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Construction and Characterization of a B. burgdorferi strain with Conditional Expression of the Essential Telomere Resolvase, ResT

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Construction and Characterization of a *B. burgdorferi* strain with
Conditional Expression of the Essential Telomere Resolvase, ResT

by

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A THESIS

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Abstract

During DNA replication in *Borrelia burgdorferi*, replication of linear elements results in an inverted dimer repeat of the telomere which is subsequently resolved by the telomere resolvase, ResT. ResT has been demonstrated as essential and a knock-out strain has never been produced. Using a conditional expression system a *resT* knock-out strain has been produced and used to investigate the effects of ResT depletion of spirochete viability as well as genome stability. ResT depleted spirochetes ceased to divide after ResT depletion, however, remained viable up to 16 days suggesting an arrested state of the spirochetes. Southern blots of the DNA revealed a shift of linear plasmid DNA to a higher molecular weight representing the circular intermediate. DNA replication did not continue in spirochetes after ResT depletion suggesting ResT may play a larger role in replication than previously thought.

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Table of Contents

Abstract	i
Acknowledgements	ii
Table of Contents	iii
List of Figures and Tables	vi
Abbreviations.....	viii
Chapter One: Introduction	1
1.1 Lyme Disease.....	1
1.1.1 Emergence.....	1
1.1.2 Etiologic agent	2
1.1.3 Transmission.....	3
1.1.4 Host Response	5
1.1.5 Signs and symptoms.....	7
1.1.6 Diagnosis and Treatment	8
1.2 <i>Borrelia burgdorferi</i>	10
1.2.1 Classification.....	10
1.2.2 Morphology	10
1.2.3 Virulence	12
1.3 Genome maintenance and replication in <i>B. burgdorferi</i>	16
1.3.1 Genome of <i>B. burgdorferi</i>	16

1.3.2	Telomeres of linear plasmids in <i>B. burgdorferi</i>	18
1.3.3	DNA replication in <i>B. burgdorferi</i>	20
1.3.3	Replication of linear plasmids in <i>B. burgdorferi</i>	21
1.3.4	Telomere resolution in <i>B. burgdorferi</i>	23
1.4	Study Introduction	26
1.4.1	General Introduction.....	26
1.4.2	Study Aims.....	29
	Chapter Two: Materials and Methods.....	30
2.1	Growth and transformation of bacterial strains	30
2.2	Extraction of <i>Borrelia</i> DNA	31
2.3	Plasmid construction	32
2.4	GFP induction and washout assays	35
2.5	Screening <i>Borrelia</i> transformants	36
2.6	Displacement of pBSV2BhresT from GCB51	37
2.7	Growth of conditional <i>resT</i> knock-outs	38
2.8	Western blotting	38
2.9	Field inversion and southern blotting of <i>Borrelia</i> DNA.....	39
2.10	Multiplex PCR for analysis of genomic content	42
2.11	ResT assays on <i>Borrelia</i> DNA.....	42
2.12	Microscopy for length measurements and genomic content	43

2.12 qPCR for quantitation of chromosomal origins and telomeres	43
Chapter Three: Results	49
3.1 Optimizing IPTG induction in <i>B. burgdorferi</i>	49
3.2 Construction of a conditional lethal <i>resT</i> mutant	53
3.3 Characterization of the conditional <i>resT</i> mutant	63
3.4 The effect of ResT depletion on the state of linear plasmids	69
Chapter Four: Discussion and Conclusions.....	79
4.1 Construction of a conditional lethal <i>resT</i> mutant	79
4.2 Characterization of the <i>resT</i> mutant	81
4.3 State of the linear DNA in ResT depleted <i>B. burgdorferi</i>	84
4.4 Future Directions	86
4.5 Summary and Conclusions	88
References	90

List of Figures and Tables

Figure 1.1 The enzootic life cycle of <i>Borrelia burgdorferi</i>	4
Figure 1.2 Global distribution of the <i>Borrelia burgdorferi</i>	11
Figure 1.3 Phase and antigenic variation in <i>B. burgdorferi</i>	14
Figure 1.4 The unusual segmented genome of <i>Borrelia burgdorferi</i>	17
Figure 1.5 Hairpin telomeres of <i>B. burgdorferi</i>	19
Figure 1.6 Replication of linear genetic elements in <i>B. burgdorferi</i>	22
Figure 1.7 Mechanism of ResT in <i>Borrelia burgdorferi</i>	25
Figure 1.8 Control of <i>resT</i> expression in <i>B. burgdorferi</i>	28
Table 2.1 Primers used in this study	45
Table 2.2 Strains used in this study	47
Table 2.3 Plasmids used in this study	48
Figure 3.1 - Induction of GFP in <i>B. burgdorferi</i>	51
Figure 3.2 – Loss of GFP fluorescence after IPTG wash-out	52
Figure 3.3 Plasmid map of pNB12.....	54
Figure 3.4 Strategies for generating a <i>resT</i> knock-out in <i>Borrelia burgdorferi</i> ...	56
Figure 3.5 Generation and demonstration of <i>resT</i> knock out.....	59

Figure 3.6 Confirmation of pBSV2BhresT displacement in <i>B. burgdorferi</i>	62
Figure 3.7 Western blot of ResT from IPTG washed-out cultures.....	64
Figure 3.8 Growth curves and Live/Dead staining of a <i>resT</i> knock-out.....	65
Figure 3.9 Determination of the average spirochete length	68
Figure 3.10 Field inversion gel of isolated <i>B. burgdorferi</i> genomic DNA.....	70
Figure 3.11 Southