minutes with ethidium bromide (0.5 µg/ml) and was visualized under UV light using FluorChem 8900 imager and AlphaEaseFC software (Alpha Innotech) (Figure 2.1A).

For the multiplex PCR, each reaction mix contained: 5 ng of *B. burgdorferi* genomic DNA, 1 unit Phusion high-fidelity DNA polymerase (New England Biolabs, Pickering, ON, Canada), 1x Phusion HF reaction buffer, 250 µM dNTPs and 1x primer mix in a 20 µl reaction. The sequence and the concentration of each primer in the 5x primer mix is listed in Table 2.3. The circular and the linear plasmids were amplified in separate reactions. The cycling conditions used were: initial 2 minutes denaturation at 95°C followed by 30 cycles of 30 seconds at 95°C, 60°C for 60 seconds, 68°C for 60 seconds and a final 5 minutes extension at 68°C at the last cycle. For each reaction, 5 µl of PCR products was analyzed by electrophoresis in a 3% Metaphor Agarose gel (Lonza, Allendale, NJ, USA)

Figure 2.1. Screen for plasmid profile in *B. burgdorferi*.

A) Example of a representative gel of a plasmid screen for *B. burgdorferi ruvB* mutants (*ruvB* KO) using a separate PCR for each plasmid in every clone to be characterized. PCRs were run in a 1.2% agarose gel, stained with ethidium bromide and DNA was visualized under UV light. For each plasmid, genomic DNA from wild-type *B. burgdorferi* B31 clone 5A4 (wt) was used as template for positive control and ddH_2O for negative control (c^-). (M) 100 bp DNA ladder was used. **B)** an example of a representative gel of a plasmid screen by multiplex PCR for *B. burgdorferi uvrA* mutants (*uvrA* KO – this construct is described in Chapter 4). For each clone to be tested, linear (Panel A) and circular (Panel B) plasmids were amplified in separate reactions. Each PCR was run on a 3% Metaphor Agarose gel, DNA was stained with GelRed and visualized under UV light. Genomic DNA from wild-type *B. burgdorferi* B31 clone 5A4 (wt) was used as template for positive control and ddH_2O for negative control. (M) 100 bp DNA ladder was used.


A)
ruvB KO (GCB)
M
c-
wt
513
514
1
0.5
0.2
(kb)
plasmid
lp25
lp28-3
lp28-2
lp21


B)
uvrA KO (GCB)
wt
565
566
M
400
300
200
(bp)
lp21
lp28-3
lp38
lp28-1
lp25
lp36
lp17
lp56
lp54
lp28-2
lp28-4
cp32-8
cp32-1
cp26
cp32-4
cp32-6
cp32-9
cp9
cp32-7
cp32-3
Lane
1
2
3
4
5
6
Panel
A
B

with 3 hours migration at 75 V in 1x RML TAE buffer. DNA was stained with 3x GelRed Nucleic Acid Gel Stain (Biotium, Hayward, CA, USA) for 30 minutes and visualized under UV light (Figure 2.1B).

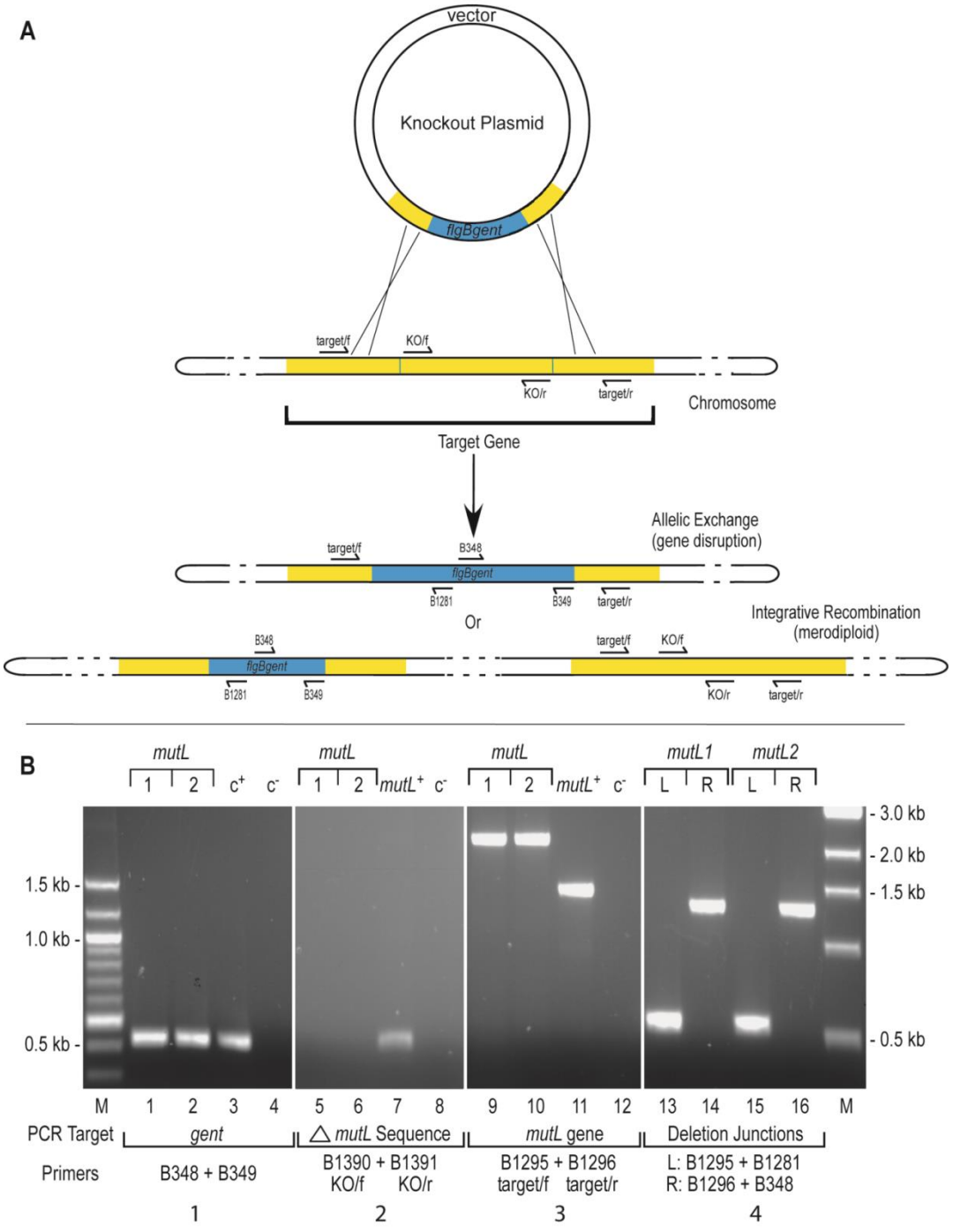
2.2.7 Gene disruption in *B. burgdorferi*

Except for *uvrD*, all *B. burgdorferi* gene targets were disrupted as described previously (see Figure 2.2) (Dresser *et al.* 2009). For each gene target, approximately 1.5 kb of the gene was amplified from *B. burgdorferi* B31 clone 5A4 (Purser and Norris 2000) gDNA using the primers listed in Table 2.1 and cloned in pJET1.2/blunt cloning vector (Fermentas, Burlington, ON, CA) or pCR BluntII-TOPO (Invitrogen, Burlington, ON, CA) following instructions from the manufacturer. The ligation product was transformed into chemically competent *E. coli* DH5α. The middle 500 bp of each target was then removed by inverse PCR using the primers listed in Table 2.1. All primers used for inverse PCR also contained an NheI restriction site that was used to ligate *PflgB-aacC1*, a *flgB*-driven gentamicin resistance cassette amplified from pBSV2G (Stewart *et al.* 2001), in the construct. In some cases, a T7 transcriptional terminator (5′ CTG CTA ACA AAG CCC GAA AGG AAG CTG AGT TGG CTG CTG CCA CCG CTG AGC AAT AAC TAG CA TAA CCC CTT GGG GCC TCT AAA CGG GTC TTG AGG GGT TTT TTG 3′), from pGEM-T easy vector (Promega), was also fused to the gentamicin resistance cassette. This was constructed using overlap extension PCR by Ashley R. Dresser (Dresser *et al.* 2009). The resulting plasmid was used to transform *B. burgdorferi* B31 clone 5A4 by electroporation (Bono *et al.* 2000; Samuels 1995). In order to ensure recovery of independent clones for

Figure 2.2. Gene disruption and confirmation.

A) Gene disruption strategy. The infectious *B. burgdorferi* strain B31, clone 5A4 (B31-5A4) was transformed with a knockout plasmid carrying a one kb gentamicin resistance cassette (blue) that replaced the central portion of the target gene (yellow) as described in Materials and Methods. The two possible outcomes of recombination events with the target gene are shown: allelic exchange would result in gene disruption while integrative recombination of the knockout plasmid would result in merodiploid formation. The positions of PCR primers used for construct verification are shown by arrows on the schematic. **B)** Construct verification of the *mutL* disruption by PCR is shown as an example. Each gene disruption was subjected to four PCR analyses. **1)** The presence of the gentamicin resistance cassette was confirmed as shown in lanes 1 and 2. The shuttle vector pBSV2G (Stewart *et al.* 2001) served as the positive control c^+ for amplification of the *gent* cassette (lane 3.) **2)** The portion of *mutL* expected to be deleted in a gene disruption was not detected in either *mutL*1 or 2 (lanes 5 and 6); however, it was detected in the positive control (*mutL*$^+$), which contained wild-type B31-5A4 DNA as a template in lane 7. **3)** The size of the target gene was compared in *mutL*1 and 2 genotypes. The expected 2.1-kb products for a gene disruption were observed (lanes 9 and 10) in comparison to the 1.5-kb product from the *mutL*$^+$ genotype (lane 11). Lanes 4, 8 and 12 are negative controls (c^-) that lacked DNA template. **4)** Confirmation of the correct insertion site was performed using combinations of the target gene primers and primers internal to the gentamicin resistance cassette to amplify the boundaries. The left

boundary in both *mutL* clones gave the expected 0.55-kb product (lanes 13 and 15). The right boundary in both clones gave the expected product of approximately 1.3 kb (lanes 14 and 16). A 100-bp ladder on the left side, relevant to the two left panels, and a 1-kb ladder on the right side, which applies to the two right panels, were the molecular weight markers (M) used. doi:10.1371/journal.ppat.1000680.g001. Figure and legend reproduced from (Dresser *et al.* 2009).


A
vector
Knockout Plasmid
flgBgent
target/f
KO/f
KO/r
target/r
Chromosome
Target Gene
Allelic Exchange
(gene disruption)
B348
B1281
B349
Or
Integrative Recombination
(merodiploid)
B
mutL
mutL1
mutL2
c+
c-
mutL+
L
R
1.5 kb
1.0 kb
0.5 kb
3.0 kb
2.0 kb
1.5 kb
0.5 kb
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 M
PCR Target
gent
△ mutL Sequence
mutL gene
Deletion Junctions
Primers
B348 + B349
B1390 + B1391
KO/f KO/r
B1295 + B1296
target/f target/r
L: B1295 + B1281
R: B1296 + B348
1
2
3
4

uvrD mutants, immediately following electroporation of *B. burgdorferi*, the culture was resuspended in 10 ml of pre-warmed culture media and separated into five aliquots. After 20 to 24 hours incubation at 35°C, each aliquot was diluted 1:12 into fresh culture media containing 100 µg/ml gentamicin and plated on a 96 well plate (250 µl/well). Each independent clone used for subsequent experiments was recovered from a separate plate.

Allelic exchange was confirmed by PCR for the replacement of the middle of the targeted gene by the gentamicin resistance cassette, the change in size of the targeted gene and the insertion junctions between the gentamicin resistance cassette and the gene sequence remaining. Gene deletion was also confirmed by a Southern blot and the plasmid content of each confirmed clone was established (see Table 2.5 and Figure 2.3).

2.2.8 Mouse infection studies

All animal studies were carried out in accordance with the principles outlined in the most recent policies and *Guide to the Care and Use of Experimental Animals* by The Canadian Council on Animal Care. Our animal protocol (AC12-0070) was approved by The Animal Care Committee of the University of Calgary.

Each three to four week old male C3H/HeNCrl mouse or C3H.C-*Prkdc*^{SCID}/IcrSmnHsd (SCID) (Charles River, St-Constant, QC or Harlan, Indianapolis, IN) was infected by subcutaneous and intraperitoneal injections of 10^4 spirochetes at each site. Mice were infected and 7 days post-infection approximately 50 µl of blood was recovered from the saphenous vein and diluted

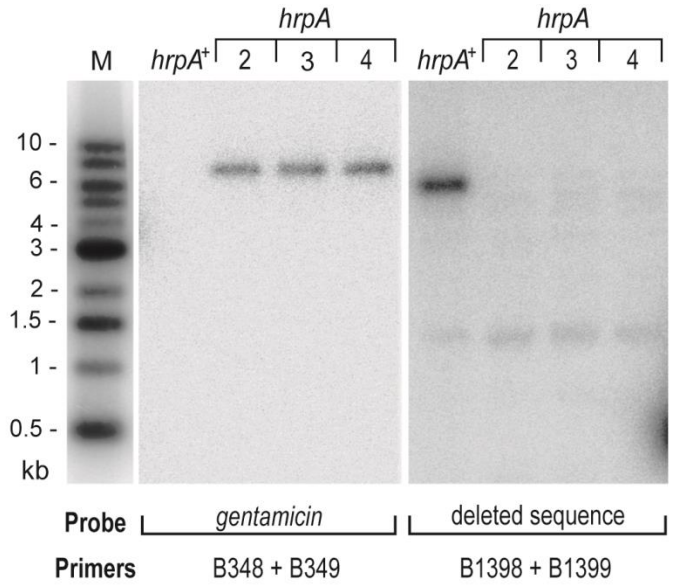


Figure 2.3. Gene disruption and the absence of additional copies of the trageted gene was confirmed by Southern hybridization.

Representative result using *B. burgdorferi hrpA* mutants as example. Genomic DNA was digested with HindIII and run on a 1.0% agarose gel with a 1-kb molecular weight ladder (M). Probes complementary to the gentamicin (*gent*) resistance cassette (left panel) and the portion of the *hrpA* gene deleted during gene disruption (right panel) were used for hybridization to duplicate blots. As expected, hybridization to the *gent* probe was not observed in the wild-type strain but was observed at the expected size (7.1 kb) for the three *hrpA* mutant strains. Conversely, hybridization to the deleted portion of *hrpA* was observed in the expected 6.5-kb fragment in the wild-type strain but not in the three *hrpA* mutant clones.

into 1.7 ml of culture media for the presence of *B. burgdorferi*. On days 14, 21 and 28 two ear punches were taken and on day 35, the heart, the ear, the bladder and the knee joint were collected and transferred into culture media for growth of spirochetes. Cultures were considered positive for *B. burgdorferi* when spirochetes could be observed by dark field microscopy.

2.2.9 Restriction fragment length polymorphism (RFLP) assay to detect switching at *vlsE*.

To detect switching, *vlsE* was amplified, either directly from *B. burgdorferi* culture or from genomic DNA, by PCR and then digested using HphI (New England BioLabs, Pickering, ON, CA) restriction endonuclease. Each 50 µl PCR mix contained either 1 µl of *B. burgdorferi* culture or 80 ng of *B. burgdorferi* gDNA, 1 unit Phusion high-fidelity DNA polymerase (New England Biolabs, Pickering, ON, Canada), 1x Phusion HF reaction buffer, 200 µM dNTPs and 0.5 µM each primer (B248 and B249). The PCR conditions used were: a 98°C for 2 minutes initial denaturation, 28 cycles of 10 seconds at 98°C and 30 seconds at 72°C, followed by a 5 minutes at 72°C final extension during the last cycle. The PCR product was purified using Qiaquick PCR purification kit following instructions from the manufacturer. Purified DNA was either left undigested or incubated with HphI for 3 hours at 37°C. Each 20 µl reaction mix contained 1x NEB reaction product was then analysed using electrophoresis in a 1.2% agarose gel migrated for 90 minutes at 80 V in 1x RML TAE buffer. DNA was stained with ethidium bromide and visualized under UV light.

2.2.10 Growth curves of *B. burgdorferi mutS* and *ruvB* mutants.

For each strain, an exponentially growing culture was diluted to a cell density of 10^6 spirochetes per ml. The cell density of each culture was determined by direct counting every 24 hours until the cultures reached stationary phase. Experiments were done in triplicate.

2.3 Results

2.3.1 Generation of mutants for DNA recombination, repair and replication genes in *B. burgdorferi*.

Genes were disrupted in *B. burgdorferi* by allelic exchange. A schematic representation of the strategy used is shown in Figure 2.2A. To disrupt *B. burgdorferi* genes, a knockout construct was first generated in *E. coli* and then used to transform *B. burgdorferi*. To generate this construct, approximately 1.5 kb of the region containing the targeted gene was first amplified by PCR from *B. burgdorferi* genomic DNA and cloned in either pJET1.2/Blunt or TOPOII. Approximately 500 bp of the center part of the targeted gene was then deleted by inverse PCR, using primers oriented outward, and replaced with a *PflgB*-driven gentamicin resistance cassette (*PflgB-aacC1*) by cloning. In order to reduce the risk of polar effect on transcription of surrounding genes, a *PflgB-aacC1* cassette fused to a T7 transcriptional terminator was used for some constructs (Table 2.1). However, this T7 terminator was later shown to not be effective in terminating transcription in *B. burgdorferi* (Samuels 2009). The resulting knockout construct was used to transform *B. burgdorferi* B31 clone 5A4 (Purser

and Norris 2000) and transformed samples were grown in the presence of gentamicin for selection. Disruption of the gene by allelic exchange in *B. burgdorferi* was confirmed by a series of PCRs (see Figure 2.2B). A first PCR to confirm the presence of *PflgB-aacC1* (Panel 1) and to verify the deletion of the middle of the target confirmed the disruption of the gene and the absence of a second copy of the gene (merodiploid) that would have resulted from an integrative recombination event. The insertion of the gentamicin resistance cassette and its orientation were then confirmed by amplifying the junctions between the target gene and the resistance cassette (Panel 4). In addition, the change in size of the targeted gene, resulting from the replacement of a part of the gene by the antibiotic resistance cassette, was verified. Finally, the allelic exchange event was confirmed by a Southern blot using probes for the deleted segment of the gene and for the gentamicin resistance cassette (Figure 2.3). For each target gene, at least two separate clones were confirmed.

This strategy was used to attempt to disrupt 14 DNA recombination, repair and replication gene targets in *B. burgdorferi* (Table 2.4) and mutants for seven of these gene targets (*uvrC*, *uvrD*, *dnaK1*, *exoA*, *ruvB*, *mutS1* and *nth*) could be recovered Table 2.5. These *B. burgdorferi* mutants are in addition to 17 gene disruption mutants generated by Ashley R. Dresser, including *recA*, *ruvA mutH*, *sbcC* and *priA* (Dresser 2009; Dresser *et al.* 2009).

2.3.2 Infectivity and persistence of *B. burgdorferi* mutants in mice

Since switching at *vlsE* only occurs during the infection of a vertebrate host and cannot be induced in culture, mice were infected with each *B.*

burgdorferi gene disruption mutant and samples were collected at various time points to establish the importance of each gene target for infectivity and persistence of *B. burgdorferi* and for switching at *vlsE*. To confirm that each clone contained the plasmids known to be essential for *B. burgdorferi* infectivity and persistence in mice (Labandeira-Rey *et al.* 2003; Labandeira-Rey and Skare 2001; Purser and Norris 2000), the plasmid content of each *B. burgdorferi* clone was determined prior to mouse infection (see Table 2.5 and Figure 2.3).

For each gene target, two separate clones were used to infect C3H/HeN mice, in which the kinetics of *B. burgdorferi* infection and dissemination have been well characterised (Coutte *et al.* 2009; Labandeira-Rey and Skare 2001; Purser and Norris 2000; Zhang and Norris 1998b). Infectivity was confirmed when spirochetes could be cultured from blood collected one week after the infection. At 21 days post-infection, ear punches were collected and persistence of *B. burgdorferi* was confirmed by the presence of spirochetes in the culture of the samples. If switching could not be verified from the samples recovered 21 days post-infection, the heart, the bladder, one ear and one joint were collected 35 days post-infection and cultured for spirochetes. Switching at *vlsE* was first verified by RFLP assay (Figure 2.4) using a PCR product from day 21 or 35 culture (Bankhead and Chaconas 2007; Ohnishi *et al.* 2003). This assay is based on testing for the generation or disruption of restriction sites by modifications in the DNA sequence. The digested product was then analysed on an agarose gel and the migration pattern of each sample was compared.

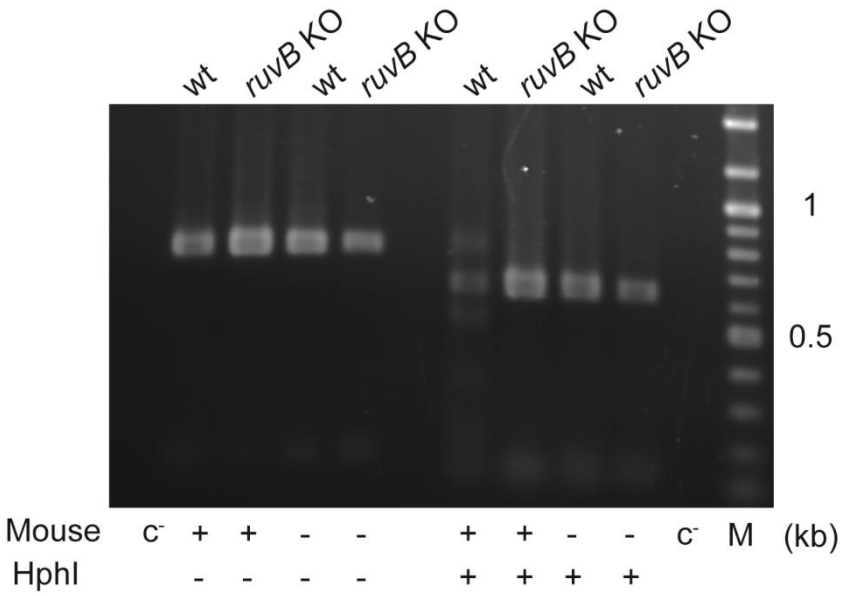


Figure 2.4. RFLP assay for switching at *vlsE* in *B. burgdorferi*.

The *B. burgdorferi vlsE* cassette was PCR amplified from the culture containing the joint tissue recovered from mice 35 days post infection from mice (+). The mice were infected with either wild-type (wt) or *ruvB* mutant (*ruvB* KO) *B. burgdorferi*. The *vlsE* cassette was also amplified from each strain, cultured *in vitro* only, as a control for switching (-). Each PCR product was then either digested with HphI (+) or left undigested (-) and run in a 1.2% agarose gel. ddH_2O was used as PCR template as negative control (c^-) and the 100-bp marker was used as DNA ladder (M).

Disruption of *nth*, *exoA*, *uvrC* and *dnaK1* did not affect *B. burgdorferi* infectivity, persistence and switching at *vlsE* in mice when 10^4 spirochetes were used for infection (Table 2.6). Indeed, similar to mice infected with wild-type *B. burgdorferi*, spirochetes were grown from all samples collected at each time point. However, the *B. burgdorferi mutS1* mutants displayed a less consistent infectivity phenotype. Whereas disruption of *mutS1* appeared to decrease the infectivity of one *B. burgdorferi* clone (1/3 mice was infected), a second clone was fully infectious, similarly to wild-type parental strain. Moreover, the attenuated *B. burgdorferi mutS* clone did not show a slower growth rate in culture (Figure 2.5A) and using a 10-fold higher infectious dose (10^5 spirochetes) did result in an infectivity and persistence phenotype similar to wild-type *B. burgdorferi* (Table 2.6). Despite a different level of infectivity, RFLP assay using samples recovered 21 days post-infection showed level of switching similar to wild type in both clones. Disruption of *B. burgdorferi ruvA* and *ruvB* did not affect the growth of *B. burgdorferi* in culture (see Figure 2.5B for growth curves of *ruvB* mutants) but resulted in a different dynamic of infection in wild-type mice. Similar to mice infected with wild-type *B. burgdorferi*, spirochetes were present in the blood collected at week one from all four mice infected with either *ruvA* or *ruvB B. burgdorferi* mutants (Table 2.7). However, no spirochetes could be grown from ear punches collected 21 days post-infection. Moreover, spirochetes were recovered only from the bladder and the joint of one mouse infected with *ruvB* mutant and from 25% of the organs recovered from mice infected with *ruvA*. This almost complete lack of persistence past the one-week infection time point is

Table 2.6. Infectivity and switching at *vlsE* in *B. burgdorferi* mutants during infection of C3H/HeN wild-type mice. Group I

B. burgdorferi genotype	*Bb* strain number (GCB)	Day 7 Blood	Day 7 Infection	Day 21 Ear	Day 21 Infection	Switching at *vlsE* day 21
5A4 wt	920	18/18	100.0%	18/18	100.0%	+
mutS1	502	1/3	66.7%	1/3	66.7%	+
	504	3/3		3/3		+
mutS1*	502	3/3	100.0%	3/3	100.0%	+
uvrC	537	2/2	100.0%	2/2	100.0%	+
	538	2/2		2/2		
uvrD	541	2/2	100.0%	2/2	100.0%	+
	542	2/2		2/2		
dnaK1	539	2/2	100.0%	2/2	100.0%	+
	540	2/2		2/2		
nth	525	2/2	100.0%	2/2	100.0%	+
	526	2/2		2/2		
exoA	531	2/2	100.0%	2/2	100.0%	+
	534	2/2		2/2		

* Mice were infected with 2 x 10^5 spirochetes instead of 2 x 10^4.

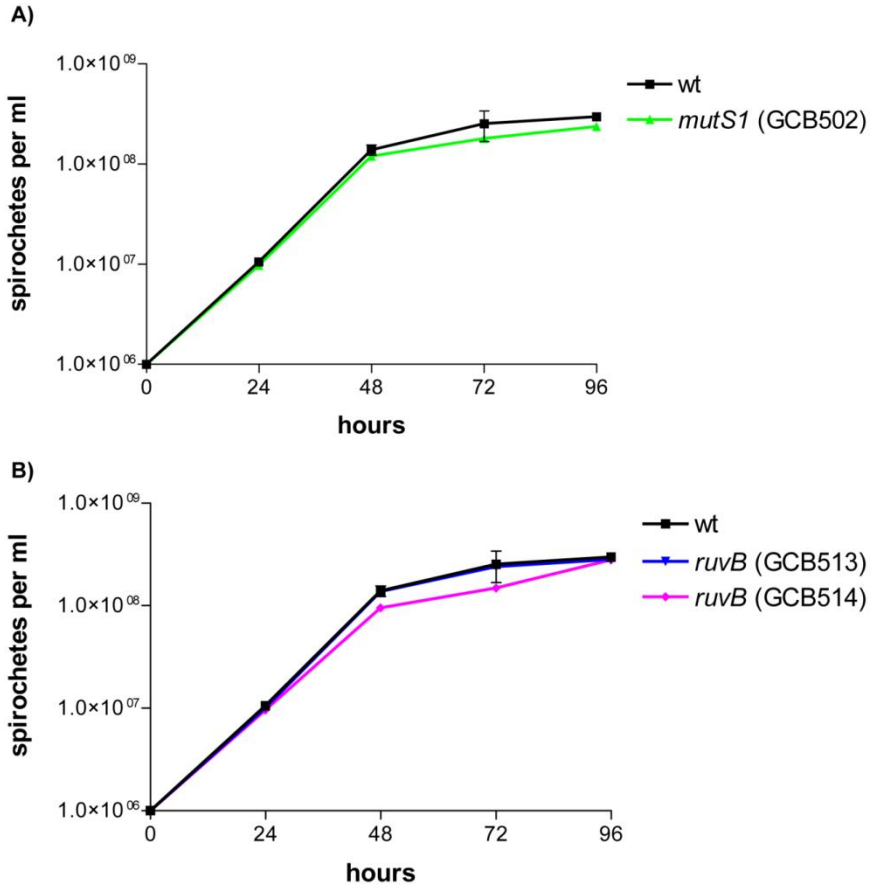


Figure 2.5. Growth curves of *B. burgdorferi mutS* and *ruvB* mutants.

For each strain, an exponentially growing culture was diluted to a cell density of 10^6 spirochetes per ml. The cell density of each culture was determined by direct counting every 24 hours until the cultures reached stationary phase. Experiments were done in triplicate.

Table 2.7. Infectivity and switching at *vlsE* in *B. burgdorferi* mutants during infection of C3H/HeN wild-type mice. Group II

B. burgdorferi genotype	*Bb* strain number (GCB)	Day 7 Blood	Day 7 Infection	Day 21 Ear	Day 21 Infection	Day 35					Persistence at day 35	Switching at *vlsE* day 35
						Heart	Bladder	Joint	Ear	Total sites		
wt	920	6/6	100.0%	6/6	100.0%	6/6	6/6	6/6	6/6	24/24	100.0%	+
ruvB1	513	2/2	100.0%	0/2	0%	0/2	0/2	0/2	0/2	0/8	12.5%	–
ruvB2	514	2/2		0/2		0/2	1/2	1/2	0/2	2/8		
ruvA1	1174	2/2	100.0%	0/2	0%	0/2	1/2	0/2	1/2	2/8	25.0%	–
ruvA2	1175	2/2		0/2		1/2	0/2	1/2	0/2	2/8		

similar to the phenotype observed in *B. burgdorferi* strains where *vlsE* is absent or cannot switch (Bankhead and Chaconas 2007; Lawrenz *et al.* 2004). When *vlsE* was amplified from day 35 cultures and digested for RLFP assay, no differences were observed between a control negative for switching and the *ruvA* and the *ruvB* mutants, compared to mice infected with wild-type *B. burgdorferi*, spirochetes were present in the blood samples from mice infected with *B. burgdorferi ruvA* and *ruvB* knockouts (Figure 2.4). These results were also confirmed by Lin *et al.* (Lin *et al.* 2009) using transposon mutagenesis in *B. burgdorferi*.

To confirm that the lack of persistence of *B. burgdorferi ruvA* and *ruvB* mutants in wild-type mice was due to the immune response, SCID mice were infected and samples were collected 7, 21 and 35 days post-infection and incubated for the presence of spirochetes. As previously reported for strains of *B. burgdorferi* that either lacked *vlsE* and the *vls* silent cassettes (Bankhead and Chaconas 2007) or that had lost the complete lp28-1 plasmid (Lawrenz *et al.* 2004), but where *vlsE* was complemented using a shuttle plasmid, spirochetes were grown from all samples recovered at each time point for *B. burgdorferi ruvA* and *ruvB* mutants (Table 2.8). However, no switching at *vlsE* could be observed by RFLP in samples recovered 35 days post-infection from mice infected with *ruvA* or *ruvB* mutants.

To confirm the absence of switching at *vlsE* in *B. burgdorferi ruvB* mutant, *vlsE* was amplified by PCR from the cultures containing the organs collected from SCID mice 35 days post-infection. The PCR products from each organ from

Table 2.8. Infectivity and switching at *vlsE* in *B. burgdorferi* mutants during infection of SCID C3H/HeN mice.

B. burgdorferi genotype	*Bb* strain number (GCB)	Day 7 Blood	Day 7 Infection	Day 21 Ear	Day 21 Infection	Day 35					Persistence at day 35	Switching at *vlsE* day 35
						Heart	Bladder	Joint	Ear	Total sites		
wt	920	6/6	100.0%	6/6	100.0%	6/6	6/6	6/6	6/6	24/24	100.0%	+
ruvB1	513	2/2	100.0%	2/2	100.0%	2/2	2/2	2/2	2/2	8/8	100.0%	–
ruvB2	514	2/2		2/2		2/2	2/2	2/2	2/2	8/8		
ruvA1	1174	2/2	100.0%	2/2	100.0%	2/2	2/2	2/2	2/2	8/8	100.0%	–
ruvA2	1175	2/2		2/2		2/2	2/2	2/2	2/2	8/8		

all four mice were then combined and cloned in *E. coli*. For each organ (heart, joint, bladder and ear), 10 clones were sent for sequencing of the *vlsE* center cassette and the sequences were compared to the unswitched *vlsE* and the *vls* silent cassettes. From mice infected with the wild-type strain, the *vlsE* sequence of all the clones from the heart and bladder contained sequences found only in the *vls* silent cassettes (**Erreur ! Référence non valide pour un signet.**). For the ear, 8/10 and 5/10 for the joint tissues contained switched sequence. Compared to the wild-type strain, all 40 clones from mice infected with the *B. burgdorferi ruvB* knockout contained only unswitched *vlsE* sequence and one clone of *ruvA* contained switched sequence.

2.4 Discussion

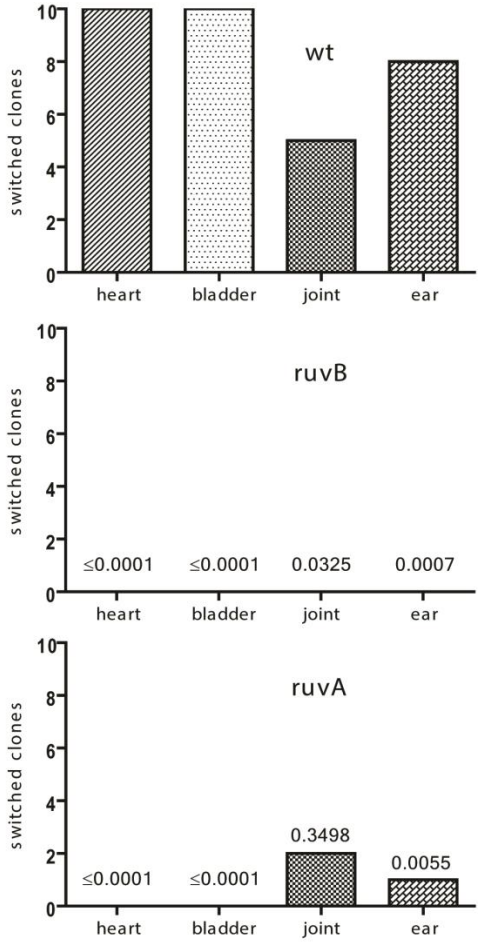
To identify genes required for switching at *vlsE* in *B. burgdorferi*, I disrupted eight gene targets in *B. burgdorferi* and verified their importance for switching at *vlsE* during mice infection. The present study was part of a wider project in collaboration with Ashley R. Dresser where a total of 28 genes have been disrupted (Dresser 2009; Dresser *et al.* 2009) and unpublished data).

Since switching at *vlsE* in *B. burgdorferi* only occurs during vertebrate host infection (Indest *et al.* 2001; Ohnishi *et al.* 2003; Zhang and Norris 1998b), each mutant was used to infect mice and switching was then verified by RFLP using spirochetes cultured from collected tissues. Although not quantitative, RFLP was used as an expeditious strategy for the initial screen to identify the mutants with a

level of switching at *vlsE* similar to wild-type *B. burgdorferi*. By combining RFLP with the kinetics of dissemination and persistence of each clone in the different

Figure 2.6. Number of switched *vlsE* clones in SCID C3H/HeN mice.

Sequencing of the cloned PCR product of the *vlsE* variable regions using primer pJET1.2/forward was performed on 10 clones from each tissue type culture for each genotype. The y-axis denotes the number of clones out of ten that contained templated nucleotide changes in variable regions 1–6 (switches) and the x-axis denotes the tissue type. The P-values above the bars indicate the level of significance of the difference between the wild-type and mutant samples, calculated using Fisher's Exact test. doi:10.1371/journal.ppat.1000680.g003. This experiment and figure were done by Ashley R. Dresser.


wt
switched clones
10
8
6
4
2
0
heart
bladder
joint
ear
ruvB
switched clones
≤0.0001
≤0.0001
0.0325
0.0007
heart
bladder
joint
ear
ruvA
switched clones
0.3498
0.0055
≤0.0001
≤0.0001
heart
bladder
joint
ear

organs, disruption of *dnaK1*, *nth* and *exoA* was shown to not affect the switching and the persistence of *B. burgdorferi*.

One clone of two *mutS1* knockouts appeared less infectious, however, the level of switching in both clones, as determined by RFLP, was similar to wild-type *B. burgdorferi*. Previous studies showed that switching at *vlsE* is only essential for persistence of *B. burgdorferi*. It does not affect the establishment of the initial infection, which is determined by the culture of spirochetes one week post-infection (Labandeira-Rey and Skare 2001; Lawrenz *et al.* 2004; Purser and Norris 2000). This *mutS1* knockout mutant was attenuated from day 7 post-infection but did persist and could be cultured from 2 mm ear punches on day 21 post-infection, which strongly suggests that the phenotype observed in one *mutS1* knockout clone resulted from a deficiency in some gene other than one required for switching at *vlsE*.

As opposed to *mutS1*, disruption of *ruvA* and *ruvB* significantly decreased the level of persistence of *B. burgdorferi* but without affecting the initial infectivity of the spirochetes. Although spirochetes could still be cultured from some organs recovered 35 days post-infection, no switching was detected by RFLP. Moreover, the persistence of both *B. burgdorferi* mutants in SCID mice was similar to the wild-type strain. Our results strongly suggest that the decrease in persistence was the result of the adaptive immune response in the host, which is consistent with absence of switching at *vlsE*.

A previous study reported that complementation of *vlsE* in a *B. burgdorferi* strain where *vlsE* and the *vls* silent cassettes had been deleted from lp28-1,

resulted in the partial clearance of *B. burgdorferi* 14 days post-infection and no spirochetes could be cultured at 35 days (Bankhead and Chaconas 2007). This suggests that the presence of the *vls* silent cassettes is required for the persistence of *B. burgdorferi*. In another study using a strain of *B. burgdorferi* that had lost the complete lp28-1 plasmid, Lawrenz *et al.* (Lawrenz *et al.* 2004) reported that spirochetes could still be recovered from the joint of one out of six mice eight weeks post-infection (Lawrenz *et al.* 2004). Since mice were infected with a significantly higher number of spirochetes in this second study, this might represent a delay in the complete clearance of the spirochetes due to the high initial number of bacteria. Spontaneous mutation in *vlsE* could also have resulted in this low level of persistence (Lin *et al.* 2009). Other studies reported a slower clearance of *B. burgdorferi* expressing parental VlsE from the joint and ear tissues (Bankhead and Chaconas 2007; Coutte *et al.* 2009; Labandeira-Rey *et al.* 2003; Labandeira-Rey and Skare 2001), suggesting that the spirochetes could be less exposed to the adaptive immune response in some tissues. In the present study, the persistence of spirochetes did not appear more important in one organ or another. Indeed, spirochetes could be cultured from all four organs tested when all mice tested are considered. However, a low level of persistence was observed similar to what was reported in the previous studies that used a *B. burgdorferi* strain expressing invariant VlsE. Our data show that the disruption of either *ruvA* or *ruvB*, strongly impair the persistence of *B. burgdorferi* in presence of an adaptive immune system.

While RFLP assay is very useful to distinguish between strains with a wild-type level of switching and strains with a reduced level of switching, it is not a quantitative technique. Moreover, switching can only be detected if the changes in the sequence generate new restriction sites. To better characterise switching in *B. burgdorferi ruvA* and *ruvB* mutants, *vlsE* from spirochetes cultured from SCID mice was sequenced. From the 80 sequences obtained, only one clone had changes in the sequence. This is in agreement with previous studies that showed that although switching occurs in SCID mice (Coutte *et al.* 2009; Crother *et al.* 2003), the absence or the expression of an invariant VlsE does not affect dissemination and persistence of *B. burgdorferi* (Bankhead and Chaconas 2007; Lawrenz *et al.* 2004). In parallel with our study, Lin *et al.* (Lin *et al.* 2009) used transposon mutagenesis in *B. burgdorferi* to identify genes required for swiching at *vlsE*. In agreement with our results, only the disruption of *ruvA* and *ruvB* also strongly impaired switching at *vlsE* during mice infection. Although in this study no switching was detected in the *B. burgdorferi ruvA* and *ruvB* mutants recovered from SCID mice, some low level of switching at *vlsE* was detected in samples collected from wild-type mice. This suggested that homologous recombination was still occurring in the absence of *B. burgdorferi ruvA* and *ruvB*, but at a significantly lower rate than in the wild-type parental strain. Although complementation of *ruvA* and *ruvB* in *B. burgdorferi* could not be achieved in both studies, the observation of the same phenotype using independent clones, a different strategy for the gene disruptions and the rescue of the mutants in SCID mice strongly suggest that the important decrease in switching at *vlsE* reported

does not result from a secondary mutation or a polar effect of the insertion. In *B. burgdorferi*, *ruvA* is part of an operon also containing *ruvB* and *queA* (Boursaux-Eude *et al.* 1998). Lin *et al.* used RT-PCR to show that *ruvB* and *queA*, located downstream of *ruvA* in the operon, are still being transcribed in the *B. burgdorferi ruvA* mutant.

Mechanisms involved in antigenic variation resulting from gene conversion are still not fully understood in any bacterium. The best-described system is the *pilS*/*pilE* pilin antigenic variation in *Neisseria gonorrhoeae*. In this system, switching results from gene conversion events where the sequence from a silent *pilS* copy replaces part of the expressed *pilE* locus. In addition to undergoing antigenic variation, *N. gonorrhoeae* is also an obligate pathogen that lacks an SOS response, like *B. burgdorferi*. In *N. gonorrhoeae*, the disruption of 13 different genes has been shown to affect antigenic variation. Among these genes, our data and results reported in other studies (Dresser 2009; Dresser *et al.* 2009; Lin *et al.* 2009; Liveris *et al.* 2008) show that only the disruption of the *B. burgdorferi* branch migrase subunits *ruvA* and *ruvB* almost completely abolishes switching at *vlsE* (Table 2.9). During homologous recombination in *E. coli*, the RuvA and RuvB subunits are responsible for translocating the Holiday junction along the DNA (Dennis *et al.* 2004; Iwasaki *et al.* 1989; Parsons *et al.* 1992; Shiba *et al.* 1991; West and Connolly 1992). However, a RuvC endonuclease is required for resolving the junction to end the recombination event (Connolly *et al.* 1991; Iwasaki *et al.* 1991) and no *ruvC* ortholog could be found in the *B. burgdorferi* sequenced genome (Fraser *et al.* 1997). In *E. coli*,

Table 2.9. Genes involved in antigenic variation in *N. gonorrhoeae pilE* and *B. burgdorferi vlsE.*

Genes	*N. gonorrhoeae pilE*	*B. burgdorferi vlsE*
recA	+	-
recX	+	absent
recJ	+	-
recO	+	absent
recQ	+	absent
recR	+	absent
recG	+	-
mutS/L	+	-
rep	+	-
rdgC	+	absent
ruvC	+	absent
ruvA	+	+
ruvB	+	+

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DnaB (Kaplan and O'Donnell 2002), RecG (Lloyd and Sharples 1993) and helicase II (UvrD) (Carter *et al.* 2012) helicases have been shown to unwind Holliday junctions *in vitro*, suggesting that they could, at least partially, complement for the deletion of *ruvAB*. However, in presence of functional RuvAB, disruption of *uvrD* and *recG* (Dresser *et al.* 2009) did not affect switching in *B. burgdorferi*. If either *uvrD* or *recG* can partially complement the activity of the RuvAB branch migrase, a double knockout would probably be required to observe a phenotype. No *E. coli* nuclease other than RuvC has been identified as a possible Holliday junction resolvase (Zhang *et al.* 2010). In a previous study, the *E. coli* nuclease RusA showed Holliday junction resolvase activity (Mandal *et al.* 1993; Sharples *et al.* 1994). The *rus-1* locus was initially identified as a *ruv* suppressor mutant because this mutation suppressed the sensitivity of *E. coli ruv* mutants to UV light in a *recG*-dependent manner (Mandal *et al.* 1993). However, further studies showed that the *rusA* locus is part of a defective prophage sequence and a mutation or an insertion increasing its normal level of expression would be responsible for this suppressor phenotype (Mahdi *et al.* 1996). Such a mutation has previously been observed in *E. coli ruv* mutants (Mandal *et al.* 1993), but there is no evidence for this gene in the *B. burgdorferi* genome. However, a similar type of *ruv*-independent recombination resulting in the low level of switching observed in *B. burgdorferi ruvA* and *ruvB* mutants cannot be ruled out.

In *E. coli*, homologous recombination also involves the activity of RecA, which in addition to activating the SOS response in presence of DNA damage,

binds ssDNA and promotes strand exchange for recombination. In *N. gonorrhoeae*, *recA* is required for DNA damage repair, homologous recombination and switching at *pilE* (Koomey and Falkow 1987; Koomey *et al.* 1987). Moreover, expression of *N. gonorrhoeae recA* can complement homologous recombination in *E. coli recA* mutant (Stohl *et al.* 2002), showing functional similarities between the two orthologs (Stohl *et al.* 2011). A previous study showed similar cross-complementation activity when *B. burgdorferi recA* is expressed in *E. coli* lacking *recA* (Liveris *et al.* 2004). Interestingly, disruption of *B. burgdorferi recA* abolishes allelic exchange activity without affecting the infectivity, the persistence or the switching at *vlsE* in mice (Dresser *et al.* 2009; Liveris *et al.* 2008). This shows that the gene conversion involved in VlsE antigenic variation in *B. burgdorferi* is a *recA*-independent recombination event, involving the RuvAB branch migrase.

In conclusion, our data show that switching at *vlsE* in *B. burgdorferi* involves the branch migrase subunits *ruvA* and *ruvB*. Results obtained by a second group (Lin *et al.* 2009) also supported our conclusions. Moreover, we have shown an important difference between *B. burgdorferi* and *N. gonorrhoeae* pilin antigenic variation, the only other well-described bacterial gene conversion system. Among the 13 genes involved in *N. gonorrhoeae* antigenic variation, only the disruption of the *ruvA* and *ruvB* genes affected switching at *vlsE* in *B. burgdorferi* (Table 2.9).

Chapter Three: Role of the *B. burgdorferi* DEAH-box RNA helicase *hrpA* upon murine infection[*].

3.1 Introduction

As part of our investigation to identify genes involved in *B. burgdorferi* antigenic switching at *vlsE,* 25 gene targets predicted to be involved in nucleic acid metabolism were disrupted and the infectivity and persistence of each mutant was then confirmed using a murine infection model (Dresser 2009; Dresser *et al.* 2009). During this study, the *hrpA* RNA helicase knockout clones were found to be non-infectious and no spirochetes could be recovered from wild-type or SCID mice at any time point post-infection (Dresser 2009; Salman-Dilgimen *et al.* 2011). According to the *B. burgdorferi* annotated genome (Fraser *et al.* 1997), the *hrpA* gene is predicted to encode a DEAH-box RNA helicase.

Helicases are ubiquitous (Anantharaman *et al.* 2002) and are classified in six superfamilies based upon their sequence similarity (SF1-6) (Fairman-Williams *et al.* 2010; Gorbalenya and Koonin 1993; Jankowsky 2011; Singleton *et al.* 2007). Members of SF1 and SF2 contain two “RecA-like” helicase domains

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[*] The text, experiments and figures presented in this chapter were done by Pierre-Olivier Hardy, with the exception of the figures 3.1, 3.2 and 3.5 and the disruption of *B. burgdorferi hrpA*. The experiments reported in the figure 3.2, and the figure 3.5, were done by Aydan Salman-Dilgimen and the disruption of *hrpA* was done by Ashley R. Dresser. The infectivity results for the disrupted *hrpA* were previously published in Salman-Dilgimen A, Hardy P-O, Dresser AR, Chaconas G (2011) HrpA, a DEAH-Box RNA Helicase, Is Involved in Global Gene Regulation in the Lyme Disease Spirochete. PLoS ONE 6(7): e22168. doi:10.1371/journal.pone.0022168. The complementation of *hrpA* and the insertion of point mutations are included in Salman-Dilgimen A, Hardy P-O, Dresser AR, Chaconas G (2013) Manuscript in preparation.

linked in tandem (Caruthers and McKay 2002; Gorbalenya and Koonin 1993) and some have been shown to be functional as monomer (Brendza *et al.* 2005; Mechanic *et al.* 1999; Nanduri *et al.* 2002; Singleton *et al.* 2007; Zhang *et al.* 2006). SF3 to SF6 contain a single RecA-like domain and form a ring-shaped oligomer (Bujalowski *et al.* 1994; Chong *et al.* 2000; Finger and Richardson 1982; Ilyina *et al.* 1992; Sawaya *et al.* 1999; Sedman and Stenlund 1998). RNA helicases are RNA-dependent ATPases with RNA unwinding activity and, most are included in the SF2 family. This superfamily regroups enzymes that share seven conserved motifs in the RecA-like core domains and most RNA helicases in SF2 are included in one of the four DExD/H families (DEAD, DEAH, DECH and DExH (Ski2)), based upon the sequence of the conserved motif II (Jankowsky 2011) (see Figure 3.1). The motifs I and II are Walker A and B motifs, respectively, and are required for the ATPase activity (Linder *et al.* 1989; Walker *et al.* 1982). Mutations in both conserved motifs abolish the activity of the proteins (Koo *et al.* 2004; Schneider *et al.* 2002). Motif III is involved in linking the ATPase and helicase activities. Mutation in the motif III of *Saccharomyces cerevisiae* DEAH-box RNA helicase Prp22 results in the loss of RNA unwinding but not of ATPase activities (Schwer and Meszaros 2000). Motifs Ia, Ib, Ic, IV and V are involved in nucleic acid binding (Cordin *et al.* 2006; Gu and Rice 2010; Lin and Kim 1999; Schneider *et al.* 2004; Story *et al.* 2001) and motif VI has been shown to be required for ATPase activity (Pause and Sonenberg 1992).

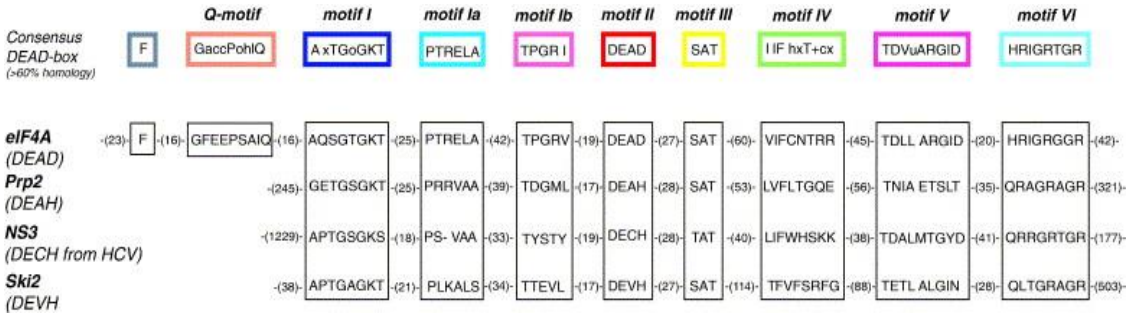


Figure 3.1. Conserved motifs in the DEAD-box and related DExD/H-box families.

Sequences of the conserved motifs from the yeast *S. cerevisiae* eIF4A (DEAD-box protein), Prp2 (DEAH-box protein), NS3 (DECH helicase from the hepatitis C virus) and Ski2 (DExH, Ski2 family). Symbols used are as follow: o: S,T;l: I, L, V;x: any residue;a: F, W, Y;c: D, E, H, K, R;h: A, F, G, I, L, M, P, V, W, Y;+: H, K, R;u: A, G. Reproduced with permission from Cordin *et al.* 2006.

In *E. coli*, HrpA is involved in processing the mRNA of the *daa* operon, which encodes F1845, a fimbrial adhesion. Point mutations in motifs I, II and VI significantly affect the activity of HrpA (Koo *et al.* 2004). In *B. burgdorferi* HrpA, ATPase and helicase activities were demonstrated *in vitro,* confirming that HrpA is an actual RNA helicase (Salman-Dilgimen *et al.* 2013). The disruption of *hrpA* was shown to significantly affect expression of a large number of proteins in *B. burgdorferi* as assessed by mass spectrometry iTRAQ analysis (Salman-Dilgimen *et al.* 2011). The current project consisted of verifying the importance of HrpA for *B. burgdorferi* murine infectivity. To achieve this, chromosomal manipulation of the *B. burgdorferi hrpA* mutant was performed to revert the mutation and complement the *hrpA* defect. This same approach was used to introduce several different point mutations in motifs II, III and V into the chromosome (see Figure 3.2) to test the importance of the ATPase and the RNA helicase activities of HrpA for infectivity and persistence in mice.

3.2 Materials and methods

3.2.1 Strains and primers used and growth conditions

All primers used are listed in Table 3.1 and were synthesized by the University of Calgary Core DNA services (Calgary, AB, CA). Plasmids and *E. coli* strains used in this study are listed in Table 3.2 and *B. burgdorferi* strains in Table 3.3. All *E. coli* and *B. burgdorferi* cultures and transformations were done as described in the Materials and methods section of Chapter 2.

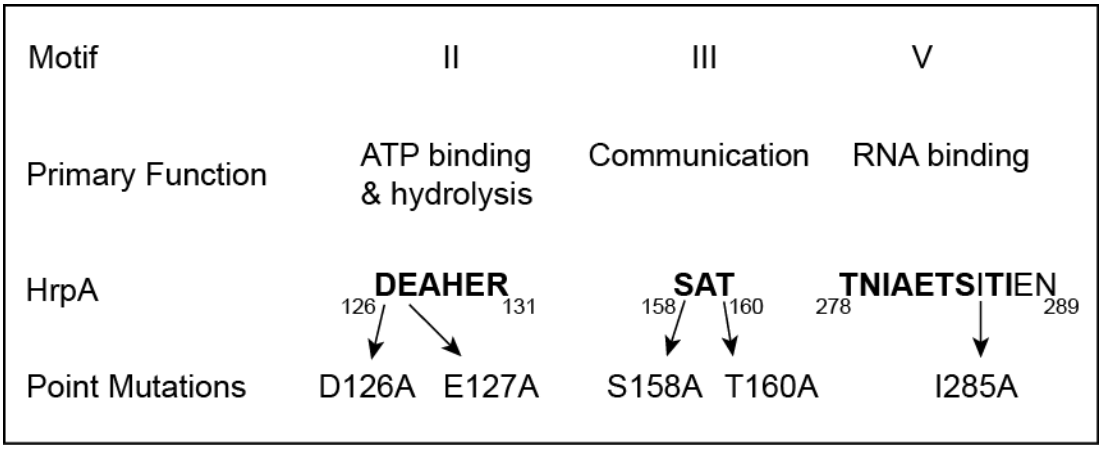


Figure 3.2. Conserved DEAH-box RNA helicase motifs in the *B. burgdorferi* HrpA protein used for point mutations.

Conserved sequence motifs II, III and V of DEAH box RNA helicases are shown, along with their primary functions. The numbers below the motif sequences represent the position of the conserved motifs in *Borrelia burgdorferi* HrpA. Amino acid residues that are conserved in the mentioned motifs are shown in bold and the residues mutated in this study are indicated.

3.2.2 Construction of *B. burgdorferi* knockouts

B. burgdorferi bb0826 gene target was disrupted as described previously (see Figure 3.3) (Dresser *et al.* 2009). Approximately 1.5 kb of the gene was amplified from *B. burgdorferi* B31 clone 5A4 (Purser and Norris 2000) and cloned in pCR BluntII-TOPO (Invitrogen, Burlington, ON, CA) following instructions from the manufacturer. The ligation product was transformed into chemically competent *E. coli* DH5α. The middle 500 bp of each target was then removed by inverse PCR. All primers used for inverse PCR also contains a NheI restriction site that was used to ligate *PflgB-aacC1*, a *flgB*-driven gentamicin resistance cassette amplified from pBSV2G (Stewart *et al.* 2001), in the construct. The resulting plasmid was used to transform *B. burgdorferi* B31 clone 5A4 by electroporation (Bono *et al.* 2000; Samuels 1995).

Allelic exchange was confirmed by PCR for the replacement of the middle of the targeted gene by the gentamicin resistance cassette, the change in size of the targeted gene and the insertion junctions between the gentamicin resistance cassette and the gene sequence left (Figure 3.3B). Gene deletion was also confirmed by Southern blot and the plasmid content of each confirmed clone was established (see Table 3.3). Southern blot and plasmid screen were done as described in Materials and methods section of Chapter 2.

3.2.3 RT-PCR analysis for the genes downstream of *hrpA*; *bb0825* and *bb0826*

The RT-PCR experiment was done by Aydan Salman-Dilgimen. *B. burgdorferi* cultures were harvested by centrifugation when they reached the

concentration of ~1×10^8 cells/ml in 10 ml BSK-II medium prepared in-house. RNA was extracted using an Aurum Total RNA Mini Kit as per manufacturer's instructions. RNA concentrations were determined using a NanoDrop spectrophotometer and the integrity of the RNA was assessed by agarose gel electrophoresis. cDNA was generated for *bb0825* and *bb0826* by The RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, Burlington, ON, CA) using gene specific primers (Table 3.1) according to manufacturer's instructions. For subsequent PCR reactions, 1 µl of cDNA was used as a template in 50 µl reactions with 10 pmoles of each primer. PCR were run for 25, 30 and 35 cycles to ensure that saturation had not been reached.

3.2.4 Complementation of *B. burgdorferi* knockout

Complementation of *hrpA* was achieved by allelic exchange (see Figure 3.6A in the results section). The *hrpA* ORF was PCR amplified from *B. burgdorferi* B31 clone 5A4 and fused in order with a *flgB*-driven kanamycin resistance cassette (*PflgB-kan*) and the 500-bp sequence downstream of *hrpA* by overlap extension PCR. The *PflgB-kan* gene was first amplified from pBSV2 (Stewart *et al.* 2001). For the overlap extension PCR, all primers were designed so approximately each half of the primer has a similar length, a melting temperature (Tm) around 60°C (calculated by adding 2°C for each A or T and 4°C for each G or C) and anneals on each target to be fused. For the first round of PCR, the *hrpA* and the *PflgB-kan* resistance cassette were amplified in separate reactions. Each 50 µl PCR mix contained either 80 ng of *B. burgdorferi* genomic DNA or 10 ng of pBSV2, 1 unit Phusion high-fidelity DNA polymerase

Table 3.1. Primers used in this study.

Primers used for *bb0826* knockout plasmid construction[1,2]	
Target PCR	B1845-GCATAAAAATATAACGGTTTTTAACA
	B1846-CCTATTATTGTTCTTGGGTGC
Inverse PCR	B1848-NheI-GCGCTAGCTAGCTAGGTTCATAATTTATTATAAACTTCATTGCTG
	B1849-NheI-GCGCTAGCTAGCTAGGATGTCAAAATAATTTATCAAATATTCAG
Knockout PCR	B1876-GGCTATCCTTTTTTTATGTTTTATT
	B1877-CTTCATTTATTCTTAATTTTAGCG

Primers used for *hrpA* complementation plasmid construction	
Annealing site	**Primers**[1,2]
hrpA + *PflgB-kan*	B1952-TTACAAAAAATTAAATTACTTTTTTTAAtacccgagcttcaaggaaga
between *hrpA* and *bb0826* + *PflgB-kan*	B1953-TCAAAGTTTAGTTTTTAAAAAAAGTAttagaaaaactcatcgagcatc
PflgB-kan + between *hrpA* and *bb0826*	B1954-gatgctcgatgagtttttctaaTACTTTTTTTAAAAACTAAACTTTGA
bb0826	B1955-EcoR1-CCGGAATTCCGGTTATTCATTTCTTTTTATTTTATAAGA

Primers used for screening *hrpA* mutants		
	Annealing site	**Primers**[3]
Point mutations in *hrpA*	D126A	B2176-CGTGTGCTTCG**g**
	E127A	B2173-TTCTTTCGTGTGCT**g**
	S158A	B2201-GTTTATTGTAGCAG**c**
hrpA complementation	*hrpA*	B1219-GTTATTTTTGTATTCCGCTTT
		B1220-TTCGGCTGCTACAATAAACAC
	Sequence deleted in *hrpA* mutant	B1398-TTAAAATCTTCAAAGATATTAACAA
		B1399-GCAGGAAGACTTTCAAAA
	PflgB-aacC1 (gent)	B348-CGCAGCAGCAACGATGTTAC
		B349-CTTGCACGTAGATCACATAAGC
	PflgB-kan	B70-CATATGAGCCATATTCAACGGGAAACG
		B71-AAAGCCGTTTCTGTAATGAAGGAG

Primers used for RT-PCR of *bb0825* and *bb0826*	
Purpose	**Primers**
bb0825 RT	B1762-CACTGCTAGTATTGATTTTAAG
bb0825 For	B1755-CTACGGAGAGATTGTAATAAAGC
bb0825 Rev	B1756-GACACCCCTTCCTTCTATTG
bb0826 RT	B1763-GCTACATATGCTTTATGATTTAACC
bb0826 For	B1757-GACTTAGAACAACATTATCTGCTAATTAC
bb0826 Rev	B1758-GAATAAATGCTTTTGGGATAGTTTG

[1] Upper and lower case letters indicate sequence from *B. burgdorferi* genome and *PflgB-kan* resistance cassette, respectively.

[2] Sequence added for the indicated restriction site is underlined.

[3] Lower case letters denotes mismatches with the wild-type *hrpA* sequence.

Table 3.2. Plasmids and *E. coli* strains used in this study

Plasmids for disruption of *bb0826*		
Knockout plasmid	**Orientation of *gent* cassette**	***E. coli* strain (GCE)**
pPOH57-1	Forward	2149
pPOH57-2	Reverse	2150

Plasmids for insertion of point mutation in *B. burgdorferi*		
Construct description	**Plasmid**	***E. coli* strain (GCE)**
hrpA complementation	pPOH62-1	2159
hrpA-D126A in pPOH62-1	pPOH86	3213
hrpA-E127A in pPOH62-1	pPOH87	3214
hrpA-S158A in pPOH62-1	pPOH88	3215
hrpA-I285A in pPOH62-1*	pPOH90*	3217

* Mutation was generated by Aydan Salman-Dilgimen

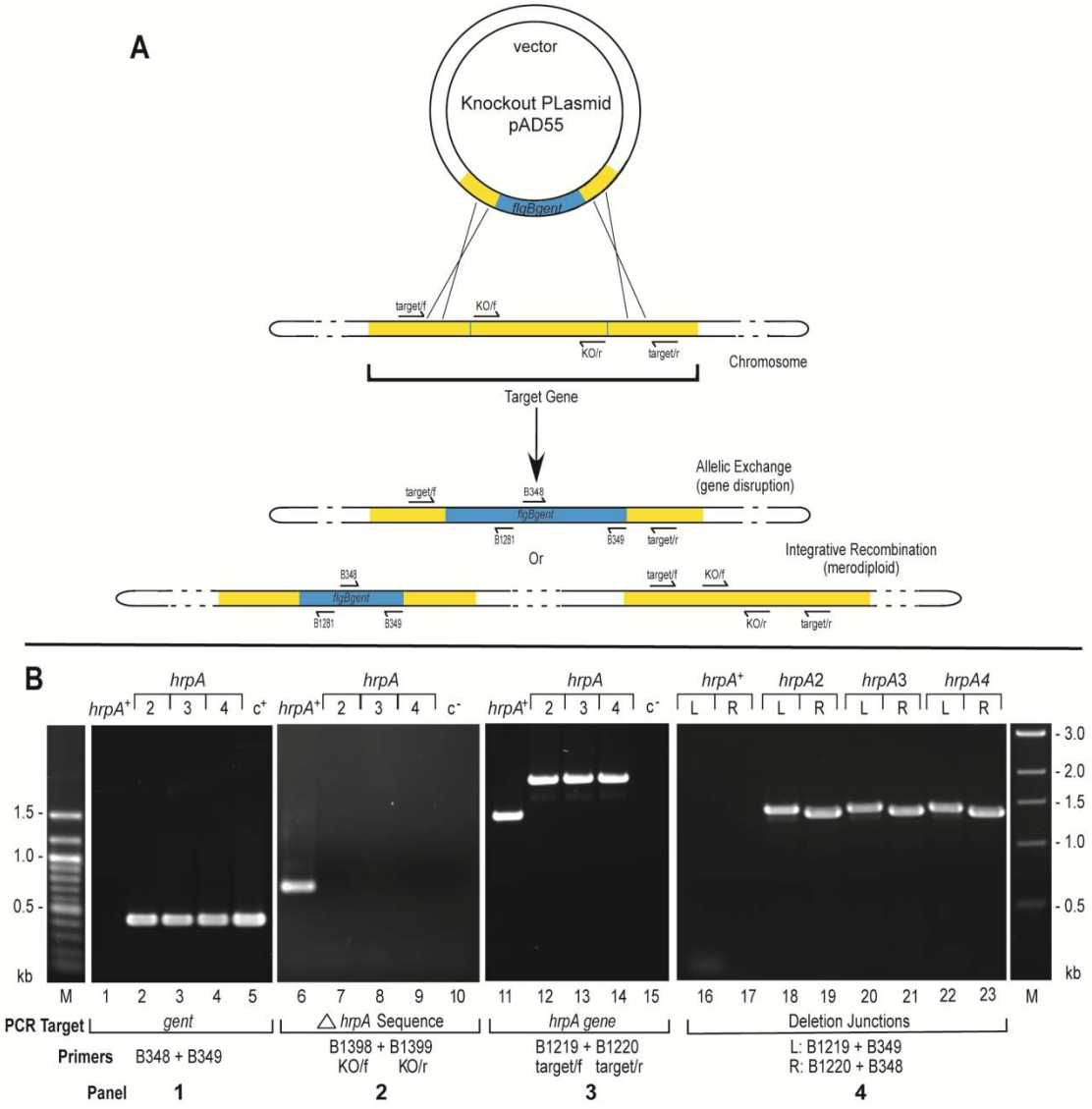
Table 3.3 *B. burgdorferi* strains used in this study

Genotype	Target locus	Description	Strain (GCB)	Missing plasmids	Reference
wt		*B. burgdorferi* strain B31 clone 5A4	920		(Purser and Norris 2000)
knockout strains					
bb0826	*bb0826*	hypothetical protein	543		(Salman-Dilgimen *et al.* 2011)
			544		
hrpA	*bb0827*	RNA helicase	1164	lp28-2, cp9	
			1165		
			1166	cp32-3, cp32-6	
complemented strains					
hrpA^{R+}			548	lp28-2, cp9	(Salman-Dilgimen *et al.* 2013)
			549	lp28-2, cp9	
strains containing point mutation in *hrpA*					
hrpA-D126A			572	lp28-2, cp9	(Salman-Dilgimen *et al.* 2013)
			573	lp28-2, cp9	
hrpA-E127A			574	lp28-2, cp9	
			575	lp28-2, cp9	
hrpA-S158A			576	lp28-2, cp9	
			577	lp28-2, cp9	
hrpA-I285A			578	lp28-2, cp9	
			579	lp28-2, cp9	

Figure 3.3 *hrpA* gene disruption and confirmation.

A) Gene disruption strategy. The infectious *B. burgdorferi* strain B31, clone 5A4 (B31-5A4) was transformed with a knockout plasmid carrying a 1-kb gentamicin resistance cassette (blue) that replaced the central 500 bp of the *hrpA* gene (yellow) as described in Materials and Methods. The two possible outcomes of recombination events with the target gene are shown: allelic exchange would result in gene disruption while integrative recombination of the knockout plasmid would result in merodiploid formation. The positions of PCR primers used for construct verification are shown by arrows on the schematic. **B)** PCR verification of the *hrpA* disruption. Each gene disruption was subjected to four PCR analyses. **Panel 1)** The presence of the gentamicin resistance cassette was confirmed as shown. The shuttle vector pBSV2G (Stewart *et al.* 2001) served as the positive control (c^+) for amplification of the *gent* cassette (lane 5). **Panel 2)** The portion of *hrpA* expected to be deleted in a gene disruption was not detected in *hrpA2, 3* or *4* (lanes 7, 8 and 9). Lane 10 was a negative control (c^-) that lacked DNA template. **Panel 3)** The size of the *hrpA* gene was compared in the three mutant strains. The expected 2.1-kb gene disruption products were observed (lanes 12, 13 and 14) in comparison to the 1.5-kb product from the wild-type *hrpA* gene (lane 11). Lane 15 was a negative control (c^-) that lacked DNA template. **Panel 4)** Confirmation of the correct insertion site was performed using combinations of the target gene primers and primers internal to the gentamicin resistance cassette to amplify the *hrpA* boundaries. The left boundary in the *hrpA* knockout clones displayed the expected 1.4-kb product (lanes 18, 20

and 22) and the right boundary showed the expected product of approximately 1.3 kb (lanes 19, 21 and 23). A 100-bp ladder on the left side of Fig. 1B is relevant to the two left panels, and a 1-kb ladder on the right side applies to the two right panels (M). The schematic in part A of the figure is modified from (Dresser *et al.* 2009).


A
vector
Knockout PLasmid
pAD55
flgBgent
target/f
KO/f
KO/r
target/r
Chromosome
Target Gene
Allelic Exchange
(gene disruption)
B348
B1281
B349
Or
Integrative Recombination
(merodiploid)
B
hrpA
hrpA+
2
3
4
c+
c-
hrpA2
hrpA3
hrpA4
L
R
1.5 -
1.0 -
0.5 -
kb
- 3.0
- 2.0
- 1.5
- 1.0
- 0.5
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 M
PCR Target
gent
△ hrpA Sequence
hrpA gene
Deletion Junctions
Primers
B348 + B349
B1398 + B1399
KO/f KO/r
B1219 + B1220
target/f target/r
L: B1219 + B349
R: B1220 + B348
Panel
1
2
3
4

(New England Biolabs, Pickering, ON, CA), 1x Phusion HF reaction buffer, 250 µM dNTPs and 0.25 µM of each primer. After the final extension step, 2.5 µl from each reaction was pooled for six more PCR cycles and 2.5 µl for this last reaction was used as template for a second complete 50-µl PCR mix. For this second PCR, only primers flanking the fused product were used. At the same time, 500 bp of the sequence downstream from the gene to be complemented was amplified in a separate reaction using 80 ng of *B. burgdorferi* strain B31 clone 5A4 gDNA as template. Following this second PCR, 2.5 µl from each reaction was pooled for 6 additional cycles and 2.5 µl from this last reaction was then used as template for a third and last PCR. For this third PCR, only primers flanking the final PCR product were used. The PCR conditions were as followed: 3 minutes at 98°C, 25 cycles of 98°C for 15 seconds, 63°C for 15 seconds and 72°C for 30 seconds/kb to be amplified and then a final extension of 72 °C for 7 min. The PCR product was then purified by electrophoresis in a 1% agarose gel with 90 minutes migration at 80 V in 1x RML and the DNA was extracted using Qiaquick gel extraction kit (Qiagen, Toronto, ON, CA) following instructions from the manufacturer. The purified overlap PCR product was then cloned into pJET1.2/blunt vector (Fermentas, Burlington, ON, CA) and used to transform the *B. burgdorferi* knockout strain. Transformants were cultivated in the presence of kanamycin (200 µg/ml) for selection. Furthermore, potential clones were tested for growth in the presence of gentamicin to confirm the replacement of the gentamicin resistance cassette by the wild-type sequence. Allelic exchange was confirmed by sequencing (University of Calgary Core DNA services, Calgary, AB,

CA) and by PCR for *PflgB-kan*, the absence of the gentamicin resistance cassette, the presence of the sequence that was deleted in the knockout mutant and for the size of the gene target (see Figure 3.6B).

3.2.5 Insertion of point mutations into the *hrpA* gene in the *B. burgdorferi* chromosome

To insert point mutations in *B. burgdorferi hrpA*, the mutation was inserted in the complementation plasmid pPOH62-1 (see Figure 3.6 in the results section). The D126A, E127A, S158A and T160A mutations were transferred from their original construct, where they were generated by site-directed mutagenesis (done by Aydan Salman-Dilgimen), to pPOH62-1 using PacI and AgeI restriction sites (see Figure 3.6 in the results section). This strategy could not be used for the I285A mutation because the site is located outside the segment flanked by the PacI and AgeI restriction sites. To insert I285A mutation in the complementation plasmid, pPOH62-1 was used as the template for site-directed mutagenesis as previously described (done by Aydan Salman-Dilgimen). The resulting constructs were methylated *in vitro* with M.SssI CpG methyltransferase (New England Biolabs, Pickering, ON) as described by Chen *et al.* (Chen *et al.* 2008). Briefly, 52 µg of DNA was incubated with 1x NEB buffer 2, 160 µM S-adenosylmethionine (SAM) and M.SssI CpG Methyltransferase (40 units) in a 400-µl reaction mix for 4 hours at 37°C. To purify the DNA, 400 µl of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the reaction and the sample was incubated with inversion during 20 minutes. Following a 1-minute centrifugation at 16,110 x g, the upper phase was transferred to a new 1.5 ml

microcentrifuge tube for a second extraction. Following the second 1-minute centrifugation, the upper phase was transferred in a new 1.5 ml microcentrifuge tube already containing 1 ml of isopropanol. After inversion, the sample was centrifuged for 20 minutes at 16,110 x g and the pellet was washed with 600 µl of 70% ethanol and centrifuged during 10 minutes at 16,110 x g. The dried pellet was then resuspended in 15 µl of ddH_2O. Methylation was confirmed by resistance to SalI digestion. Methylated DNA was used to transformed *B. burgdorferi* GCB1164 (*hrpA* KO) strain by electroporation as described previously. Possible clones were grown in presence of kanamycin (200 µg/ml) for selection. Despite many attempts, no *B. burgdorferi hrpA* T160A mutants were recovered.

3.2.6 Screening for point mutations in *B. burgdorferi*

A PCR strategy to screen for point mutations in *B. burgdorferi* was adapted from (Newton *et al.* 1989) (see Figure 3.7). A primer containing the mutated base as last (3') nucleotide was used in pair with a primer (B1950) containing only sequence that is conserved between mutated and wild-type *B. burgdorferi hrpA*. The primer containing the mismatched base was designed to be relatively short, having a melting temperature (Tm) of approximately 40°C to decrease the stability of annealing during the PCR. The Tm was calculated by adding 2°C for each A or T and 4°C for each G or C nucleotide. The primer pairs were designed to recover a PCR product only if the mutation is present. Each 50 µl PCR mix contained: 1 to 5 ng of *B. burgdorferi* genomic DNA, 1 unit Phusion high-fidelity DNA polymerase (New England Biolabs, Pickering, ON, Canada), 1x

Phusion HF reaction buffer, 250 µM dNTPs and 0.25 µM each primer. The PCR conditions used were: an initial 2 minutes denaturation at 94°C, 25 cycles of 94°C for 30 seconds, 50°C for 30 seconds and 68°C for 1 minute, followed by a final 7 minutes extension at 68°C. The product from the PCR was analysed by electrophoresis in a 1.2% agarose gel with migration during 90 minutes at 80 V in 1x RML TAE buffer. The DNA was stained for 30 minutes with ethidium bromide and visualized under UV light. Restitution of a complete *hrpA* gene was confirmed by PCR as described for *hrpA* complementation (see Figure 3.6 in the results section) and by sequencing.

3.2.7 Mouse infection studies

All animal studies for *hrpA* experiments were carried as described in the Materials and methods section of Chapter 2.

3.3 Results

3.3.1 Disruption of *hrpA* and *bb0826* genes in *B. burgdorferi*

The disruption of the *hrpA (bb0827)* and *bb0826* genes in *B. burgdorferi* was accomplished by allelic exchange. The disruption of *hrpA* was done by Ashley R. Dresser. The same strategy was used for both gene targets. To generate *B. burgdorferi bb0826* mutant, a knockout plasmid (see Figure 3.3A in Materials and Methods section) was constructed in *E. coli*, in which the central 500 bp of the *hrpA* gene was deleted and replaced with a gentamicin resistance cassette (*aacC1)* under the control of the *B. burgdorferi flgB* promoter. The orientation of the resistance cassette relative to the target genes is shown in Table 3.2. A

knockout plasmid was also constructed for *bb0826,* the gene downstream from *hrpA,* which contains an RNA binding motif and therefore, was considered as a protein that might function together with HrpA. The constructs were used to transform infectious *B. burgdorferi* B31 clone 5A4 (Purser and Norris 2000). The transformants recovered were screened by PCR as shown in Figure 3.3B (results for *bb0826* not shown). First, the presence of the gentamicin resistance cassette was verified (Figure 3.3 - Panel 1, lanes 2-4), followed by confirmation that the central 500 bp of the target gene was no longer present (panel 2, lanes7-9). To confirm that the recovered mutants carried only the disrupted *hrpA* gene and were not merodiploids, the presence of only the disrupted gene carrying the *gent* cassette (2.1 kb) was verified (Panel 3, lanes 12-14) along with the absence of the wild-type gene (1.5 kb, lane 11). Finally, the correct insertion site was confirmed using PCR primers to uniquely amplify left and right side deletion junctions (Panel 4, lanes 18-23). In addition, the structural integrity of the gene disruptions and the presence of only a single disrupted gene were independently demonstrated by Southern hybridization using probes specific for the gentamicin resistance cassette and the deleted portion of the *hrpA* or *bb0826* gene (see Figure 3.4 for the Southern blot of the *hrpA* gene disruption).

All genetic constructs were analyzed for plasmid content, which can affect infectivity. No plasmid loss was observed for GCB 1165 (*hrpA3*). GCB 1164 (*hrpA2*) is missing lp28-2 and cp9. GCB 1166 (*hrpA4*) was lacking cp32-3 and cp32-6; cp32-3 is not required for infectivity (Purser and Norris 2000) and the

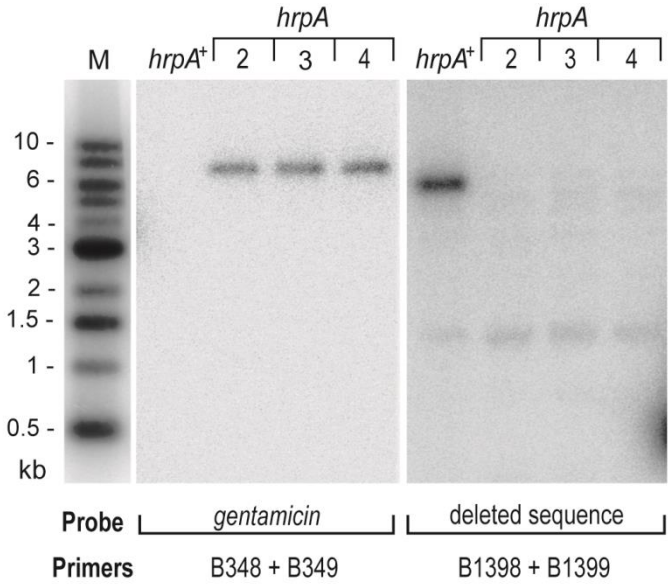


Figure 3.4 Gene disruption and the absence of additional copies of the *hrpA* gene was confirmed by Southern hybridization.

Genomic DNA was digested with HindIII and run on a 1.0% agarose gel with a 1-kb molecular weight ladder (M). Probes complementary to the gentamicin (*gent*) resistance cassette (left panel) and the portion of the *hrpA* gene deleted during gene disruption (right panel) were used for hybridization to duplicate blots. As expected, hybridization to the *gent* probe was not observed in the wild-type strain but was observed at the expected size (7.1 kb) for the three *hrpA* mutant strains. Conversely, hybridization to the deleted portion of *hrpA* was observed in the expected 6.5-kb fragment in the wild-type strain but not in the three *hrpA* mutant clones.

effect of loss of cp32-6 has not been previously reported (Purser and Norris 2000) (Table 3.3). GCB 543 (*bb0826-3*) and GCB 544 (*bb0826-11*) both contained a full plasmid complement. Finally, RT-PCR was used to show that the disruption of *hrpA* did not affect the transcription of *bb0825* and *bb0826*, the two genes downstream of *hrpA*. (RT-PCR experiments were realized by Aydan Salman-Dilgimen – see Figure 3.5). All three clones displayed wild-type morphology and normal growth in culture (data not shown).

3.3.2 Effect of *hrpA* and *bb0826* gene disruptions on the infectivity of *B. burgdorferi* in C3H/HeN mice.

The mutant strains were each used to infect C3H/HeN mice using an inoculum of $1x10^4$ spirochetes at two locations (see Materials and Methods). At seven days post-infection spirochetes were not recovered from the blood of any of the mice inoculated with the *hrpA* mutant clones, in contrast to the *bb0826* mutants and the control group where all the cultures were positive for spirochetes (Table 3.4). Similarly, ear cultures at day 21 were all negative for the mice inoculated with *hrpA* mutant spirochetes, but 100% positive for the *bb0826* mutants and the wild-type spirochetes. Finally, at day 35 when all cultures from heart, bladder, ear and joint were positive for wild-type and *bb0826* mutant *B. burgdorferi*, no positive cultures were recovered from the mice infected with the *hrpA* mutant clones. Mutation of the *hrpA* gene, therefore, appeared to obliterate spirochete infectivity.

Figure 3.5. Region view and transcription patterns of *hrpA*, *bb0825* and *bb0826*.

A) Schematic representation of *hrpA* and the two downstream genes on the *B. burgdorferi* chromosome. Arrows represent the direction of transcription and the numbers denote the coordinates on the chromosome. **B**) Ethidium bromide stained 1.4% agarose gel showing the products of RT-PCRs to assess the transcription patterns of genes *bb0825* and *bb0826* in the three *hrpA* mutant clones, along with a 100 bp molecular weight ladder (M). **Panel 1**) RT-PCRs for *bb0825* in the wild-type parent strain (*B. burgdorferi* B31, clone 5A4) and in the *hrpA2*, *3* and *4* mutants. The expected product size was 210 bp. **Panel 2**) RT-PCRs for *bb0826* in the strains described in Panel 1. The expected product was 310 bp. [1]The experiments and the figure were done by Aydan Salman-Dilgimen.

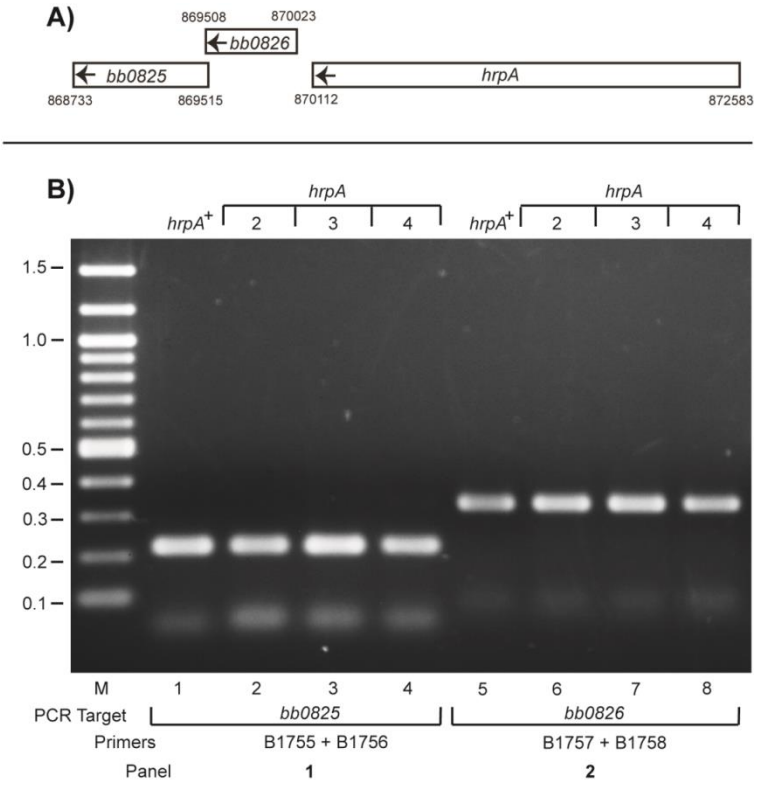

A)
869508
870023
bb0826
bb0825
hrpA
868733
869515
870112
872583
B)
hrpA
hrpA
hrpA+
2
3
4
hrpA+
2
3
4
1.5
1.0
0.5
0.4
0.3
0.2
0.1
M
1
2
3
4
5
6
7
8
PCR Target
bb0825
bb0826
Primers
B1755 + B1756
B1757 + B1758
Panel
1
2

Table 3.4. Infectivity of *B. burgdorferi hrpA* (*bb0827*) and *bb0826* mutants in C3H/HeN mice.

B. burgdorferi genotype	Strain	Total mice[a]	Day 7 Blood[b]	Day 7 Infection	Day 21 Ear	Day 21 Infection	Day 35[c] Heart	Bladder	Joint	Ear	Total sites[d]	Day 35 Infection
B31 5A4 (wt)	GCB933	18	18/18	100,0%	18/18	100.0%	4/4	4/4	4/4	4/4	16/16	100.0%
hrpA2[e]	GCB1164	9	0/3	0,0%	0/3	0.0%	0/3	0/3	0/3	0/3	0/12	0.0%
hrpA3[e]	GCB1165		0/3		0/3		0/3	0/3	0/3	0/3	0/12	
hrpA4	GCB1166		0/3		0/3		0/3	0/3	0/3	0/3	0/12	
bb0826-3	GCB543	4	2/2	100,0%	2/2	100.0%	2/2	2/2	2/2	2/2	8/8	100.0%
bb0826-11	GCB544		2/2		2/2		2/2	2/2	2/2	2/2	8/8	

[a] There is a large number of mice in the control group because the *hrpA* mutants were initially assessed as part of a larger group of mutants.

[b] Values listed correspond to number of cultures positive / number of sites tested.

[c] Four mice infected with *B. burgdorferi* 5A4 were chosen as positive controls for organ harvests at day 35.

[d] Number of positive tissue sites/ number of sites tested.

[e] Clones that were originally found as non-infectious by Ashley R. Dresser.

3.3.3 Complementation of *hrpA* in *B. burgdorferi*

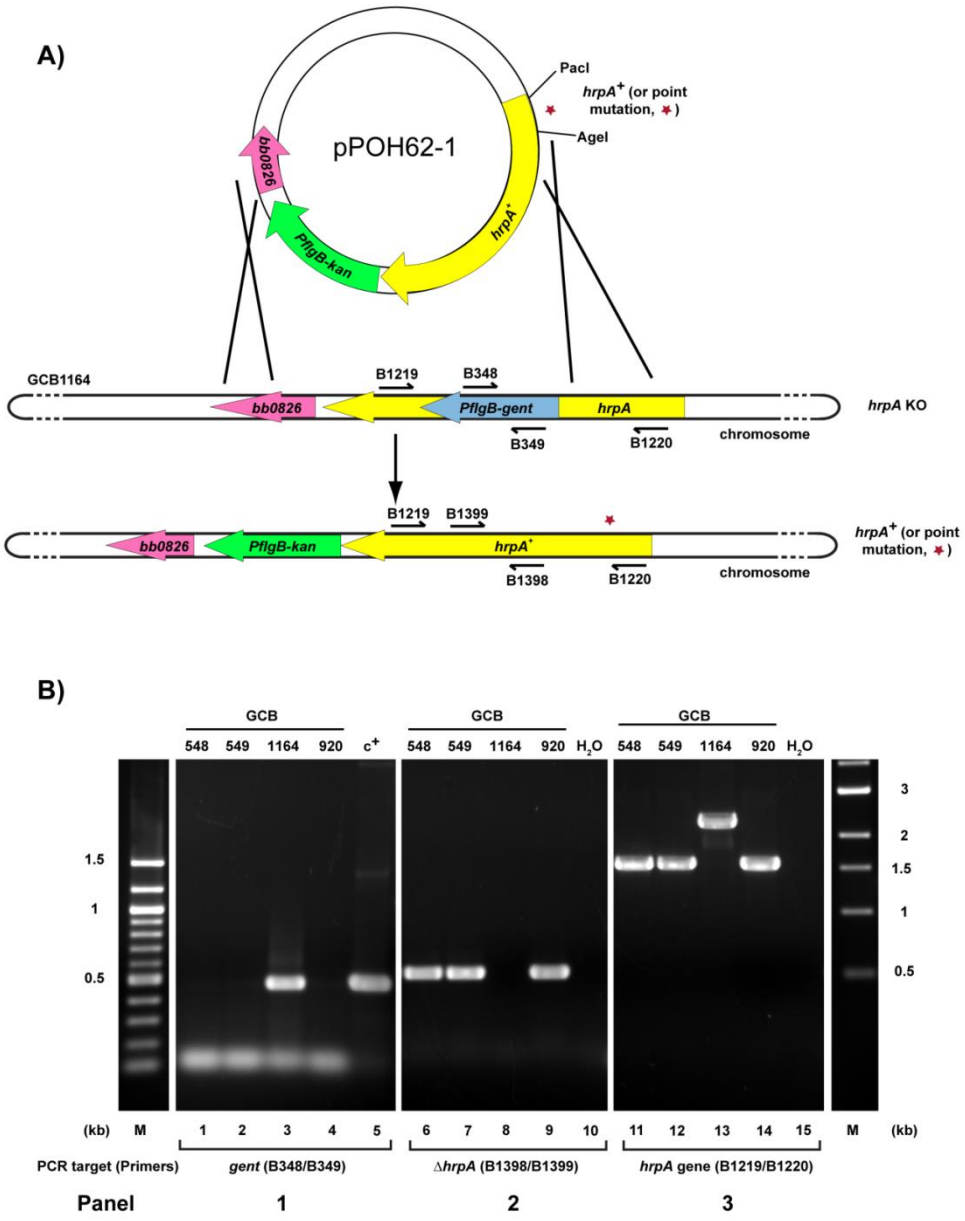
Attempts for complementing *B. burgdorferi hrpA in trans* using the *B. burgdorferi* shuttle plasmid pBSV2 remained unsuccessful. This strategy had previously been used to restore complete infectivity of *B. burgdorferi* lacking *bbe22*, which encodes for the nicotinamidase PncA and has been shown to be required for *B. burgdorferi* infectivity in mice (Purser *et al.* 2003). In the present study, an alternative strategy based on allelic exchange was attempted. A complementation plasmid lacking a *B. burgdorferi* origin of replication but containing a wild-type copy of *B. burgdorferi hrpA* fused to a *PflgB*-driven kanamycin resistance cassette and 500 bp of the genomic sequence downstream of *hrpA* was constructed (see Figure 3.6A). The resulting plasmid was sequenced to confirm the absence of mutation in *hrpA* and then used to transform *B. burgdorferi hrpA* mutant strain GCB 1164. Restoration of a wild-type *hrpA* gene in *B. burgdorferi* chromosome was confirmed by PCR (see Figure 3.6B) and sequencing. The *hrpA* gene in the complemented clones was shown to be of the same size than wild-type *hrpA*, the sequence that had been deleted in the knockout clone was restored.

3.3.4 Complementation of *hrpA* in *B. burgdorferi* restored infectivity in mice

To confirm the complementation of *hrpA* in *B. burgdorferi*, two complemented clones containing a plasmid profile similar to the parental knock-out strain (missing only lp28-2 and cp9) were used to infect wild-type C3H/HeN mice. Each mouse was infected by subcutaneous and intraperitoneal injections and infectivity of *B. burgdorferi* was assessed by culture from samples collected

Figure 3.6. Strategy for complementation and insertion of point mutations in *B. burgdorferi hrpA*.

A) Schematics of the strategy used. The *B. burgdorferi hrpA* KO strain GCB 1164 was transformed with a construct carrying either wild-type or mutated *hrpA* (*hrpA*$^{+}$, a red star indicates the point mutation), a *PflgB*-driven kanamycin resistance cassette (green) and 500 bp of sequence downstream from *hrpA*, to replace the disrupted *hrpA* gene. The point mutations were transferred in the complementation plasmid pPOH62-1 using PacI and AgeI restriction sites. Allelic exchange was confirmed by PCR using primers indicated by an arrow. **B)** PCR verification of the allelic exchange. Each construct was confirmed with four PCR analyses. **Panel 1)** The loss of gentamicin resistance cassette following allelic exchange was confirmed as shown. The shuttle vector pBSV2G served as the positive control (c^{+}) for amplification of the gent cassette (lane 5). **Panel 2)** The replacement of the gentamicin resistance cassette by *hrpA* was confirmed by amplification of the segment of *hrpA* that was deleted in the KO strain GCB1164 (lane 8). **Panel 3)** The size of the *hrpA* gene was compared between wild-type (lane 14), *hrpA* KO (lane 13) and complemented clones (lanes 11 and 12). Lane 10 and 15 was a negative control containing ddH_2O as template. A 100 bp and a 1 kb DNA ladder were used (M). The schematic in **A** of the figure is modified from (Dresser *et al.* 2009).


A)
pPOH62-1
PacI
hrpA+ (or point mutation, *)
AgeI
bb0826
PflgB-kan
hrpA+
GCB1164
B1219
B348
bb0826
PflgB-gent
hrpA
hrpA KO
B349
B1220
chromosome
B1219
B1399
bb0826
PflgB-kan
hrpA+
hrpA+ (or point mutation, *)
B1398
B1220
chromosome
B)
GCB
548 549 1164 920 c+
GCB
548 549 1164 920 H2O
GCB
548 549 1164 920 H2O
1.5
1
0.5
3
2
1.5
1
0.5
(kb) M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 M (kb)
PCR target (Primers)
gent (B348/B349)
ΔhrpA (B1398/B1399)
hrpA gene (B1219/B1220)
Panel 1 2 3

at different time points post-infection. At one week post-infection, spirochetes were present in the blood collected from the every mice infected with the wild-type and the complemented clones (Table 3.5). At weeks 2, 3 and 4, *B. burgdorferi* was also cultured from ear punches recovered from every mouse infected with *B. burgdorferi* encoding a wild-type *hrpA*. Similar to wild-type *B. burgdorferi*, spirochetes were cultured from the ear, the heart, the bladder and the joint of mice infected with the complemented clones. As previously reported, all samples collected from mice infected with *B. burgdorferi hrpA* knockout were negative for spirochetes.

3.3.5 Effect of point mutations in *hrpA* on murine infection

HrpA is an 823-amino acid protein and may conceivably contain functions other than RNA helicase activity. It was therefore of interest to test point mutations affecting RNA helicase activity on murine infection to determine whether the inability of a *hrpA* mutant to support infection was a result a loss of RNA helicase activity. To introduce point mutations into the chromosomal *B. burgdorferi hrpA* gene, each mutation was first inserted in pPOH62-1, the construct previously used for the complementation of *hrpA* (Figure 3.6A). The resulting plasmid was then used to transform the *B. burgdorferi hrpA* knockout strain GCB1164. To screen transformants for the presence of the desired point mutation, a PCR strategy was adapted from a previously described method developed to screen for known SNPs between human alleles (Newton *et al.* 1989). For each mutation introduced in *B. burgdorferi hrpA*, a primer with the mutated nucleotide as the 3' base was used in conjunction with a primer

containing sequence that is conserved between the wild-type and the mutant allele (Figure 3.7A). Using this approach, a PCR product was recovered from a mini-genomic DNA prep only if the mutation was present. This strategy was used to screen and recover for *hrpA*-D126A, E127A, S158A and T160A mutations (Figure 3.7B). However, *hrpA*-T160A could not be recovered and the primers designed for *hrpA*-I285A did not produce a sufficiently clear result to be used for screening *B. burgdorferi* genomic DNA. Following the transformation, 68 clones for D126A, 93 clones for E127A, 21 clones for S158A, 30 clone for T160A (from 4 transformation and 41 clones for I285A (from 4 transformations) were recovered. For the D126A mutation, four out of the six clones tested by PCR contained the mutation. From the 12 clones tested for the D127A mutation, nine appeared to contain the mutation. For T160A mutation, 30 clones were recovered over four transformation and all were shown to contain only the wild-type *hrpA* sequence both by PCR and by sequencing. For the I285A, 41 clones were recovered over four transformations and only two contained the expected mutation, as confirmed by sequencing. Two clones for each *B. burgdorferi* D126A and E127A mutants were subsequently confirmed by DNA sequencing.

The *hrpA*-D126A and *hrpA*-E127A mutations (Motif II, ATP binding and hydrolysis - Figure 3.2) resulted in a complete loss of *B. burgdorferi* infectivity in wild-type mice, similar to the *hrpA* knockout strain. All tissue samples recovered were negative for spirochetes (Table 3.5). In contrast, *hrpA*-S158A (Motif III, communication) resulted in a slight delay in the infection, where samples from one of four mice were positive for spirochetes for weeks one and two.

Figure 3.7. Strategy used to screen for point mutation in *B. burgdorferi*.

The strategy is adapted from Newton *et al.* 1989. **A)** For each mutant, a primer with a sequence that is conserved between the mutants and the wild-type *hrpA* (B1220) was used in pair with a second primer containing the mutated base as last nucleotide (B2176 is shown as an example). The nucleotide mismatch is indicated by a box. **B)** Example of a PCR screen for *B. burgdorferi hrpA*-D126A point mutant (clones 1 and 2). The construct containing the mutated *hrpA* (pPOH87) and genomic DNA from wild-type *B. burgdorferi* GCB920 strain were used as templates for positive and negative controls, respectively. A PCR using ddH_2O as template was also included. Presence of the point mutation was confirmed by sequencing.

A)

***hrpA* - conserved sequence**

```
B1220 - ATGAATGATTTCAAACTCCCAAT
   5' - ATGAATGATTTCAAACTCCCAATTTATAAATACAAAGATGAATTAATTAAAGTACTAAAAAACCACAATGTTTTA - 3'
   3' - TACTTACTAAAGTTTGAGGGTTAAATATTTATGTTTCTACTTAATTAATTTCATGATTTTTTGGTGTTACAAAAT - 5'
```

***hrpA* D126A mutation**

```
   5' - TATGAATATGATGTAATAATAATAGACGCAGCACACGAAAGAAGTTTAAACATTGATTTTATATTGGGTCTTATC - 3'
   3' - ATACTTATACTACATTATTATTATCTGCGTCGTGTGCTTTCTTCAAATTTGTAACTAAAATATAACCCAGAATAG - 5'
                             GTCGTGTGCTTTCTT - B2176
```

***hrpA* wild-type**

```
   5' - TATGAATATGATGTAATAATAATAGACGAAGCACACGAAAGAAGTTTAAACATTGATTTTATATTGGGTCTTATC - 3'
   3' - ATACTTATACTACATTATTATTATCTGCTTCGTGTGCTTTCTTCAAATTTGTAACTAAAATATAACCCAGAATAG - 5'
                             GTCGTGTGCTTTCTT - B2176
```

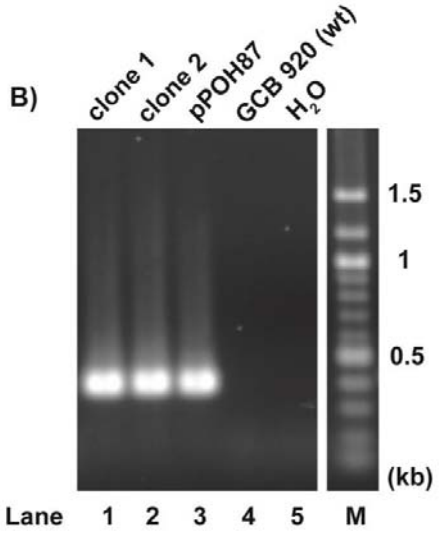


Table 3.5. Effect of complementation and mutations in *hrpA* on *B. burgdorferi* infectivity in C3H/HeN mice.

Genotype	Strain (GCB)	Total mice	Day 7 Blood[a]	Day 7 %	Day 14 Ear	Day 14 %	Day 21 Ear	Day 21 %	Day 28 Ear	Day 28 %	Day 35 Organs					Day 35 %
											Ear	Bladder	Joint	Heart	Total sites	
wt	920	6	6/6	100%	6/6	100%	6/6	100%	6/6	100%	6/6	6/6	6/6	6/6	24/24	100%
hrpA KO	1164	3	0/3	0%	0/3	0%	0/3	0%	0/3	0%	0/3	0/3	0/3	0/3	0/12	0%
[b]*hrpA*$^{R+}$	548	4	2/2	100%	2/2	100%	2/2	100%	2/2	100%	2/2	2/2	2/2	2/2	8/8	100%
	549		2/2		2/2		2/2		2/2		2/2	2/2	2/2	2/2	8/8	
hrpA-D126A	572	4	0/2	0%	0/2	0%	0/2	0%	0/2	0%	0/2	0/2	0/2	0/2	0/8	0%
	573		0/2		0/2		0/2		0/2		0/2	0/2	0/2	0/2	0/8	
hrpA-E127A	574	4	0/2	0%	0/2	0%	0/2	0%	0/2	0%	0/2	0/2	0/2	0/2	0/8	0%
	575		0/2		0/2		0/2		0/2		0/2	0/2	0/2	0/2	0/8	
hrpA-S158A	576	4	1/2	75%	1/2	75%	2/2	100%	2/2	100%	2/2	1/2	2/2	2/2	7/8	93.75%
	577		2/2		2/2		2/2		2/2		2/2	2/2	2/2	2/2	8/8	
hrpA-I285A	578	4	2/2	50%	1/2	25%	1/2	50%	2/2	75%	2/2	1/2	2/2	2/2	7/8	81.25%
	579		0/2		0/2		1/2		1/2		2/2	0/2	2/2	2/2	6/8	

[a]Values listed correspond to number of cultures positive/number of sites tested.

[b]Complemented *hrpA* mutant where the mutant chromosomal gene was replaced with a wild-type gene.

At weeks three, four and five post-infection, all four mice were positive for *B. burgdorferi*. Finally, *hrpA*-I285A (Motif V, RNA binding) resulted in a more intermediate infectivity phenotype. Only 50% of blood samples collected 7 days post-infection were positive for spirochetes. For ear punches collected 14, 21 and 28 days post-infection, a delay in dissemination of *B. burgdorferi hrpA*-I285A was observed, compared to wild-type *B. burgdorferi*. Indeed, in contrast to wild-type *B. burgdorferi* that were grown from all ear punches collected 14, 21 and 28 days post-infection, spirochetes were observed in samples from only 25, 50 and 75% of the mice, respectively with the *hrpA*-I285A mutant. When the complete ear, bladder, joint and heart were recovered on day 35 post-infection, the difference between wild-type and *hrpA*-I285A mutant *B. burgdorferi* was attenuated. At this time point, 81.25% of the organs collected were positive for spirochetes, only a decrease in the dissemination to the bladder appeared to remain.

3.4 Discussion

As part of a study on switching at *vlsE*, the *B. burgdorferi hrpA* (*bb0827*) gene was disrupted and then used to infect wild-type C3H/HeN mice. This resulted in a complete loss of infectivity of *B. burgdorferi* in mice. While attempts to complement *hrpA in trans*, using a shuttle plasmid were not successful, allelic exchange to replace the disrupted chromosomal gene with a wild-type copy of *hrpA* restored complete infectivity and persistence in mice to a level similar to wild type. This complementation experiment confirms that *hrpA* is indeed

essential for the infectivity of *B. burgdorferi* and the defect observed in the knockout clones is not the result of a secondary mutation in the genome. The possibility that the loss of infectivity resulted from a polar effect on the expression of surrounding genes was disproved by RT-PCR. Moreover, the disruption of the gene downstream of *hrpA*, *bb0826*, had no effect on *B. burgdorferi* infectivity, demonstrating further that *hrpA* is essential for infectivity. For complementation, a kanamycin-resistance cassette was inserted downstream of *hrpA*. This suggests that the expression of the gene downstream of *hrpA* might be disrupted. However, since the disruption of *bb0826* was shown to not affect *B. burgdorferi* infectivity, this was not expected to affect the complementation.

In order to verify the importance of the ATPase and the RNA-binding activities of *B. burgdorferi* HrpA, point mutations that were shown *in vitro* to either abolish or significantly decrease the activities of HrpA were inserted into the *B. burgdorferi* genome. To our knowledge, the insertion of point mutations into *B. burgdorferi* genome had been done few times in the past (Knight *et al.* 2000; Samuels and Garon 1997; Samuels *et al.* 1994b; Yang *et al.* 2003). Each point mutation was inserted into the plasmid used for the complementation of the knockout strain and inserted into *B. burgdorferi* chromosome by allelic exchange, to replace the knockout copy by a mutated copy of *hrpA*. Because *hrpA* is a relatively long gene (2472 bp) and all the mutations were in the first kilobase, the knockout strain was used instead of the wild-type parental strain for the transformation. This was expected to increase the chances of the sites of mutation being included in the allelic exchange.

Insertion of each point mutation into the *B. burgdorferi* genome was ultimately confirmed by sequencing. However, in order to first screen potential clones, a PCR screen assay was adapted from a strategy previously used to screen for single nucleotide polymorphism in different human alleles. This strategy consisted in using a primer with the mutation as last nucleotide instead of the wild-type sequence, in conjunction with a primer that contains sequence that is conserved between the mutant and the wild-type alleles. This strategy allowed efficient screening of multiple potential clones for the presence of each mutation. Using these strategies, four point mutations (D126A, E127A, S158A and I285A) were inserted into *B. burgdorferi hrpA*. However, only two clones containing I285A could be recovered from four different transformations and no clones containing *hrpA* T160A were recovered from four transformations.

All four mutants were used to infect wild-type mice to verify their impact on *B. burgdorferi* infectivity. As expected, mutations in motif II (D126A and E127A), which almost completely abrogate both ATPase and helicase activities of HrpA *in vitro*, resulted also in a complete loss of *B. burgdorferi* infectivity. However, mutation in the motif III (S158A) only slightly decreased infectivity and dissemination of *B. burgdorferi*, while mutation in the RNA binding motif V (I285A) resulted in a more intermediate infectivity phenotype. During *in vitro* activity assays, mutation in the motif III affected more importantly the HrpA ATPase activity than the RNA unwinding function. However, mutation in motif V affected the activities of HrpA the opposite way, with a stronger decrease of helicase than of ATPase activity. In general the severity of the mutants *in vitro*

was reflected in the infectivity phenotype suggesting that *B. burgdorferi* requires the helicase activity of HrpA for mouse infection and that only the ATPase activity is not sufficient. However, it also shows that only partial HrpA helicase activity is sufficient to restore an infectivity level similar to the wild-type strain. It is possible that a more important difference in infectivity would have been observed by using a lower dose of infection. However, the I285A mutant could have failed to infect and the intermediate phenotype of I285A mutant clone would have been missed.

The biological function of HrpA that is essential for *B. burgdorferi* infectivity but dispensable for growth in culture remains unknown. The growth of *B. burgdorferi hrpA* mutants was shown to be reduced when cultured at 23°C instead of 35°C. However, this temperature sensitivity would be expected to affect the growth of *B. burgdorferi* in the ticks but not in mice infected by needle injection. Moreover, the disruption of *hrpA* did not affect the growth of *B. burgdorferi* when cultured in presence of 5% of human blood (data not shown).

RNA helicases are involved in RNA processing, ribosome biogenesis, mRNA transport, transcription, translation and RNA degradation. Data from iTRAQ experiments showed that the disruption of *B. burgdorferi hrpA* significantly affected the level of expression of at least 180 proteins, including the surface protein P66 (Salman-Dilgimen *et al.* 2011), which was recently shown to be required for *B. burgdorferi* infectivity in mice (Ristow *et al.* 2012). However, despite the large number of proteins which expression level was affected by the disruption of *hrpA* in *B. burgdorferi*, the transcription level of only 30 genes presented a 1.5 or more fold changes in a microarray experiment (Salman-

Dilgimen *et al.* 2013). This suggests that *B. burgdorferi* HrpA influences protein expression primarily at the post-transcriptional level. In *E. coli*, the expression of the F1845 fimbrial adhesion involves differential levels of expression of genes organized in a single operon, *daaA-E*. To achieve differential level of translation, the polycistronic transcription product is cleaved in the stable *daaE* and the unstable *daaA-D* mRNAs rapidly degraded (Bilge *et al.* 1993a; Bilge *et al.* 1993b). A previous study showed that processing of the *daaA-E* transcript requires HrpA (Koo *et al.* 2004) possibly allowing the cleavage of the transcript by altering its conformation, interacting with the ribosomal RNA or by removing proteins from the transcript. However, the precise function of HrpA remains to be identified. Similar post-transcriptional interactions with mRNA transcript could explain the differences observed between the microarray and the iTRAQ results. As opposed to *E. coli*, which encodes five DEAD-box RNA helicases in addition of an ortholog of *hrpA*, the only RNA helicase predicted in the *B. burgdorferi* genome is *hrpA*. This suggests that *B. burgdorferi* HrpA might have functions that are filled by other helicases in *E. coli*. For example, the SrmB and the CsdA DEAD-box RNA helicases have been shown to be involved in the ribosome biogenesis in *E. coli* (Charollais *et al.* 2004; Charollais *et al.* 2003). Disruption of these helicases leads to a cold-sensitive phenotype in *E. coli* and a similar function filled by *B. burgdorferi* HrpA could explain the cold-sensitive phenotype.

In conclusion, a *B. burgdorferi hrpA* knockout strain was successfully complemented to confirm that HrpA is required for *B. burgdorferi* infectivity in mice and point mutations were inserted into the *B. burgdorferi* genome and used

to demonstrate that the helicase activity of HrpA is essential for the infectivity of *B. burgdorferi*.

Chapter Four: Importance of *B. burgdorferi* nucleic acid metabolizing genes for survival to DNA damage[*].

4.1 Introduction

All living organisms encounter DNA damage that, if left unrepaired, would cause mutation or lethality. Damage in the DNA can result from spontaneous base alteration or exposure to a DNA damaging agent (Aruoma *et al.* 1989a; Friedberg 2006). Bacteria encode multiple pathways for detecting and repairing damaged bases. The main pathways, base excision repair (BER), methyl-directed mismatch correction (MMC), nucleotide excision repair (NER) (including transcription-coupled repair) and homologous recombination, have been best characterized in *E. coli* (Friedberg 2006; Hanawalt and Spivak 2008; Iyer *et al.* 2006; Lu *et al.* 1983; Modrich 1989; Sancar 1996; Smith 2012; Symington and Gautier 2011; Truglio *et al.* 2006). In addition to naturally occurring DNA damage, pathogens also encounter stress from the host tissue environment and immune response. Capacity to detect and repair DNA damage is essential for a pathogen to successfully infect its host (Chakravortty and Hensel 2003; Fang 1997; O'Rourke *et al.* 2003).

The Lyme disease spirochete *Borrelia burgdorferi* is an obligate parasite transmitted to a vertebrate host, including human and mouse, by *Ixodes* ticks (Burgdorfer *et al.* 1982; Levine *et al.* 1985; Radolf *et al.* 2012; Steere *et al.*

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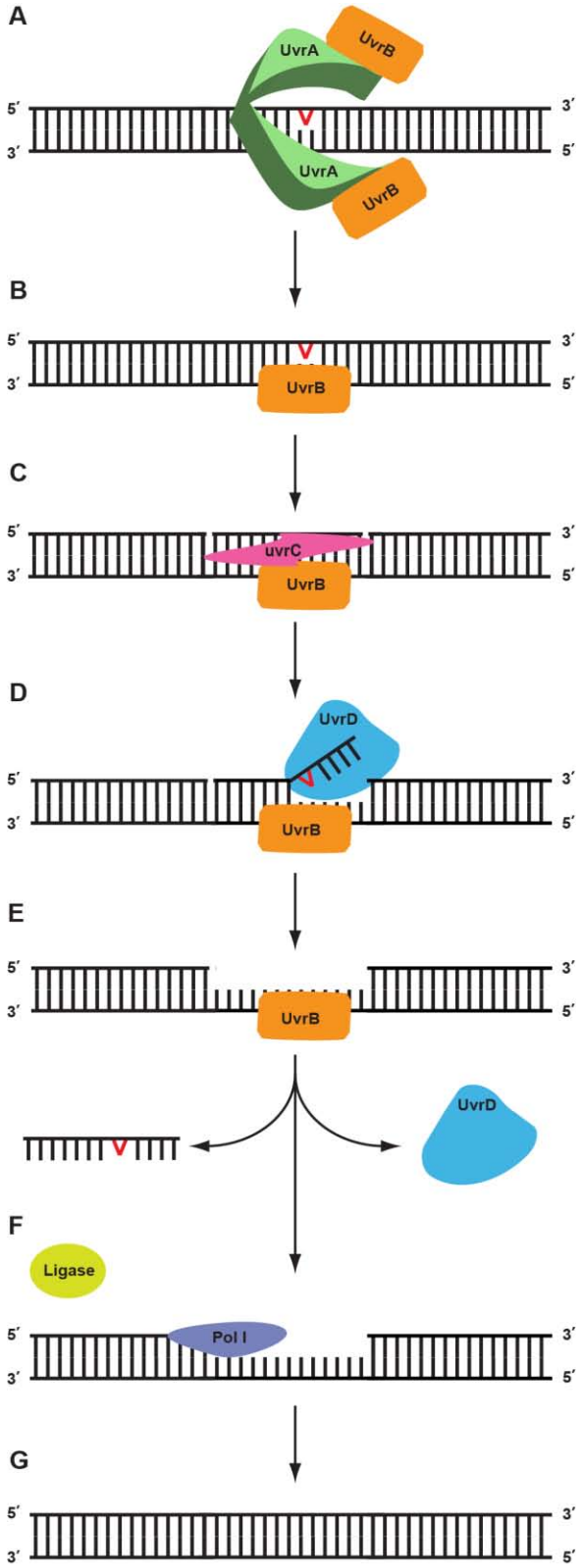
[*] All the text, experiments and figures were done by Pierre-Olivier Hardy and are included in Hardy, P.-O. and G. Chaconas (2013). "The nucleotide excision repair system of *B. burgdorferi* is the sole pathway involved in repair of DNA damage by UV light." Journal of Bacteriology, with the exception of the figures 4.8 and 4.9 and the text related, which have not been previously published.

1983b). During murine infection, *B. burgdorferi* disseminates haematogenously to multiple organs, including joint, heart, skin and bladder (Barthold *et al.* 1991). Previous studies suggested that during its infectious cycle, *B. burgdorferi* could be exposed to DNA damaging conditions and agents (Brown and Reiner 1998; Levick 1990; Li *et al.* 2007b; Menten-Dedoyart *et al.* 2012; Modolell *et al.* 1994; Ruderman *et al.* 1995; Sawyer *et al.* 1991; Sojka *et al.* 2008; Ward and Steigbigel 1978). However, little is known about the pathways used by *B. burgdorferi* to repair damaged DNA and if these systems are required for infection in mice.

Sequencing of *B. burgdorferi* revealed a relatively small but highly segmented 1.5 Mbp genome (Fraser *et al.* 1997). Interestingly, no ortholog of *lexA*, *ruvC*, *mutH*, *sbcB* and *recFOR* DNA repair/recombination genes have been identified. However, sequence comparison revealed that all four genes of the nucleotide excision repair (NER) pathway appeared to be present. The *E. coli* NER pathway is involved in detecting and removing various types of damaged bases, including damage caused by UV light (Friedberg 2006) (see Figure 4.1 for a schematic representation of the *E. coli* NER pathway). Briefly, *E. coli* UvrB is loaded at the damage site by UvrA and recruits the UvrC endonuclease, which incises the damaged DNA strand. The DNA helicase II (UvrD) displaces the damaged strand and then, Polymerase I and DNA ligase fill the gap. Amino acid sequences of *B. burgdorferi* and *E. coli* UvrA, UvrB, UvrC and UvrD are similar in

Figure 4.1. Schematic of the bacterial NER pathway in response to DNA damage.

(A) The damage site (red nucleotides) is recognised by the UvrA dimer, as part of the $UvrA_2B_2$ tetramer complex. UvrB is then loaded by UvrA to scan the DNA for damage. **(B)** Once an altered base is found, UvrB remains bound to the undamaged strand at the site of damage, forming the UvrB:DNA pre-incision complex, while $UvrA_2$ is released. **(C)** Following damage recognition, UvrB recruits the UvrC endonuclease which first cleaves the phosphodiester backbone on the damaged strand four or five nucleotides 3' from the damage and then eight nucleotides 5' from the damage site. **(D)** The damaged strand is then removed by the DNA helicase II (UvrD), leaving a gap of single-stranded DNA **(E)**. **(F)** DNA Polymerase I and DNA ligase are then recruited to fill the gap **(G)**.


A
UvrA
UvrB
5′
3′
V
3′
5′
UvrA
UvrB
B
UvrB
C
uvrC
UvrB
D
UvrD
UvrB
E
UvrB
UvrD
F
Ligase
Pol I
G

length and have 53, 50, 29 and 30% identity and 72, 68, 53 and 49% similarity, respectively (Table 4.1). Moreover, microarray results show a constitutive level of expression similar to *B. burgdorferi flgB* (Salman-Dilgimen *et al.* 2013). However, DNA repair mechanisms in *B. burgdorferi* and their importance for infectivity remains poorly understood.

In the present study, we assessed the importance of 25 *B. burgdorferi* DNA recombination/repair genes for survival to DNA damage. A previously described fluorescence assay (Kim and Surette 2004) was optimized for *B. burgdorferi* and used to expediently quantify survival of multiple *B. burgdorferi* mutants after exposure to DNA damaging agents. Surprisingly, only disruption of the *B. burgdorferi* nucleotide excision repair pathway resulted in a significantly increased sensitivity to UV light. Moreover, all four NER genes were shown to be required for repairing nitrosative, but not oxidative, damage in *B. burgdorferi*. Finally, disruption of *B. burgdorferi uvrA*, *uvrC* and *uvrD* only slightly decreased dissemination time in mice.

Table 4.1. Comparison between select *E. coli* and *B. burgdorferi* proteins involved in the homologous recombination and NER repair pathways.

Gene	*Bb* locus[1]	*E. coli* locus[2]	*Bb/Ec* protein length	% identity	% similarity	Percent expression[6] relative to *flgB*
uvrA	*bb0837*	*b4058*	950/940	52.6	72.2	264
uvrB	*bb0836*	*b0779*	673[3]/673	49.9	67.8	111
uvrC	*bb0457*	*b1913*	603[4]/588	28.7	52.5	30
uvrD	*bb0344*	*b03813*	699[5]/720	30.1	48.9	37
recA	*bb0131*	*b2699*	365/353	51.1	70.3	444
ruvA	*bb0023*	*b1861*	197/203	25.5	47.6	7
ruvB	*bb0022*	*b1860*	347/336	46.3	66.0	228
recG	*bb0581*	*b3652*	696/693	32.4	49.2	320

[1]From (Fraser *et al.* 1997).
[2]From (Blattner *et al.* 1997).
[3]An alternative met start would give a length of 668. Actual start point is unknown.
[4]An alternative met start would give a length of 580. Actual start point is unknown.
[5]An alternative met start would give a length of 698. Actual start point is unknown.
[6]Expression levels relative to *flgB* were calculated based upon micro-array data (Salman-Dilgimen *et al.* 2013).

4.2 Materials and methods

4.2.1 Strains and primers used and growth conditions

All primers used are listed in Table 4.2 and were synthesized by the University of Calgary Core DNA services (Calgary, AB, CA). Plasmids and *E. coli* strains used in this study are listed in Table 4.3 and *B. burgdorferi* strains in Table 4.4. All *E. coli* and *B. burgdorferi* cultures were done as described in the Materials and methods section of Chapter 2.

4.2.2 Disruption of *B. burgdorferi* NER genes.

The disruption of *B. burgdorferi uvrC* and *uvrD* is described in the section Materials and Methods of Chapter 2. The disruption of *uvrB* was done as described for *uvrD*. Disruption of *uvrA* was done essentially using the same strategy but the complete *uvrA* gene flanked by an additional 500 bp of upstream and 500 bp of downstream sequence was PCR amplified. The complete *uvrA* gene was replaced by a gentamicin resistance cassette. To avoid polar effects from the insertion of the gentamicin resistant cassette, gene inactivation constructs were used with the *gent* cassette in both orientations. For all the inactivated *uvr* genes at least two clones were recovered with transcriptional readthrough from the inserted gentamicin resistance cassette in the direction of the adjacent downstream gene.

Allelic exchange was confirmed by PCR and Southern blot as described in Materials and Methods of Chapter 2. *B. burgdorferi* mutants for *uvrA* and *uvrB* contain all the *B. burgdorferi* plasmids tested. Both *uvrC* mutant strains are

Table 4.2. Primers used in this study.

Primers used for knockout plasmid construction			
Genotype	**Target**	**Inverse[1]**	**Confirmation of knockout**
uvrA	B1946-GCTTAGAGAGGGCTTAGATATTCC B1947-CCAATAGCATAAAGTTTTTTAGAGTTC	B1948-NheI-gcgctagctagctagATGCGAGAATTCCTATACAGG B1949-NheI-gcgctagctagctagAAATTACACCTCTCTTTTTTTATTACG	B1921-GCAAAGGTAAACGCCTAAGC B1922-CCCATATCAATAATATAGTCCGC
uvrB	B1915-GGTGTTACAGGCAGTGGAAAG B1916-CATTAGAATTTCTAGCAGCCCTACC	B1919-NheI-gcgctagctagctagGCGCTTGAAAACAGACC B1920-NheI-gcgctagctagctagGCCAAATTTCAACAATATCTCC	B1923-GGAGAGTTTGCTTATCGAATTTG B1924-GCAGGAAGTCTAAACCCAAAG
uvrC	B1470-GCGAATACGAAGCATTGCTT B1471-GGCAATATATCTTTATAGGTTCCAAT	B1472-NheI-ctagctagctagGGGAATGCCTTTTAAAGAAAACTA B1473-NheI-ctagctagctagTTTCTCCTGGATGAACATGG	B1995-GGAAAATTAGTTGAAAGAGATGC B1996-CCATAGAAGCTACTGTTTCTTGAC
uvrD	B1178-GGGATACTAATGATGTTGTTAAATTTG B1833-CTCAGGGATAAAGATAATGTCATAAG	B1834-NheI-gcgctagctagctagGAGGAGCTGGGTAAAAAACTTTTTG B1835-NheI-gcgctagctagctagCCTCATTTGCAATGCCCAC	B1874-CAACTCAAAATAGTTCTAATAAAAGG B1875-AAATAATAAAAGAGACTCTTTGGC

Primers used for screening
B348-CGCAGCAGCAACGATGTTAC
B349-CTTGCACGTAGATCACATAAGC
B70-CATATGAGCCATATTCAACGGGAAACG
B71-AAAGCCGTTTCTGTAATGAAGGAG

[1] Lower case letters indicate sequences added for the indicated restriction site except sequences indicated by [4].

[2] Modified from (Sambir *et al.* 2011).

[3] Primer used in (Sambir *et al.* 2011).

[4] Upper and lower case letters indicate sequence from *B. burgdorferi* genome and *PflgB-kan* resistance cassette, respectively.

Primers used for complementation plasmid construction		
Gene complemented	**Target site**	**Primers**[1]
uvrA	*PflaB*	B2163-SacI-taagagctcTGTCTGTCGCCTCTTGTGGCTTC[3]
		B2164-KpnI-tgtggtaccTCATTCCTCCATGATAAAATTTAAATTTCTGAC[3]
	uvrA	B2165-KpnI-tcaggtaccTTGGAAAAAAGTTTGAAAAAAAAAATTATTGTCAGAG[3]
	bb_0838	B2166-PstI-gcattggctgcagAAGGCCCATTTTTTTAAATCCTCATC[2]
	PuvrA	B2167-SacI-gtagagctcTGCTTAGAGAGGGCTTAGATATTCCAGAAGT[3]
	bb_0838	B2168-PstI-tcatcctgcagCTGGGCAAAAATTGCATTAGAAAATGTT[3]
uvrB	*uvrB*	B2019-KpnI-ggggtaccccATGATAGATTTTTTTTTGAAGTCAG
	PflgB-kan + *uvrB*	B2020-tcttccttgaagctcgggtaTTACACCTCTCTTTTTTTATTACG[4]
	uvrB + *PflgB-kan*	B2021-CGTAATAAAAAAAGAGAGGTGTAAtacccgagcttcaaggaaga[4]
	uvrA + *PflgB-kan*	B2022-TTTTTTTTCAAACTTTTTTCCAAAAAttagaaaaactcatcgagcatc[4]
	PflgB-kan + *uvrA*	B2023-gatgctcgatgagtttttctaaTTTTTGGAAAAAAGTTTGAAAAAAAA[4]
	uvrA	B2024 -EcoRI-ccggaattccggTACCAAGCTTAATCCTATCAAC
uvrC	*uvrC*	B2083-NotI-ataagaatgcggccgctaaactatATGAAAGAGAACCTAACAAATTTATTC
		B2084r-XhoI-ccgctcgagcggTTATTTATCTTGATTATTATTTTTTATGGAG
	between *uvrC* and *bb_0456*	B2087-BamHI-cgggatcccgATTTTAAACTAGATTATTATTTATTAATATTTTTTAAAATAAA
	bb_0456	B2030-EcoRI-ccggaattccggCCAAGTGATGATGATCTGAAG
uvrC and *uvrD*	*PflgB-kan*	B2085-XhoI-ccgctcgagcggTACCCGAGCTTCAAGGAAG
		B2086-BamHI-cgggatcccgTTAGAAAAACTCATCGAGCATC
uvrD	*uvrD*	B2088-NotI-ataagaatgcggccgctaaactatGTGATGGATAAAATAAAAAAATTTTTTTC
		B2089-XhoI-ccgctcgagcggCTAAACTTTGACAATTTTTTTAATATACTCA
	bb_0343	B2092-BamHI-cgggatcccgAGGTTTATTTTGAAAGATATACATTTAAAAA
	bb_0342	B2111-XbaI-gctctagagcGGCAAATCTTCTAATTCACAATT

[1] Lower case letters indicate sequences added for the indicated restriction site except sequences indicated by [4].

[2] Modified from (Sambir *et al.* 2011).

[3] Primer used in (Sambir *et al.* 2011).

[4] Upper and lower case letters indicate sequence from *B. burgdorferi* genome and *PflgB-kan* resistance cassette, respectively.

Table 4.3. Plasmids and *E. coli* strains used in this study

Gene disruption targets and knockout plasmid attributes					
Gene target	**Locus**	**Gene description**	**Plasmid**	***E. coli* strain number (GCE)**	**Polarity of *gent* cassette**
uvrA	*bb0837*	excinuclease ABC, subunit A	pPOH64-3	2166	forward
uvrB	*bb0836*	excinuclease ABC, subunit B	pPOH61-1	2157	forward
			pPOH61-2	2158	reverse

Plasmids for gene complementation			
Gene target	**Construct description**	**Plasmid**	***E. coli* strain number (GCE)**
uvrA complementation	*PflaB-uvrA*	pPOH85-1	3200
	PuvrA-uvrA	pPOH83-2	2197
uvrB complementation		pPOH69	2175
uvrC complementation		pPOH80-2	2190
uvrD complementation		pPOH82-1	2193

Table 4.4. *B. burgdorferi* strains used in this study.

Genotype	Target locus	Description	Strain (GCB)	Missing plasmids	Reference
wt		*B. burgdorferi* strain B31 clone 5A4	920		(Purser and Norris 2000)
knockout strains					
uvrA	bb_0837	Excinuclease ABC, subunit A	565		(Hardy and Chaconas 2013)
			566		
uvrB	bb_0836	Excinuclease ABC, subunit B	545		
			546		
uvrC	bb_0457	Excinuclease ABC, subunit C	537	cp9	
			538	cp9	
uvrD	bb_0344	DNA helicase	541	lp38, lp28-4	
			542	lp38	
*recD**	bb_0623	Exodeoxyribonuclease V, alpha chain	1217		
			1218		
dnaK1	bb_0264	Heat shock protein – Chaperone	539		
			540		
exoA	bb_0534	Exonuclease III	531		
			534		
*hrpA**	bb_0827	RNA helicase	1164	lp28-2, cp9	(Salman-Dilgimen *et al.* 2011)
			1165		
			1166	cp32-3, cp32-6	
ruvB	bb_0022	Holliday junction helicase	513		(Dresser *et al.* 2009)
			514	lp28-4, cp9	
nth	bb_0745	endonuclease III	525		
			526		
mutS1	bb_0797	mismatch repair protein	502		
			504		
*recJ**	bb_0254	ssDNA-specific exonuclease	1153	lp28-2	
			1154		
*priA**	bb_0014	helicase	1205		
			1206	cp9	
*sbcD**	bb_0829	exonuclease	1251		
			1252		
*ruvA**	bb_0023	Holliday junction helicase	1174		
			1175		
*mutL**	bb_0211	mismatch repair protein	1178		
			1179	cp9, cp32-3	
*sbcC**	bb_0830	exonuclease	1248		
			1249		

Genotype	Target locus	Description	Strain (GCB)	Missing plasmids	Reference
knockout strains					
*BBG32**	bb_G32	putative helicase	1233		(Dresser *et al.* 2009)
			1234		
*mutS2**	bb_0098	mismatch repair protein	1135		
			1136		
*recA**	bb_0131	DNA-dependent ATPase	1284	cp9	
			1285	cp9	
*recG**	bb_0581	ATP-dependent helicase	1155		
			1156		
*rep**	bb_0607	ssDNA-dependent ATPase helicase	1158		
			1159		
*nucA**	bb_0411	exonuclease involved in competency	1176	lp21,cp32-3	
			1177		
*mag**	bb_0422	3'-methyladenine DNA glycosylase	1161		
			1162	lp28-4	
*mfd**	bb_0623	transcription-repair coupling factor	1180		
			1181		
complemented strains					
$uvrA^{R+}$			*PflaB-uvrA* - 567	lp21	(Hardy and Chaconas 2013)
			PuvrA-uvrA - 568		
$uvrB^{R+}$			562		
			563		
$uvrC^{R+}$			553	cp9	
			554	lp28-4, cp9	
$uvrD^{R+}$			555	lp28-4, lp36, lp38	
			557	lp36, lp38	

* *B. burgdorferi* mutants were made by Ashley R. Dresser (Dresser 2009).

missing *cp9*, the *uvrD* mutant clone 3 is missing lp38 and lp28-4 and clone 4 is missing only lp38.

4.2.3 Complementation of *uvrA*, *uvrB*, *uvrC* and *uvrD*

Complementation of *uvrB*, *uvrC* and *uvrD* was achieved by allelic exchange, as described for *hrpA* in Materials and Methods of Chapter 3. For all complementing clone constructions the DNA sequence of the *uvr* gene from the transforming plasmids was verified. For *uvrB*, the ORF was PCR amplified from *B. burgdorferi* B31 clone 5A4 and fused in order with a *flgB*-driven kanamycin resistance cassette (*PflgB-kan*) and the 500 bp sequence downstream of *uvrB* by overlap extension PCR. The *PflgB-kan* gene was first amplified from pBSV2 (Stewart *et al.* 2001). The PCR product was then cloned into pJET1.2/blunt vector (Fermentas, Burlington, ON, Canada) and used to transform the *B. burgdorferi uvrB* mutant. For *uvrC* and *uvrD*, the first 500 bp of DNA downstream from the target gene was PCR amplified from *B. burgdorferi* B31 clone 5A4 and inserted into the pJET1.2/blunt vector. *PflgB-kan* was then PCR amplified from pBSV2 (Stewart *et al.* 2001) and cloned into the construct using BamHI and XhoI restriction sites. Finally, *uvrC* and *uvrD* ORFs were amplified from *B. burgdorferi* B31 clone 5A4 and cloned into their respective constructs using BamHI and XbaI restriction sites. Each construct was used to transform its respective *B. burgdorferi* mutant and cultivated in the presence of kanamycin (200 µg/ml) for selection. Furthermore, potential clones were tested for growth in the presence of gentamicin to confirm the replacement of the gentamicin resistance cassette by the wild-type sequence.

Allelic exchange was confirmed by PCR for *PflgB-kan*, the absence of the gentamicin resistance cassette, the presence of the sequence that was deleted in the knockout mutant and for the size of the gene target. Since all our complementing constructs resulted in restoration of a wild-type phenotype in *B. burgdorferi,* DNA sequencing of the *uvr* genes in the *B. burgdorferi* constructs was not undertaken.

For *uvrA*, reversion of the mutant to wild type could not be accomplished. Therefore, we generated plasmids for complementation *in trans*. Two constructs similar to the plasmids previously described by Sambir et al. (Sambir *et al.* 2011) were assembled. The first construct contained a *PflaB*-driven *uvrA* gene and the second plasmid contained the *uvrA* ORF with a region upstream of the gene, possibly encoding the native promoter. For the first construct, the *PflaB* promoter was amplified from *B. burgdorferi* B31 clone 5A4 using primers B2163 and B2164 and cloned into the shuttle plasmid pBSV2 using SacI and KpnI restriction sites. The *uvrA* ORF was then PCR amplified from *B. burgdorferi* B31 clone 5A4 using primers B2165 and B2166 and cloned into the construct using KpnI and PstI restriction sites, to obtain pPOH85-1. For the second construct, the *uvrA* ORF with 500 bp of DNA upstream were PCR amplified using primers B2167 and B2168 and cloned in pBSV2 using SacI and PstI restriction sites, respectively, to obtain pPOH83-2. The complementation constructs were used to transform *B. burgdorferi uvrA* mutant. One clone complemented with each construct was used in all experiments testing for *uvrA* complementation. The *uvrA* complemented clone *PflaB-uvrA* is missing lp21, the *uvrC* complemented clone 2 is missing cp9,

the clone 3 is missing lp28-4 and cp9, the *uvrD* complemented clone 2 is missing lp28-4, lp36 and lp38 and the clone 9 is missing lp36 and lp38.

4.2.4 Growth curve of *B. burgdorferi uvr* mutants.

Each *B. burgdorferi uvr* mutant was grown to a density of 1 x 10^7 to 5 x 10^7 spirochetes/ml and diluted to 1 x 10^4 spirochetes/ml in fresh culture medium with 1x *Borrelia* antibiotics cocktail. Each mutant was grown with or without 5% blood that was obtained from BALB/c mice (Charles River, St-Constant, QC) by cardiac puncture using a 1 ml syringe coated with heparin (100 units/ml). Tubes were inverted twice a day. Every 48 hours, cultures were centrifuged 5 minutes at 600 x g at 4°C. The upper phase was transferred in a new tube with fresh blood. Cell densities were determined by counting using a dark-field microscope at the indicated time points. Statistical significance was determined using a Student's *t*-test comparing the culture grown in presence or in absence of blood for a particular mutant at the last three time points.

4.2.5 UV light survival assay

To test the sensitivity of various *B. burgdorferi* mutants to UV light, cultures were grown to a density of 1 x 10^7 to 5 x 10^7 spirochetes/ml, harvested and resuspended at 1 x 10^8 bacteria/ml in PBS. For each sample, 10^7 cells were exposed on a 35-mm petri dish to 0, 1, 2, or 3 mJ/cm^2 of 254 nm UV light (Stratalinker UV crosslinker 1800, Stratagene, La Jolla, CA, USA). UV exposure conditions were empirically chosen to result in about 80% survival of the wild-type *B. burgdorferi* strain at the maximal dose, with our starting point based upon previously reported conditions (Lin *et al.* 2009; Liveris *et al.* 2008; Sambir *et al.*

2011). The UV exposure range was similar to that typically used in *E. coli*. Immediately following exposure to UV light, the samples were diluted 1:100 in fresh culture media and incubated 36 to 48 hours.

The growth of each clone was determined using a method adapted from a previously reported protocol (Kim and Surette 2004) (see Figure 4.2A). Cell density of the untreated wild-type *B. burgdorferi* was determined by counting with a dark-field microscope. When the culture reached 5 x 10^7 to 1 x 10^8 cells/ml, the same volume from each sample (equivalent to 10^8 spirochetes for the counted culture) was centrifuged and spirochetes where washed twice with PBS. Cells were resuspended in 90 µl of water and incubated 95°C for 5 minutes. The DNA was stained by adding 10 µl of 34 µM Syto-9 (Invitrogen, Burlington, ON, Canada) in a 96 or 384-well plate and incubated 30 minutes at room temperature. The fluorescence was measured using a plate reader (Wallac 1420 VICTOR^2, PerkinElmer, Waltham, MA, USA) with 0.5 second exposure time using a fluorescein filter. The percent relative fluorescence represents the fluorescence after UV light exposure compared to no exposure for a particular strain. Two independent clones were used for each mutated gene target. Experiments were done in triplicate.

Alternatively, the cell density of each culture was determined by direct counting using a dark-field microscope. Each clone was exposed to 0 and 1 mJ/cm^2 of UV light and cultured as described when the fluorescence assay was used to compare sensitivity to UV light. When the culture of untreated wild-type

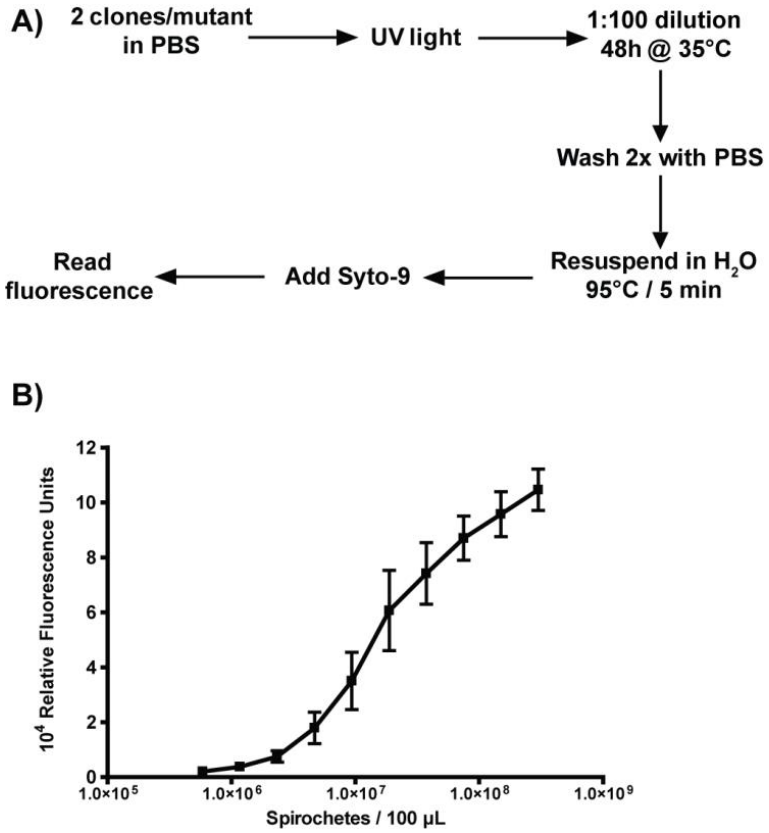


Figure 4.2 Method used to assess sensitivity of *B. burgdorferi* to UV light.

(A) Flow chart of the method used. Each *B. burgdorferi* clone was resuspended in PBS and exposed to the indicated dose of UV light. Each sample was then diluted 1:100 in BSK and cultured until the untreated control reached late exponential phase. Spirochetes were lysed, the DNA was stained with Syto-9 and the fluorescence of each sample was measured using a plate reader. The method was adapted from (Kim and Surette 2004). (**B**) Correlation between number of spirochetes per sample and the level of fluorescence observed in the assay described above. Samples were analyzed in triplicate and the mean and standard deviation were plotted.

B. burgdorferi reached a cell density between 5 x 10^7 and 1 x 10^8 spirochetes per ml, the cell density of every culture was determined. Cell density could not be determined if less than 5 x 10^4 spirochetes per ml were present. Three experiments done using two clones per mutated gene.

4.2.6 Endonuclease sensitivity site (ESS) assay

The ESS assay was adapted for *B. burgdorferi* from (Sutherland and Shih 1983) and (Oguma *et al.* 2001) (see Figure 4.4A in Results section). When a culture of *B. burgdorferi* lacking *uvrC* (GCB 537) reached 5 x 10^7 spirochetes per ml, cells were resuspended in PBS at 1.5 x 10^8 spirochetes per ml. Cells were either left untreated or were exposed to 15 mJ/cm^2 of 254 nm UV light (Stratalinker UV crosslinker, Stratagene, La Jolla, CA, USA) in 100 µl aliquots in 35 mm cell culture dishes (BD Falcon, Franklin Lakes, NJ USA). Immediately following exposure cells were lysed (100 mM Tris pH 8.5, 10 mM EDTA, 30 mM NaCl and 0.5% SDS), treated with RNase A (60 µg/ml), then Proteinase K (75 µg/ml). Genomic DNA was extracted with phenol/chloroform. To detect pyrimidine dimers, 1.2 µg of genomic DNA was either left undigested or incubated with either 10 or 15 units of T4 pyrimidine dimer glycosylase (PDG, New England BioLabs, Pickering, ON, Canada), following the supplier instructions, for 30 minutes at 37°C. The reaction was then stopped by addition of an alkaline loading buffer (100 mM NaOH, 1 mM EDTA, 2.5% Ficoll and 0.05% bromocresol green) and loaded on a 10 cm, 0.5% alkaline agarose gel (Oguma *et al.* 2001) run in alkaline buffer (300 mM NaOH, 10 mM EDTA) for 16 hours at 15 V with buffer recirculation. Following electrophoresis, the gel was

incubated 2 hours in neutralization buffer (1 M Tris, 1.5 M NaCl, pH 7.4) and the DNA was stained with SYBR Gold (Invitrogen, Burlington, ON, Canada).

4.2.7 Reactive nitrogen survival assay

Sensitivity to RNS damage was done as previously described (Bourret *et al.* 2011). Cultures were grown to a density of $1x10^7$ to $5x10^7$ cells ml^{-1}, then spirochetes were pelleted by centrifugation at 6,000 x g for 15 minutes at 4°C and resuspended in culture media at $5x10^7$ spirochetes per ml with 2.5 mM DEA/NO (Diethylamine NONOate diethylammonium salt, Sigma-Aldrich, Oakville, ON, Canada) or with culture media only. Samples were then incubated 4 hours at 35°C, diluted 1:100 in fresh culture media and incubated at 35°C until the culture of treated wild-type *B. burgdorferi* B31 clone 5A4 reached $5x10^7$ spirochetes per ml. Spirochete densities were determined by direct counting on a dark-field microscope. The percent survival for each mutant strain was determined as the number of surviving spirochetes divided by the number of surviving spirochetes in the treated wild-type strain. Experiments were done in triplicate using two clones for each mutated gene target.

4.2.8 Sensitivity of *uvr* mutants to oxidative damage.

Sensitivity to ROS damage was done as previously described (Boylan *et al.* 2008). Cultures were grown to a density of $1x10^7$ to $5x10^7$ cells ml^{-1}, then spirochetes were pelleted by centrifugation at 6,000 x g for 15 minutes at 4°C and resuspended in culture media at $5x10^7$ spirochetes per ml with 10 mM *tert*-Butyl hydroperoxide, Sigma-Aldrich, Oakville, ON, Canada) or with culture media only. Samples were then incubated 4 hours at 35°C, diluted 1:100 in fresh culture

media and incubated at 35°C until the culture of treated wild-type *B. burgdorferi* B31 clone 5A4 reached $5x10^7$ spirochetes per ml. Spirochete densities were determined by direct counting on a dark-field microscope. The percent survival compares spirochete densities of treated and untreated culture for each particular mutant strain. Error bars represent standard deviation from three experiments done using two clones for each mutated gene target.

4.2.9 Mouse infection studies

All animal studies were carried out in accordance with the principles outlined in the most recent policies and *Guide to the Care and Use of Experimental Animals* by The Canadian Council on Animal Care. Our animal protocol (AC12-0070) was approved by The Animal Care Committee of the University of Calgary. Each three to four week old male C3H/HeNCrl mouse (Charles River, St-Constant, QC) was infected by both subcutaneous and intraperitoneal injection of 10^3 spirochetes at each site. Three mice were infected with each clone and two independent clones were used for each NER gene disrupted, resulting in a total of 6 mice infected per gene target. On day 7 post-infection approximately 50 µl of blood was recovered from the saphenous vein and diluted into culture media for the presence of *B. burgdorferi*. On days 14 and 21 two ear punches were taken and on day 23, the heart, the ear, the bladder and the knee joint were collected and transferred into culture media for growth of spirochetes. Cultures were considered positive for *B. burgdorferi* when spirochetes could be observed by dark-field microscopy.

4.3 Results

4.3.1 An expeditious fluorescence assay for *B. burgdorferi* sensitivity to DNA damage.

Previous reports on testing DNA damage in *B. burgdorferi* were based on a protocol designed for *E. coli* where treated samples were plated and cultured until colonies could be counted (Lin *et al.* 2009; Liveris *et al.* 2008; Miller 1992). However, solid plating requires incubation for two to three weeks before individual *B. burgdorferi* colonies can be observed. To more expediently monitor the DNA damage of two clones for each of 25 mutated target genes, a more efficient strategy was adapted from Kim and Surette (Kim and Surette 2004) where the growth of swimmer and swarmer populations of *Salmonella enterica* serovar Typhimurium were followed and compared by measuring the total concentration of DNA using a fluorescent stain.

The adaptation of this strategy for *B. burgdorferi* (Figure 4.2A) is described in Materials and Methods. As an easy, controllable and reproducible method of inducing DNA damage, we chose exposure to UV light. Briefly, following UV light irradiation, cells were diluted 1:100 in culture medium and incubated 48 hours until they reached late exponential phase. Cells were then washed with PBS, lysed and the DNA stained by the addition of the fluorescent dye Syto-9. The level of fluorescence was measured in 100 µl samples using a plate reader. PBS washes prior to lysis were required because residual BSK medium, even if lacking phenol red, generated a strong fluorescence background. It is important to note that the fluorescence assay quantifies only living cells that have grown

after a 48 hour incubation period following the UV irradiation. Dead cells do not grow and even when all spirochetes are killed by irradiation, following the 1:100 dilution their concentration is too low to contribute any background fluorescence. The cell density of a given UV light-treated strain versus the untreated strain, after 48 hours of outgrowth, was used as a measure of the UV sensitivity of the strain.

Before using this strategy to compare the growth of cultures treated with UV light, serial dilutions of a growing *B. burgdorferi* culture were used to confirm a correlation between the number of cells and the amount of fluorescence measured (Figure 4.2B). When lysed samples contained between 10^7 and 10^8 spirochetes per 100 µl, the level of fluorescence detected reflected the number of cells lysed. This positive correlation established that the fluorescence assay could be used as an alternative strategy to rapidly estimate the growth of multiple liquid cultures of *B. burgdorferi*.

4.3.2 Most replication/repair genes do not affect UV sensitivity in *B. burgdorferi.*

A wide variety of *E. coli* genes are involved in resistance to UV radiation. However, only a few *B. burgdorferi* mutants have been tested for UV sensitivity (Lin *et al.* 2009; Liveris *et al.* 2008). Surprisingly, disruption of neither the *recA* recombinase nor the Holliday junction branch migrase subunit *ruvA* resulted in a significant effect upon *B. burgdorferi* survival. In the present study, sensitivity to UV light of 21 mutant genes not part of the Nucleotide Excision Repair pathway in *B. burgdorferi* was assessed. For each mutated gene, two independent clones

were exposed to 0, 1, 2 or 3 mJ/cm^2 UV light and their capacity to grow was determined using the fluorescence assay. For each UV dose, the relative fluorescence of the mutant was compared to the wild-type strain to establish its level of sensitivity (see Figure 4.3). The disruption of *dnaK1*, *bbg32*, *exoA*, *hrpA*, *mag*, *mfd*, *mutL*, *mutS1*, *mutS2*, *nth*, *nucA*, *priA*, *recA*, *recD*, *recG*, *recJ*, *rep*, *ruvA*, *ruvB*, *sbcC* or *sbcD* did not result in a significant increase in *B. burgdorferi* sensitivity to UV light. Disruption of *mutS1* and *sbcC* (but not *sbcD*) resulted in a significant decrease in *B. burgdorferi* sensitivity to UV radiation, but only at the highest dose used (3 mJ/cm^2). The magnitude of the decrease was only a 0.7 and 1.5 fold difference from wild type for *sbcC* and *mutS1*, respectively.

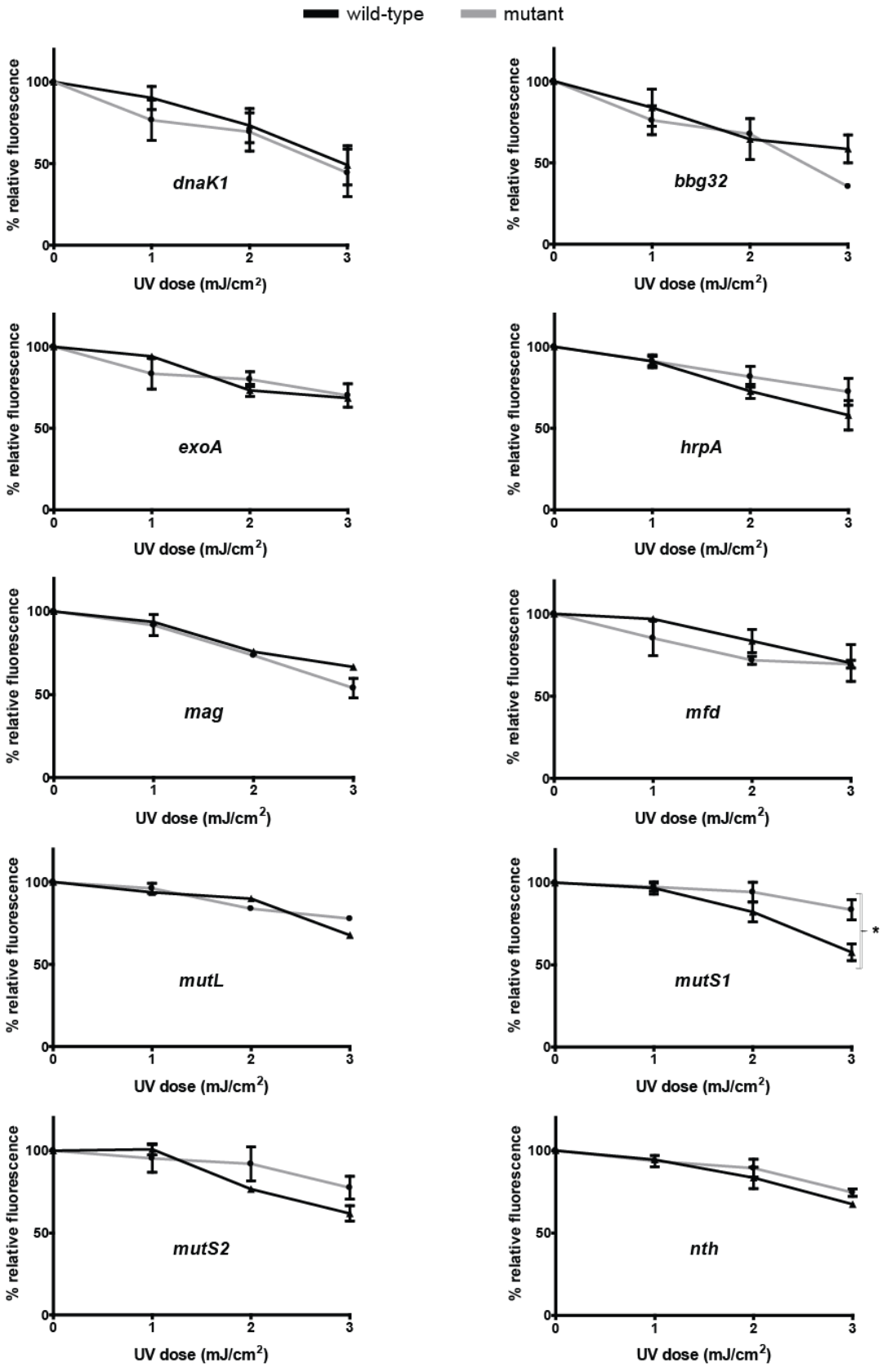
Mutants for 12 genes known to increase *E. coli* sensitivity to UV light have not been tested in *B. burgdorferi* for various reasons. No mutants could be recovered for *ssb*, *recB*, *recC* and *ligA*. *dnaX* encodes the τ and γ subunits of DNA polymerase III and so, was expected to be essential as it is in *E. coli* (Blinkova *et al.* 1993; Gerdes *et al.* 2003), and disruption of *pnp* was not attempted because its impact on *E. coli* resistance to UV light has only been shown more recently (Rath *et al.* 2012). Finally, no orthologs of *recX*, *ruvC*, *recF*, *recR*, *recO* and *mutH* could be found in *B. burgdorferi* sequenced genome (Fraser *et al.* 1997).

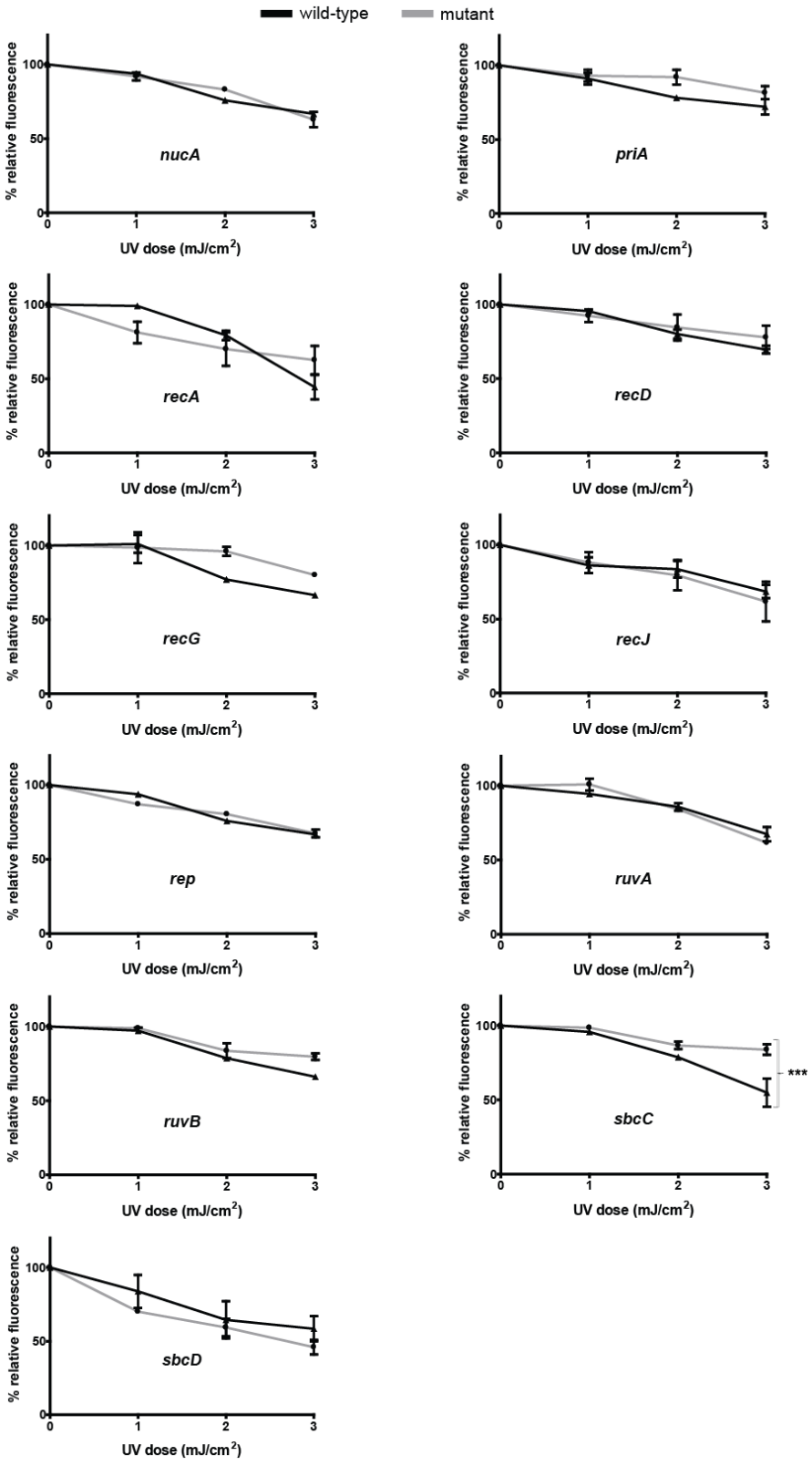
4.3.3 *B. burgdorferi* DNA is susceptible to UV damage

The lack of an effect from the mutation of 21 replication/repair genes on UV sensitivity in *B. burgdorferi* suggested the possibility that *B. burgdorferi* was significantly resistant to UV damage at the DNA level. In order to detect

Figure 4.3. Sensitivity of *B. burgdorferi* mutants to UV light.

The fluorescence assay was used to determine the level of sensitivity to UV light for 21 *B. burgdorferi* DNA replication/repair mutants. Each mutant was treated with 0, 1, 2 and 3 mJ/cm^2 of 254nm UV light. The percent relative fluorescence represents the percent fluorescence of a treated sample compared to the untreated culture for a particular clone. Each graph presents a particular mutant (grey line) and the wild-type control that was run at the same time (black line). The background level of fluorescence (~5%) was not subtracted from the plotted values. Error bars represent standard error from at least three experiments done with two clones for each mutated gene. *P denotes a P value corresponding to < 0.05 for a given UV dose and ***P a P value of < 0.001.


wild-type
mutant
% relative fluorescence
UV dose (mJ/cm2)
dnaK1
bbg32
exoA
hrpA
mag
mfd
mutL
mutS1
mutS2
nth
*


wild-type
mutant
% relative fluorescence
UV dose (mJ/cm²)
nucA
priA
recA
recD
recG
recJ
rep
ruvA
ruvB
sbcC
***
sbcD

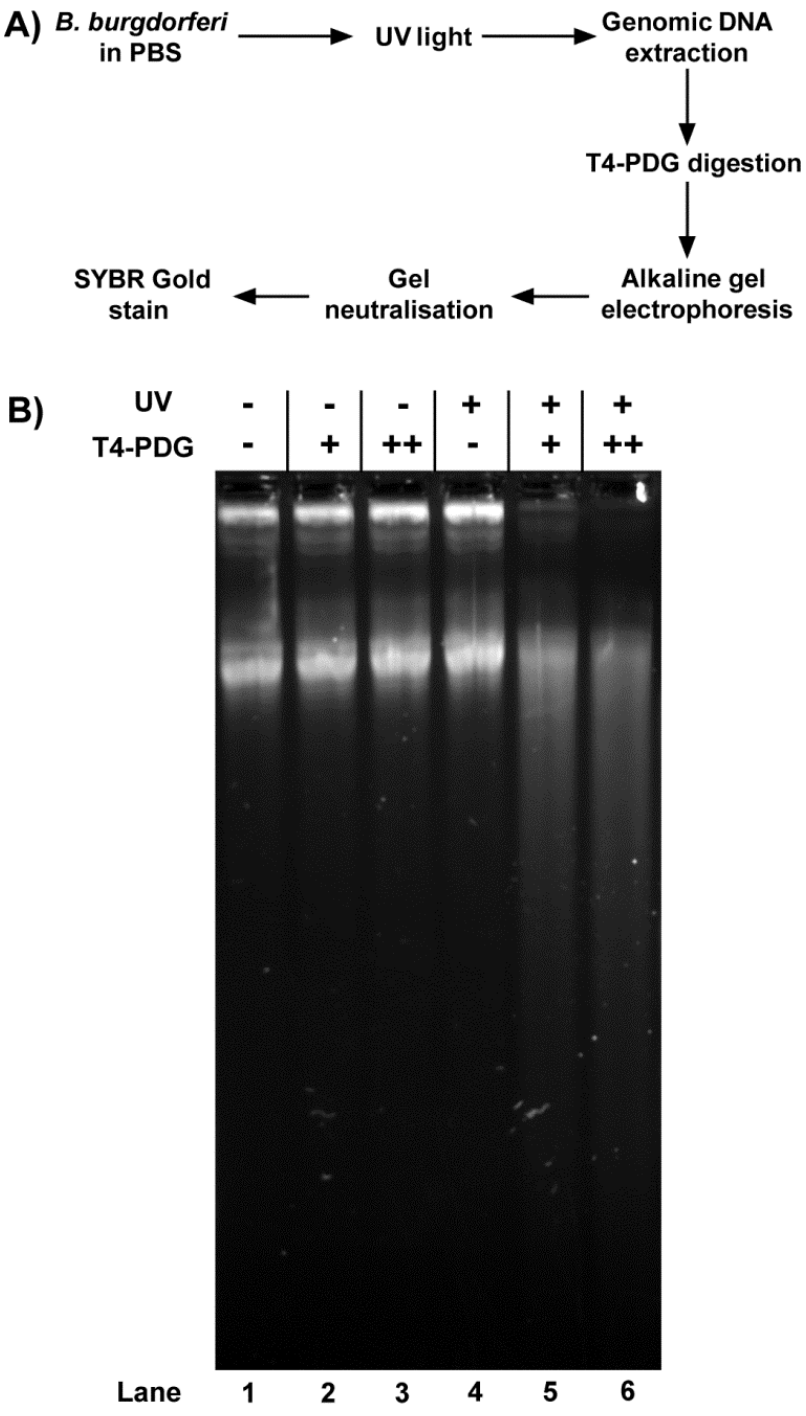
UV-induced DNA damage, *B. burgdorferi* genomic DNA was extracted after exposure to UV light and analyzed using an endonuclease sensitive site (ESS) assay (Figure 4.4A) (Achey *et al.* 1979; Oguma *et al.* 2001; Sutherland and Shih 1983). In this assay, the damaged DNA is incubated with T4 pyrimidine dimer glycosylase (PDG), an endonuclease that recognizes *cis-syn* cyclobutane pyrimidine dimers (CPD), the most common type of DNA damage generated by UV-C light (Dodson and Lloyd 1989; Friedberg 2006; Gordon and Haseltine 1980). The PDG-treated DNA is then separated by alkaline gel electrophoresis to detect single-strand DNA breaks. In *E. coli*, the ESS assay allows determination of the number of breaks/damaged sites generated in the DNA. However, because of the segmented nature of its genome, this was not possible in *B. burgdorferi*. Nonetheless, exposing *B. burgdorferi* to both UV light and PDG (Figure 4.4B, lanes 5 and 6) clearly increased the migration rate of the DNA compared to DNA from UV-treated *B. burgdorferi* that was not treated with PDG (lane 4) or DNA from non-UV treated cells that was incubated with PDG (lanes 2 and 3). This confirms that UV light does generate pyrimidine dimers in *B. burgdorferi* DNA.

4.3.4 All four nucleotide excision repair (NER) genes are required to repair UV-induced and nitrosative DNA damage

Although many genes influence *E. coli* resistance to UV light, disruption of the NER excinuclease UvrABC have the most dramatic impact and disruption of *uvrD* results in a more intermediate sensitivity phenotype (Kuemmerle and Masker 1980). To confirm if the NER pathway has a similar role in *B. burgdorferi*,

Figure 4.4. UV-induced DNA damage in *B. burgdorferi*.

(A) Flow chart representation of the endonuclease sensitive site (ESS) assay used to detect DNA damage in *B. burgdorferi* DNA after exposure to UV light (Oguma *et al.* 2001; Sutherland and Shih 1983). Briefly, *B. burgdorferi* was resuspended in PBS and exposed to UV light. Genomic DNA was then extracted and digested with T4 pyrimidine dimer glycosylase (PDG) before being run on an alkaline gel to determine the presence or absence of damage induced by UV light. **(B)** Ethidium bromide stained agarose gel from an ESS assay to monitor *B. burgdorferi* DNA damage by UV light. Genomic DNA recovered from a *B. burgdorferi uvrC* mutant exposed to 0 or 15 mJ/cm^2 of UV light as indicated was either left undigested or digested with 10 (+, Lanes 2 and 5) or 15 (++, Lanes 3 and 6) units of T4 PDG.


A)
B. burgdorferi in PBS
UV light
Genomic DNA extraction
T4-PDG digestion
Alkaline gel electrophoresis
Gel neutralisation
SYBR Gold stain
B)
UV - - - + + +
T4-PDG - + ++ - + ++
Lane 1 2 3 4 5 6

mutants for the *uvrA*, *uvrB*, *uvrC* and *uvrD* genes were tested for their resistance to UV light using the fluorescence assay. As described for the other replication/repair gene mutants, each clone was exposed to 0, 1, 2 and 3 mJ/cm^2 of UV light and assayed for their level of survival as described in Figure 4.2A. The fluorescence for all four mutants treated with UV light was similar to the background level, even at 1 mJ/cm^2, the lowest dose of UV light used (Figure 4.5). The *uvrA* data confirms a previous report by Sambir *et al.* showing that this gene is required for *B. burgdorferi* survival after exposure to UV light (Sambir *et al.* 2011). Complementation of each NER gene restored *B. burgdorferi* UV resistance to a level similar to the wild-type strain. We conclude that all four *uvr* genes are required for survival of *B. burgdorferi* after UV light exposure.

Previous studies showed that an *E. coli uvrD* mutant is not as sensitive to UV light as the *uvrA*, *uvrB* and *uvrC* mutants (Kuemmerle and Masker 1980), but such a difference was not observed in *B. burgdorferi* when the fluorescence assay was used to compare culture densities (Figure 4.5). However, this assay has a limited dynamic range. We therefore used direct dark-field counting to investigate growth differences greater than tenfold in *B. burgdorferi* wild-type and *uvr* knockout strains. Spirochetes were exposed to either 0 or 1 mJ/cm^2 UV light and cultured as described for the fluorescence assay. When the untreated wild-type *B. burgdorferi* reached a cell density between 5 x 10^7 to 1 x 10^8 spirochetes per ml, the density of each culture was determined by direct counting using a dark-field microscope. The level of survival of each clone treated with

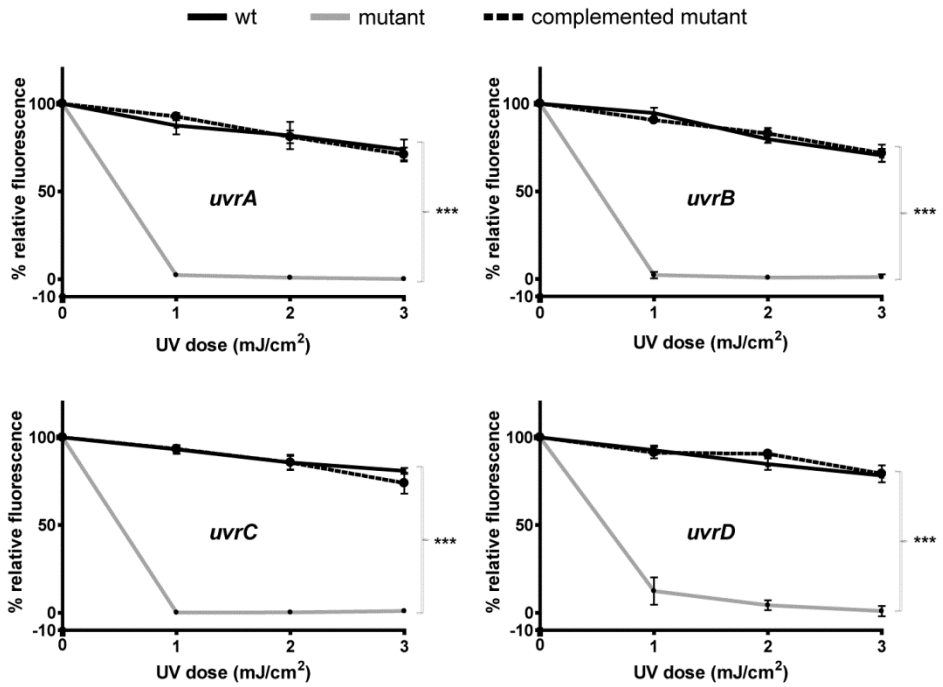


Figure 4.5. Sensitivity of *uvr* mutants to UV light.

The fluorescence assay was used to evaluate the sensitivity of *uvrA*, *uvrB*, *uvrC* and *uvrD* mutants (grey line) and of their respective complemented strains (black dotted line) compared to the wild type (solid black line). Two clones for each mutant and their respective complemented clones were exposed to 0, 1, 2 and 3 mJ/cm^2 of 254nm UV light. For the complemented *uvrA* mutant both complemented clones were used and the data from the two averaged. The percent relative fluorescence represents the fluorescence after UV light exposure compared to no exposure for a particular strain. Error bars represent the standard deviation from at least three experiments done with two clones per mutant. ***P denotes a P value corresponding to < 0.001 for the comparison between a given mutant and the wild-type control for a given UV dose.

UV light was then compared to the untreated culture for a particular strain. Similarly to *E. coli*, disruption of *B. burgdorferi uvrD* resulted in a 10 to 20-fold increase in sensitivity to UV light while exposure of *uvrA*, *uvrB* and *uvrC* mutants resulted in a more than 1000-fold increase in sensitivity (Figure 4.6). The cell density of the treated *uvrA*, *uvrB* and *uvrC* mutant cultures was below 5 x 10^4 spirochetes per ml, the lowest density that can be determined by direct counting. The correspondence of UV sensitivity for the *uvrA,B,C* versus *uvrD* mutants in both *B. burgdorferi* and *E. coli* underscores the functional similarity of the NER pathway in both organisms.

Although reactive nitrogen species (RNS) have been shown to kill *B. burgdorferi* primarily through S-nitrosylation of proteins, disruption of *uvrB* or *uvrC* (Bourret *et al.* 2011), but not *uvrA* (Sambir *et al.* 2011), has also been shown to significantly reduce *B. burgdorferi* survival to RNS-induced DNA damage. We assessed the importance of all four genes in the NER pathway in response to RNS stress by comparing the growth of *B. burgdorferi* lacking *uvrA*, *uvrB*, *uvrC* or *uvrD* with a wild-type strain following exposure to the nitric oxide (NO) donor DEA/NO. For this experiment, spirochete numbers were determined by direct counting using a dark-field microscope. This allowed for detection of a greater range of effect than the fluorescence assay but limits the number of cultures that can be analyzed. All four *B. burgdorferi* NER mutants showed between a 10 and 20-fold decrease in survival when compared to the wild-type strain (Figure 4.7). There was no significant difference between the wild-type strain and all complemented clones. In addition to *B. burgdorferi* NER mutants,

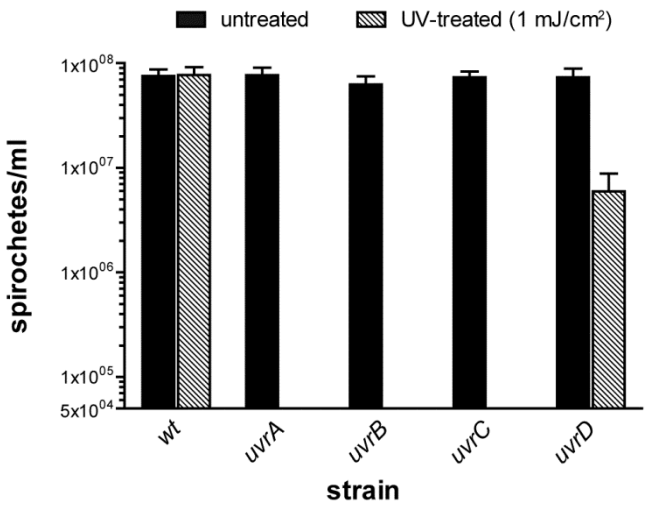


Figure 4.6. Survival of *B. burgdorferi uvr* mutants after exposure to UV light.

To detect a greater range of effect than possible with the fluorescence assay, the cell density of each culture was determined by direct counting using a dark-field microscope. Each clone was exposed to 0 and 1 mJ/cm^2 of UV light and cultured as described when the fluorescence assay was used to compare sensitivity to UV light. When the culture of untreated wild-type *B. burgdorferi* reached a cell density between 5 x 10^7 and 1 x 10^8 spirochetes per ml, the cell density of every culture was determined and is shown on the graph. Cell density could not be determined if less than 5 x 10^4 spirochetes per ml were present. Error bars represent standard deviation from three experiments done using two clones per mutated gene.

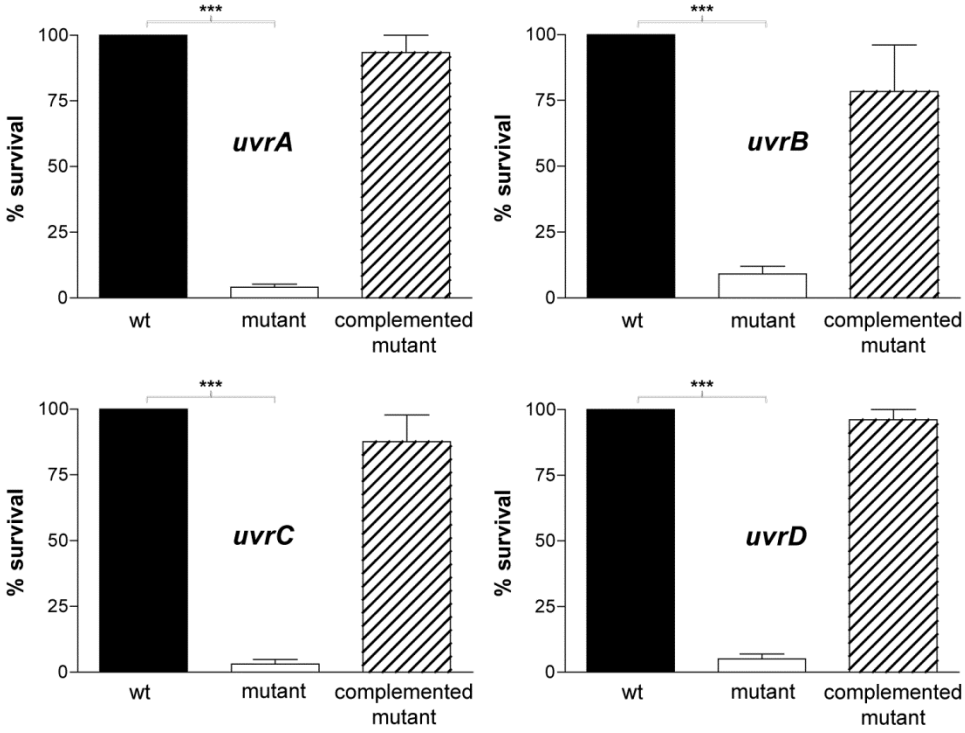


Figure 4.7. Sensitivity of *uvr* mutants to nitrosative damage.

Survival of *uvrA*, *uvrB*, *uvrC* and *uvrD* (white bar) compared with their respective complemented clones after *B. burgdorferi* exposure to 2.5 mM DEA NONOate. After treatment, the samples were diluted in fresh culture media and cultivated until the treated wild-type culture reached $5x10^7$ cells/ml. Spirochete densities were determined using a counting chamber in a dark-field microscope. The percent survival compares each particular mutant strain to the wild-type *B. burgdorferi* after treatment. Error bars represent standard deviation from three experiments done using two clones per mutant. ***P denotes a P value corresponding to < 0.001 for a given UV dose.

the sensitivity of *mfd*, *sbcC*, *exoA*, *ruvB*, *recG*, *mutS*, *recA*, *nth*, *recJ* and *priA* mutants was also tested but none of the mutants displayed an increased sensitivity to DEA/NO (data not shown).

Our results point to a requirement for all genes of the NER pathway for *B. burgdorferi* to repair nitrosative DNA damage and once again suggest that as for UV damage, the NER system in the sole pathway for repair of nitrosative DNA damage. There was no effect of oxygen radicals on the survival of mutants in the NER pathway (Figure 4.8) as expected based upon previous work that established that oxidative damage is limited to the outer membrane and does not damage DNA in *B. burgdorferi* (Boylan *et al.* 2008). This is because of a lack of iron in the cell and a corresponding absence of Fenton chemistry (Posey and Gherardini 2000). However, the disruption of *bicA*, which encodes for the iron and copper-binding protein BicA, was recently shown to increase the sensitivity of *B. burgdorferi* to oxidative stress (Wang *et al.* 2012). The generation of a double mutant by the disruption of *uvrA*, *uvrB* or *uvrC* in addition of the disruption of *bicA* could result in a more important increase in sensitivity to oxidative stress.

4.3.5 Disruption of *B. burgdorferi* NER system had a minor effect on murine infection.

The nucleotide excision repair pathway is required for survival to reactive nitrogen stress, which is known to affect infectivity of pathogens (Darwin and Nathan 2005). Although previous studies have described the role of some NER genes for resistance to DNA damage in *B. burgdorferi* (Bourret *et al.* 2011; Sambir *et al.* 2011), our current study is the first time that the importance of the

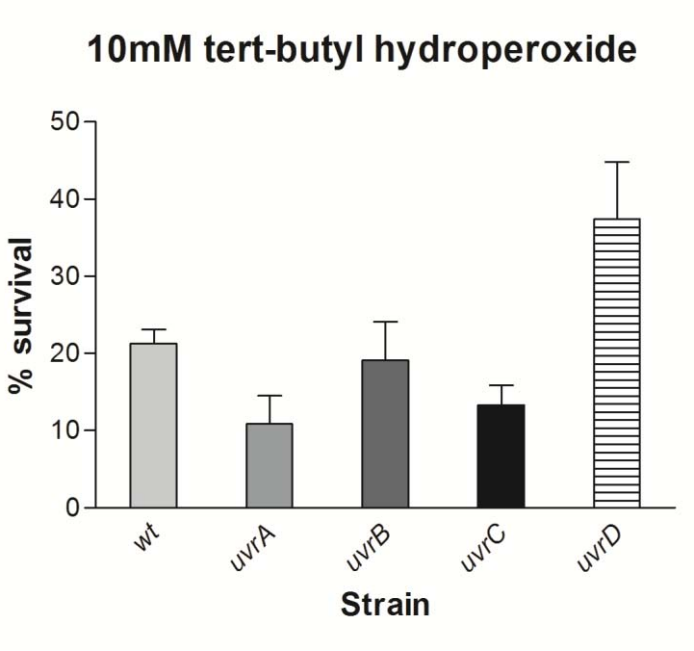


Figure 4.8 Sensitivity of *uvr* mutants to oxidative damage.

Survival of *uvrA*, *uvrB*, *uvrC* and *uvrD* (white bar) compared with the wild-type parental *B. burgdorferi* strain after exposure to 10 mM *tert*-Butyl hydroperoxide. After treatment, the samples were diluted in fresh culture media and cultivated until the untreated wild-type culture reached $5x10^7$ cells/ml. Spirochete densities were determined using a counting chamber in a dark-field microscope. The percent survival compares spirochete densities of treated and untreated culture for each particular mutant strain. Error bars represent standard deviation from three experiments done using two clones per mutant. There was no statistically significant difference observed between the wild-type strain and any of the mutants.

NER pathway for infectivity has been tested. In order to detect a difference in infectivity between each mutant, mice were infected with 10^3 spirochetes at each of two sites, close to the minimum infectious dose required for infection of all mice and for dissemination to all target organs tested with strain B31 (Labandeira-Rey and Skare 2001). For each *uvr* gene, two independent clones were used to infect three mice each, resulting in six mice per NER gene mutant. Infectivity was determined from a blood sample taken 7 days post-infection and dissemination was monitored in ear punches collected 14 and 21 days post-infection. On day 23, the ear, the heart, the bladder and the knee joint were recovered to determine if the NER genes were required for the invasion of specific organs.

At one week post-infection, spirochetes were cultured from the blood of all mice infected with wild-type and with *uvrA* and *uvrB* mutant strains. The blood from one mouse inoculated with a *uvrC* mutant was culture negative and only 50% of mice infected with *uvrD* mutants contained spirochetes in the blood at day 7 (Table 4.5 Panel A). These data indicate full infectivity of spirochetes carrying mutations in *uvrA* or *B* with perhaps a small decrease in infectivity for *uvrC* and *uvrD*.

Cultures of 2 mm ear punches at week two are a sensitive indicator of dissemination efficiency as the small amount of tissue cultured will be negative without a substantial spirochete density in the ear. Ear punch cultures for wild-type and *uvrB* mice were positive for 67% of the mice infected while *uvrC, D* and *A* mutants displayed 50%, 17% and 0% positive ear cultures at day 14,

Table 4.5. Infectivity of *B. burgdorferi* carrying mutant NER genes in C3H/HeN mice

		A		B		C		D					
Genotype	Strain	Day 7 Blood	Day 7 (%)	Day 14 Ear	Day 14 (%)	Day 21 Ear	Day 21 (%)	Day 23 Organs					Day 23 (%)
								Ear	Bladder	Heart	Joint	Total sites	
wt	920	6/6	100%	4/6	66.7%	6/6	100%	6/6	5/6	6/6	6/6	23/24	95.83%
uvrA	564	3/3	100%	0/3	0%	0/3	50%	3/3	3/3	3/3	3/3	12/12	100%
	565	3/3		0/3		3/3		3/3	3/3	3/3	3/3	12/12	
uvrB	545	3/3	100%	2/3	66.7%	3/3	100%	3/3	1/3	3/3	3/3	10/12	87.5%
	546	3/3		2/3		3/3		3/3	3/3	2/3	3/3	11/12	
uvrC	537	3/3	83.3%	1/3	50%	2/3	83.3%	3/3	3/3	3/3	3/3	12/12	83.3%
	538	2/3		2/3		3/3		2/3	2/3	2/3	2/3	8/12	
uvrD	541	1/3	50%	0/3	16.7%	0/3	16.7%	2/3	2/3	1/3	2/3	7/12	70.83%
	542	2/3		1/3		1/3		3/3	3/3	1/3	3/3	10/12	

respectively (Panel B). These data suggest reduced dissemination efficiency for *uvrA* and D mutants. This reduced dissemination into the ear persisted at day 21 (50% for *uvrA* and 17% for *uvrD*, see Panel C).

Organ cultures collected on day 23 post-infection provide a general picture for dissemination to a variety of sites but with greater sensitivity than 2 mm ear punches, as the entire organ is cultured. At day 23 (Panel D) 100% of the needle inoculated mice were infected and mutants for all four NER genes had successfully invaded the majority of the organs recovered.

To ensure that the difference in infectivity observed between the different *B. burgdorferi* clones is not the result of a change in growth rate, each clone was grown in the presence or absence of blood. Even if a significant decrease in the cell density during stationary phase, the presence of blood did not significantly affect the growth rate of *B. burgdorferi* NER mutants and all mutants showed a similar growth (

Figure 4.9).

In summary, at a low infectious dose, mutations in the NER pathway resulted in a minor decrease in spirochete burden in the blood at day 7 and in the efficiency of dissemination to ear. Nonetheless, by day 23, invasion of ear, bladder, heart and joint had occurred for all the *uvr* mutants. The attenuation in infectivity observed in all cases remained minor and did not affect the overall dissemination of the spirochetes.

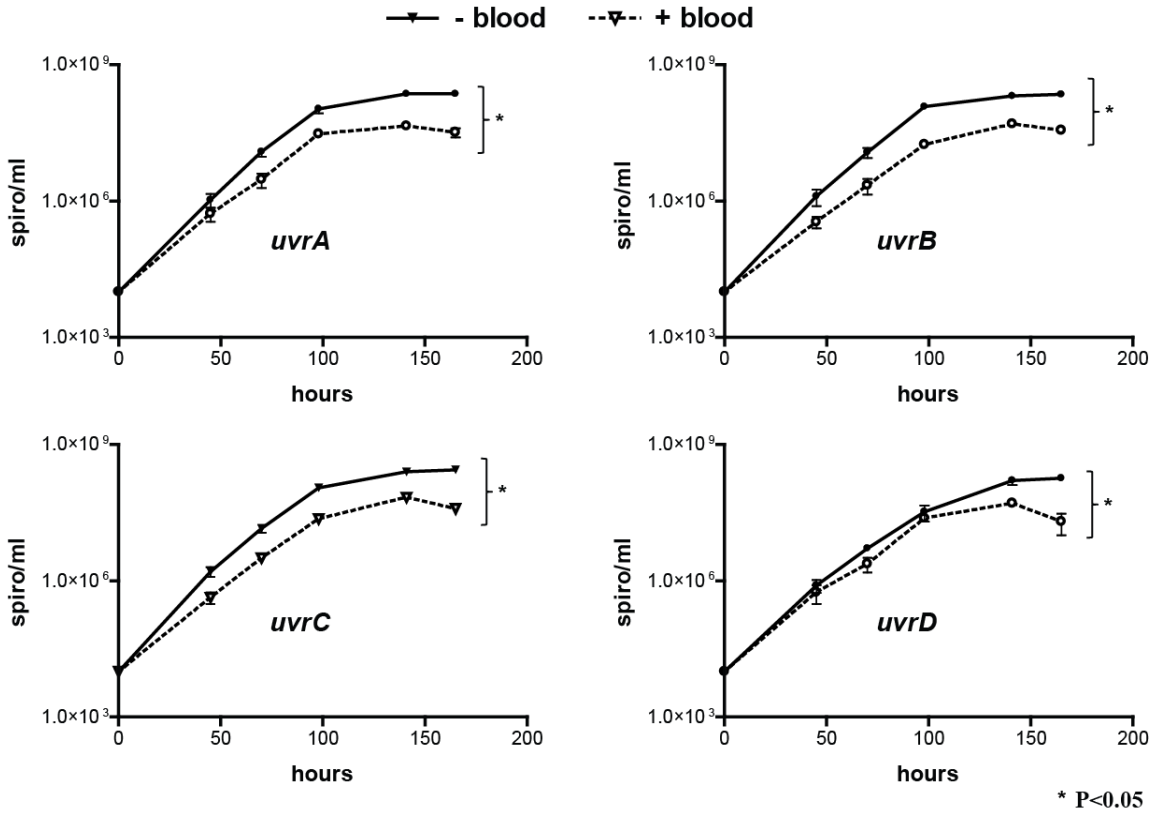


Figure 4.9. Growth curves of *B. burgdorferi* NER mutants.

Growth of *uvrA*, *uvrB*, *uvrC* and *uvrD* in absence or in presence of blood. Spirochete densities were determined using a counting chamber in a dark-field microscope. Error bars represent standard deviation from three experiments

done using two clones per mutant. *P denotes a P value corresponding to < 0.05 for a given time point.

4.4 Discussion

4.4.1 Sensitivity of *B. burgdorferi* nucleotide excision repair mutants to DNA damage

A fluorescence assay, originally described to compare the growth of *Salmonella enterica* serovar Typhimurium cultures (Kim and Surette 2004), was adapted to expediently compare multiple *B. burgdorferi* liquid cultures. This assay was used to evaluate sensitivity to DNA damage of two clones for each of the 29 *B. burgdorferi* constructs described in this study. Although solid plating and direct microscopic counting can detect variations in a wider range of cell densities, both techniques require significant effort, limiting the number of samples that can be analyzed. Plating also requires a two-week turnaround for results due to slow growth of *B. burgdorderi.*

In this study, the importance of various *B. burgdorferi* DNA replication/repair genes for survival to DNA damage was assessed. While the disruption of *B. burgdorferi uvrA*, *uvrB* or *uvrC* genes resulted in a complete loss of measurable survival following UV-induced DNA damage, an intermediate sensitivity was observed in the *uvrD* mutant, similar to what has been observed in *E. coli* (Figure 4.6). Although sensitivity of a *B. burgdorferi uvrA* mutant was previously reported (Sambir *et al.* 2011), this is the first time that the importance

of all four genes of *B. burgdorferi* NER pathway is shown to be similar to *E. coli* for survival to UV light-induced DNA damage.

The importance of the NER pathway to repair DNA damage in *B. burgdorferi* was also demonstrated for survival to nitrosative stress. A previous study has reported that disruption of *B. burgdorferi uvrB* and *uvrC* results in decreased survival following 4 hours exposure to the NO donor, DEA/NO (Bourret *et al.* 2011). However, in another study, using a different strain of *B. burgdorferi* exposed for 1 hour to different sources of NO, disruption of *uvrA* did not result in a significant increased sensitivity (Sambir *et al.* 2011). The difference between the results of these two studies might be explained by the use of different NO donors or different exposure times to the damaging agent. In the work reported here, disruption of any of the four *B burgdorferi* NER genes resulted in a similar significant increase in sensitivity to NO. These results further demonstrate the importance of the NER pathway for *B. burgdorferi* survival to chemical DNA damage.

Compared to UV- induced and nitrosative damages, the disruption of all four *B. burgdorferi* NER genes did not increase the sensitivity of the spirochete to oxidative damage. This result is in agreement to observations previously reported for *E. coli* lacking NER genes (Imlay and Linn 1987).

4.4.2 Lack of sensitivity of *B. burgdorferi* replication/repair mutants to DNA damage

Surprisingly, disruption of 21 replication/repair genes not part of the NER pathway did not result in significant increases of sensitivity to DNA damage. This

includes mutation of 11 genes previously reported to increase *E. coli* sensitivity to a dose of UV light similar to that used for *B. burgdorferi* in the present study (Table 4.6). The absence of impact is not due to a lack of sensitivity of the method used since the fluorescence assay is sensitive enough to detect a change in cell density of less than two-fold. A small but significant increase in resistance to UV light was observed in *mutS1* and *sbcC* mutants (Figure 4.3), although the meaning and possible biological significance of the *mutS1* and *sbcC* results is unknown.

An interesting difference between *B. burgdorferi* and *E. coli* is the apparent lack of an SOS response in *B. burgdorferi.* In the presence of DNA damage, the *E. coli* SOS response increases the expression of over 40 genes, including DNA recombination/repair genes *uvrA*, *uvrB, uvrD*, *recA*, *ruvA*, *ruvB,* and *ruvC* (Courcelle *et al.* 2001; Friedberg 2006; Khil and Camerini-Otero 2002; Lobysheva *et al.* 1999; Spek *et al.* 2001). There is no ortholog for the SOS response regulator *lexA* in the *B. burgdorferi* genome (Fraser *et al.* 1997). Moreover, the expression of *recA, ruvB* and the *uvr* genes in *B. burgdorferi* (Table 4.1) occurs, without induction (Boardman *et al.* 2008; Fisher *et al.* 2005; Liveris *et al.* 2008; Ouyang *et al.* 2009b; Rogers *et al.* 2009), at a level close to that of the constitutively transcribed *flgB* gene (Salman-Dilgimen *et al.* 2013). The lack of a requirement for *recA* to promote SOS induction in *B. burgdorferi* partially explains the lack of sensitivity of *B. burgdorferi recA* mutants to DNA damage, since induction of the NER pathway is apparently not needed.

Table 4.6. Comparison of UV sensitivity in *B. burgdorferi* with *E. coli*

Mutant Gene[1]	Description	Sensitivity in *B. burgdorferi*[2]	References	Level of sensitivity in *E. coli*[3]
uvrA	Excinuclease ABC, A subunit	++	(Howard-Flanders *et al.* 1966; Lloyd *et al.* 1984; Lloyd and Buckman 1991; Sambir *et al.* 2011)	Highly sensitive (≥1,000x)
uvrB	Excinuclease ABC, B subunit	++	(Lloyd *et al.* 1984)	
uvrC	Excinuclease ABC, C subunit	++	(Lloyd *et al.* 1984)	
recA	DNA recombinase	-	(Liveris *et al.* 2008; Lloyd *et al.* 1984)	
ruvA	Holliday junction DNA helicase	-	(Li and Waters 1998; Lin *et al.* 2009; Lloyd 1991; Lloyd *et al.* 1984)	
ruvB	Holliday junction DNA helicase	-	(Lloyd 1991; Lloyd *et al.* 1984; Sharples *et al.* 1990)	
ruvC	Holliday junction resolvase	absent	(Fraser *et al.* 1997; Lloyd 1991; Sharples *et al.* 1990)	
ssb	Single-stranded DNA-binding protein	?	(Glassberg *et al.* 1979)	
recC	Exodeoxyribonuclease V, gamma chain	?	(Lloyd *et al.* 1984)	Moderately sensitive (10-1,000x)
uvrD	ATP-dependent DNA helicase II	++	(Kuemmerle and Masker 1980; Siegel 1973)	
recB	Exodeoxyribonuclease V, beta subunit	?	(Ivančić-Baće *et al.* 2003; Ivančić-Baće *et al.* 2005; Lloyd *et al.* 1984)	
recF	ssDNA and dsDNA binding, ATP binding	absent	(Courcelle *et al.* 1997; Fraser *et al.* 1997; Lloyd *et al.* 1984; Lloyd and Buckman 1991; Lloyd *et al.* 1988; Old *et al.* 1993)	
recR	Recombination and repair	absent	(Courcelle *et al.* 1997; Fraser *et al.* 1997; Ivančić-Baće *et al.* 2003)	
priA	Primosomal protein N	-	(Kogoma *et al.* 1996)	
recG	DNA helicase, resolution of Holliday junctions, branch migration	-	(Lloyd and Buckman 1991)	
hupAB (HU)	DNA binding	- (hbb)	(Li and Waters 1998)	
ligA	DNA ligase	?	(Konrad *et al.* 1973)	
dnaK	Hsp70, chaperone	-	(Goldfless *et al.* 2006; Zou *et al.* 1998)	
mutL	Methyl-directed mismatch repair	-	(Mellon and Champe 1996)	
recO	Repair/recombination protein	absent	(Fraser *et al.* 1997; Ivančić-Baće *et al.* 2005; Lloyd *et al.* 1988)	

Mutant Gene[1]	Description	Sensitivity in *B. burgdorferi*[2]	References	Level of sensitivity in *E. coli*[3]
umuC	Translesion synthesis	absent	(Bagg *et al.* 1981; Fraser *et al.* 1997)	Slightly sensitive (2-10x)
umuD	Translesion synthesis	absent	(Fraser *et al.* 1997; Opperman *et al.* 1999)	
pnp	Polynucleotide phosphorylase	?	(Rath *et al.* 2012)	
mutS	Methyl-directed mismatch repair	+	(Caillet-Fauquet *et al.* 1984; Mellon and Champe 1996)	
rep	Helicase, a single-stranded DNA-dependent ATPase	-	(Calendar *et al.* 1970; Denhardt *et al.* 1967; Uzest *et al.* 1995)	
dnaX	DNA polymerase III, tau and gamma subunits	?	(Goldfless *et al.* 2006)	
mutH	Methyl-directed mismatch repair	absent	(Caillet-Fauquet *et al.* 1984; Fraser *et al.* 1997; Mellon and Champe 1996)	
mfd	Transcription-repair coupling factor	-	(Mellon and Champe 1996; Oller *et al.* 1992; Witkin 1966)	
oraA	RecX regulator	absent	(Fraser *et al.* 1997; Stohl *et al.* 2003)	
sbcC[4]	Exonuclease	+	(Rudolph *et al.* 2010)	
sbcD	Exonuclease	-	(Rudolph *et al.* 2010)	
exoA[3]	Exodeoxyribonuclease III	-	(Cunningham *et al.* 1986)	Non-sensitive
recD	Exodeoxyribonuclease V, alpha subunit	-	(Ivančić-Baće *et al.* 2005)	
recJ	Single-stranded-DNA-specific exonuclease	-	(Ivančić-Baće *et al.* 2005)	
mag (alkA)[3]	3-methyl-adenine DNA glycosylase II	-	(Chen *et al.* 1990; Yamamoto *et al.* 1978)	
hrpA	ATP-dependent RNA helicase	-	(Moriya *et al.* 1995)	
nth	AP sites endonuclease III	-	(Cunningham *et al.* 1986; Serafini and Schellhorn 1999)	
bb0098	DNA mismatch repair protein, MutS2 protein	-	(Fukui *et al.* 2008)	
nucA	DNA/RNA non-specific endonuclease	-		Unknown
bbg32	Replicative helicase	-		

[1] Genes are listed in order of decreasing sensitivity in *E. coli*.
[2] ++: Highly sensitive, +: Slightly sensitive, -: non-sensitive, absent: No ortholog found in *B. burgdorferi*, ?: Sensitivity is not known in *B. burgdorferi.*
[3] Based on literature. Sensitivity of *exoA* and *mag* mutant is based on *Bacillus subtilis* and *Saccharomyces cerevisiae*. Mutant for *E. coli alkA*, the *mag* ortholog, does not present an increased sensitivity to UV light.
[4] Sensitivity based on *sbcCD* mutant in *E. coli*.

In contrast to our results, disruption of *recA, ruvABC*, *recG*, *recBCD*, *priA* and *mfd* in *Neisseria gonorrhoeae*, also an obligate parasite missing the SOS response (Black *et al.* 1998), results in a significant increase in sensitivity to UV light (Black *et al.* 1998; Kline and Seifert 2005a; LeCuyer *et al.* 2010; Mehr and Seifert 1998; Sechman *et al.* 2006; Sechman *et al.* 2005). These results suggest that, even in presence of a functional NER system, recombinational repair (*recA*, *ruvABC*, *recG* and *recBCD*), transcription-coupled repair (*mfd*) and repair of arrested replication forks (*recG, priA*) play a significant role in the repair of UV damage in *N. gonorrhoeae*, but not in *B. burgdorferi*. No orthologs of *sbcCD* (Rudolph *et al.* 2010) are found in the sequenced genome of *N. gonorrhoeae*, but their disruption did not affect *B. burgdorferi* survival to UV damage. Other recent studies have also reported that *B. burgdorferi recA* and *ruvA* are dispensable for repairing UV-induced DNA damage (Lin *et al.* 2009; Liveris *et al.* 2008). Recombinational repair of UV and nitrosative damage may well be occurring at low levels in *B. burgdorferi*, however, experiments to detect this would require *recA, uvr* double mutants.

Our results also show that disruption of the methyl-directed mismatch correction (MMC) pathway (*mutS* and *mutL*) does not affect survival of *B. burgdorferi* after exposure to UV light, but does affect *E. coli*. Interestingly, no potential ortholog of the MutH endonuclease has been identified in the *B. burgdorferi* genome (Fraser *et al.* 1997). *N. gonorrhoeae* is also lacking a *mutH* ortholog, and similarly to *B. burgdorferi*, the MMC system is dispensable for DNA damage repair (Criss *et al.* 2010). However, disruption of *N. gonorrhoeae* MMC

genes results in an increase of spontaneous mutation rate due to base-pairing errors, suggesting a functional MMC pathway, even in the absence of *mutH* (Criss *et al.* 2010). In *B. burgdorferi*, disruption of *mutS*, but not *mutL*, resulted in a small but significant decrease in sensitivity to UV light. Whether this small difference has any biological significance remains unknown. A similar phenotype is observed for *recD* and *recJ* mutants. As opposed to *N. gonorrhoeae* (Helm and Seifert 2009; Hill 2000), disruption of *E. coli* (Lloyd *et al.* 1988; Lovett *et al.* 1988; Viswanathan and Lovett 1998) and *B. burgdorferi recD* and *recJ* does not result in an increased sensitivity to UV light. The possible role of *dnaK* (Goldfless *et al.* 2006), *pnp* (Rath *et al.* 2012), *rep* (Denhardt *et al.* 1967) and *hbb* (related to the HU/IHF family) (Kobryn *et al.* 2000; Li and Waters 1998) in DNA damage repair also remains unclear. Sensitivity to UV light was also tested for *B. burgdorferi exoA*, *mag*, *hrpA*, *nth*, *bb0098*, *nucA* and *bbg32* mutants because of their predicted role in DNA replication, recombination and repair (Fraser *et al.* 1997). However, none of these mutants had an increased sensitivity to UV light compared to wild-type *B. burgdorferi*.

4.4.3 Effect of *B. burgdorferi* NER mutations on murine infection

The nucleotide excision pathway is required for full infectivity of various pathogens. Garbom *et al.* showed that disruption of *Yersinia pseudotuberculosis virB* gene, an ortholog of *uvrA*, results in a significant increase of LD_{50} for mice infection (Garbom *et al.* 2004). In *Mycobacterium tuberculosis*, transcription of *uvrA* is significantly increased after being internalized by human macrophages (Graham and Clark-Curtiss 1999). Also, *M. tuberculosis uvrB* mutants are

attenuated for infection in wild-type and iNOS$^{-/-}$ mice but not in iNOS/phox-deficient mice, suggesting that the nucleotide excision repair pathway might be important for *M. tuberculosis* survival to Phox-mediated stress (Darwin and Nathan 2005). In *B. burgdorferi*, our data demonstrate that all four NER pathway genes are involved in survival to nitrosative stress.

B. burgdorferi has been shown to induce release of NO from macrophages (Modolell *et al.* 1994) and recruitment of macrophages and neutrophils to organs where it disseminates (Duray and Steere 1988; Menten-Dedoyart *et al.* 2012; Ruderman *et al.* 1995). However, previous studies showed that inhibition of iNOS using N^{G}-L-monomethyl arginine (LMMA) does not significantly affect bacterial burden in the heart and joint of C3H/HeJ or BALB/c mice infected with *B. burgdorferi* (Seiler *et al.* 1995) and has only a minor effect on its survival in presence of macrophages (Modolell *et al.* 1994). Although *B. burgdorferi* appears resistant to nitrosative DNA damage, Bourret *et al.* showed that disruption of either *uvrB* or *uvrC* results in a significant increase in sensitivity to DNA damage (Bourret *et al.* 2011). Our study demonstrates that a complete NER pathway is required for *B. burgdorferi* survival to nitrosative damage. This suggests that *B. burgdorferi* NER mutants could be impaired in mouse infection. However, our data show that disruption of *uvrA* and *uvrD* had only a minor effect upon murine infection. *B. burgdorferi uvrA* and *uvrD* mutants appeared to be attenuated as determined by culture of ear punch samples but did disseminate to all the organs tested by day 23 post-infection. This suggests that during mouse

infection, *B. burgdorferi* would not be exposed to levels of nitrosative stress sufficient to prevent infectivity and dissemination of a sensitive strain.

Disruption of *uvrD* had the most noticeable effect with decreased proportions of blood and ear biopsy specimens that were culture positive on days 7-21. This could reflect a role for the UvrD helicase in other repair pathways, as described in *E. coli*. Previous studies showed that UvrD is also involved in MMC in *E. coli* (Lu *et al.* 1983; Nevers and Spatz 1975), but it was shown that disruption of *B. burgdorferi mutS* and *mutL* does not affect *B. burgdorferi* infectivity and dissemination to the ear (Dresser *et al.* 2009). However, mice in that study were infected with a higher number of spirochetes and, in the presence of a functional NER system, disruption of MMC might not have generated a discernible phenotype.

In conclusion, this study demonstrated that *B. burgdorferi* requires a functional NER system for survival of nitrosative and UV light-induced DNA damage and that the pathway appears to be conserved between *E. coli* and *B. burgdorferi*. Moreover, disruption of *uvrA* and *uvrD* had only a minor effect upon infectivity of *B. burgdorferi* in mice. Further studies will be required to investigate the role of the NER pathway during the tick/mammal interphase or in the tick.

Chapter Five: Concluding remarks and future directions

5.1 Concluding remarks

In summary, the genetic analysis presented in the previous chapters demonstrate the importance of the RuvAB branch migrase for switching at *vlsE* in *B. burgdorferi* and is required for the persistence of the spirochetes in the murine host. Moreover, 11 other genes previously shown to affect switching and antigenic variation in *N. gonorrhoeae*, are either absent from the genome of *B. burgdorferi* or their disruption did not have a significant effect on switching at *vlsE*. In addition of genes disruption, genetic manipulations was used to successfully complement the disruption of *hrpA*, which encodes for a DEAH-box ATP-dependent RNA helicase shown to regulate the expression of genes involved in a wide array of cellular functions. In *B. burgdorferi*, disruption of *hrpA* results in a complete loss of infectivity in mice and complementation by the restoration of a wild-type allele by allelic exchange restored the infectivity of the spirochete to a level similar to wild type. Moreover, allelic exchange was used to insert point mutation in *hrpA*, in motifs required either for the ATPase activity, the binding of RNA or for the communication between the two function. These mutations were used to demonstrate that the loss of the helicase activity of HrpA affects the infectivity of *B. burgdorferi* more profoundly than the loss of the ATPase activity. The genes required for the resistance of *B. burgdorferi* to DNA damage were also determined. Following the demonstration that UV light does generate CPD damage in the DNA in *B. burgdorferi*, an expeditious fluorescence assay was adapted to compare the growth of different *B. burgdorferi* cultures.

This assay and complementation by allelic exchange were then used to demonstrate that the nucleotide excision repair system is the sole pathway involved in repair of DNA damage induced by UV light. The disruption of 11 other genes involved in the survival of *E. coli* to UV light was shown to not have a significant effect in *B. burgdorferi*.

Genetic manipulations in *B. burgdorferi* are constantly evolving. Traditionally, genes were disrupted by allelic exchange using a construct containing approximatively 1.5 kb of the targeted sequence. Approximately 500 bp from the middle of the targeted gene was then deleted and replaced by a *PflgB*-driven kanamycin or gentamicin resistance cassette. This strategy was used to disrupt most of the genes targeted in this study. However, when a gene target is longer than 500 bp, this implies that some sequence from the target remains in the genome, raising the possibility that the remaining sequence could encode for a partially active protein. To avoid this potential problem, the complete ORF of *uvrA*, the most recently targeted gene, was deleted and replaced by the antibiotic resistance cassette. This strategy ensures that the complete gene is deleted and that no partially active product remains.

A second question was raised by the original strategy used to disrupt genes in *B. burgdorferi*. Since complementation of disrupted genes is not always possible, at least two clones for each gene target were used in every experiment to decrease the chances that the phenotype observed would be caused by a secondary mutation in the genome. This strategy implies that the spirochetes have not replicated during the 20 to 24 hours recovery incubation, because this

would result in the plating of clonal siblings in more than one well and not obtaining completely independent clones. *B. burgdorferi* has a six to eight hours doubling time and following electroporation, it is incubated for 24 hours before plating with the antibiotic. Therefore, one cannot exclude possible replication during the incubation. To eliminate the risks of recovering non-independent clones, immediately following the electroporation to generate the *uvrA* and *uvrB* mutants, the cells were divided into aliquots and then incubated for recovery. Each aliquot was then plated on a single plate. Clones used for subsequent experiments were recovered from different plates, ensuring that each clone used was independent.

Alternatively, two transformations were done for the majority of the gene targets using two knockout constructs; each with the antibiotic resistance cassette in the opposite orientation. In this case *B. burgdorferi* clones chosen for subsequent experiments contained a different construct, whenever possible. However, for some gene targets, mutants could only be recovered if the cassette was in a specific orientation. This was the case for the disruption of *uvrB* where clones were only recovered if the *PflgB-gent* cassette was in the same orientation as the *uvrB* ORF. This result would be expected if one orientation of the selection cassette resulted in a polar effect and disruption of downstream gene(s). Although *uvrA*, the gene downstream of *uvrB*, was successfully disrupted in the present study, multiple transformations were required to obtain mutants. Moreover, Lin et al. recently generated a library of *B. burgdorferi* mutants by transposon mutagenesis. In this library, no insertion in *uvrA* could

was recovered. However, mutants with insertions in the gene upstream (*uvrB*) and in the intergenic region downstream were recovered, suggesting that although *uvrA* is not essential, its disruption in appears to be problematic. Thus, when the selection cassette could be inserted in only one orientation, making aliquots prior to the recovery incubation was the only possibility to ensure obtaining independent clones.

As previously mentioned, the complementation of disrupted genes in *B. burgdorferi* is not always possible. Previous studies have reported successful complementation of genes by transforming *B. burgdorferi* with a shuttle plasmid encoding the gene to be complemented with or without its native promoter (Bankhead and Chaconas 2007; Brisson *et al.* 2012; Lawrenz *et al.* 2004; Purser *et al.* 2003; Rosa *et al.* 2010; Rosa *et al.* 2005; Sambir *et al.* 2011). However in the present and other studies (Lin *et al.* 2009; Ristow *et al.* 2012; Tilly *et al.* 2006), this strategy did not restore a wild-type phenotype for the targeted gene. In the present study, complementation *in trans* using a shuttle plasmid was unsuccessful for complementing *B. burgdorferi ruvA*, *ruvB* (Dresser *et al.* 2009) and *hrpA* (Salman-Dilgimen *et al.* 2011) mutants. A different strategy was then used, consisting of the restoration of a wild-type copy of *hrpA* at its native location in the chromosome. This strategy has also been very recently reported for the complementation of the *B. burgdorferi* β_3-integrin ligand P66, while complementation *in trans* was unsuccessful (Ristow *et al.* 2012). In the present study, the restoration of the wild-type allele of *hrpA* into the chromosome involved the insertion of a selectable marker downstream of the ORF, potentially

disrupting the gene located after *hrpA*, *bb0826*. However, this gene was shown not to influence the infectivity of *B. burgdorferi* and, so, was not expected to affect the complementation of *hrpA*. This strategy was also used for the complementation of *uvrB*, *uvrC* and *uvrD*. In each case, the restoration of the wild-type allele restituted the resistance to DNA damage to a level similar to wild type.

Allelic exchange to replace the disrupted copy of *hrpA* in the chromosome of *B. burgdorferi* was also used to introduce point mutations in *hrpA*. A similar strategy was previously reported for the insertion of mutations in the wild-type *B. burgdorferi* response regulator Rrp2 (Yang *et al.* 2003) and for the insertion of point mutations the *gyrA* and *gyrB* genes, encoding for the DNA gyrase subunits (Knight *et al.* 2000). In the first study, the mutated site was located approximately 650 bp from the selectable marker. However, in the present study the mutated sites to be inserted were located as far as 2 kb from the antibiotic resistance cassette, which was expected to reduce the chances of the sites being included in the recombination event. Knight *et al.* reported the insertion of mutations, separated by approximatively 4 kb, in the *gyrA* and *gyrB* genes. However, the authors reported that only three out of 400 clones recovered contained mutations in both genes (Knight *et al.* 2000). Thus, the transformation of the *B. burgdorferi* clone encoding the disrupted *hrpA* allele significantly reduced the distance between the mutated sites and the selection marker. Moreover, in the work reported here we used a PCR assay to conveniently screen drug resistant transformants for the presence of the desired mutation. This saved significant

time and energy in identifying mutants that were subsequently confirmed by sequencing. As an example, the PCR screening approach did not work for the *hrpA*-I285A mutant. It was necessary in this case to sequence 23 transformants obtained from three different transformations to recover two mutants.

Assessing the growth or the sensitivity of multiple cultures of *B. burgdorferi* is limited by the long doubling time of the bacteria and by the techniques that are available for determining the spirochete density in a culture. While direct counting of liquid cultures is time consuming, plating on solid media requires a two-to-three-week incubation before CFUs can be counted. To expediently compare the growth of multiple cultures of *B. burgdorferi*, a fluorescence assay was adapted from a previous study where a similar assay was developed to compare cultures of *Salmonella* (Kim and Surette 2004). This assay was adapted for *B. burgdorferi* to compare relative amount of DNA in a large number of cultures and was applied to determine the level of sensitivity of two clones for each of 25 *B. burgdorferi* mutants to UV light. This assay allows for simultaneous and quantitative comparison of relative growth of multiple cultures of *B. burgdorferi,* in only two days. This assay is expected to find use in a variety of studies where quantification of many samples of *B. burgdorferi* are required in a short period of time.

5.2 Future directions

5.2.1 Identification of a functional ortholog of *ruvC*.

Switching at *vlsE* is required for the persistence of *B. burgdorferi* during mouse infection. We have successfully identified that the recombination events that generate the switching at *vlsE* involve the activity of the RuvAB branch migrase. However, to complete a recombination reaction, the Holliday junction must be resolved. In *E. coli*, the Holliday junction resolvase is *ruvC* but no ortholog was identified by sequencing of the genome of *B. burgdorferi.* A project has already been started to generate a library of the *B. burgdorferi* genes to be expressed in an *E. coli ruvC* deficient strain and to identify a gene from *B. burgdorferi* that complements the absence of *E. coli ruvC*.

5.2.2 Generation of double mutants in *B. burgdorferi*

We have observed that the disruption of *B. burgdorferi* genes involved in methyl mismatch correction pathway does not significantly affect infectivity in mice. However, a reduced infectivity phenotype of *B. burgdorferi mutS* but not *mutL* mutants has previously been reported (Botkin *et al.* 2006; Lin *et al.* 2009; Lin *et al.* 2012) and we have observed only a slight effect on *B. burgdorferi* infectivity in the NER mutants. The NER and the MMC pathways have been previously shown to interact during DNA damage repair in *E. coli* and the possibility that the NER and the MMC pathways are involved in the repair of similar DNA damage during mouse infectivity could be verified by generating *mut uvr* double mutants in *B. burgdorferi*. Disruption of *mutS* has previously been shown to slightly increase the sensitivity to UV light of *E. coli* lacking *uvrA* (Siegel

1973). Moreover, MutS and UvrD have both been shown to stimulate the degradation of dsDNA by exonuclease VII, suggesting overlapping activities between the two proteins (Noothi *et al.* 2009). The UvrD helicase has also been shown to interact with the MMC pathway during DNA repair.

5.2.3 Characterisation of the function of HrpA required for infectivity

The precise function of the *B. burgdorferi* HrpA protein, required for infectivity in mice remains unknown. However, experiments have been started to characterize a possible role for HrpA in the processing of *p66* and *bb0241* mRNA transcripts by HrpA. The P66 integrin ligand was shown to be required for infectivity in mice but dispensable in ticks (Ristow *et al.* 2012), and the *bb0241* is part of the predicted glycerol utilization operon (Fraser *et al.* 1997). The disruption of this operon decrease bacterial burden in the tick (Pappas *et al.* 2011). Previous, iTRAQ results showed a significant decrease in the expression of *p66* and *bb0241* in the *B. burgdorferi hrpA* mutant, compared to the wild-type strain. However, transcriptional analysis did not revealed significant changes in the levels of transcripts of both genes in the mutant *B. burgdorferi*, suggesting a post-transcriptional activity of HrpA.

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APPENDIX A: LIST OF PUBLICATIONS

1. **Hardy, P.-O.** and G. Chaconas (2013). "The nucleotide excision repair system of *B. burgdorferi* is the sole pathway involved in repair of DNA damage by UV light." Journal of bacteriology.

2. Salman-Dilgimen, A., **P.-O. Hardy** and G. Chaconas (2013). Manuscript in preparation.

3. Woo-Yong Lee, Maria-Jesus Sanz, Connie H Y Wong, Tara J. Moriarty, **Pierre-Olivier Hardy**, Aydan Salman-Dilgimen, George Chaconas, Roman Krawetz, Christopher H Mody and Paul Kubes. "Visualizing iNKT cells in joints: An extravascular cytotoxic barrier for invading Lyme *Borrelia*." Manuscript in preparation.

4. Moriarty, T. J., Shi, M., Lin, Y.-P., Ebady, R., Zhou, H., Odisho, T., **Hardy, P.-O.**, Salman-Dilgimen, A., Wu, J., Weening, E. H., Skare, J. T., Kubes, P., Leong, J. and Chaconas, G. (2012), "Vascular binding of a pathogen under shear force through mechanistically distinct sequential interactions with host macromolecules." Molecular Microbiology, 86: 1116–1131. doi:10.1111/mmi.12045.

5. Salman-Dilgimen, A., **P.-O. Hardy**, A. R. Dresser and G. Chaconas (2011). "HrpA, a DEAH-box RNA helicase, is involved in global gene regulation in the Lyme disease spirochete." PLoS ONE 6(7): e22168.

6. Dresser AR, **Hardy P-O**, Chaconas G (2009) "Investigation of the genes involved in antigenic switching at the *vlsE* locus in *Borrelia burgdorferi*: An essential role for the RuvAB branch migrase". PLoS Pathog 5(12): e1000680. doi:10.1371/journal.ppat.1000680.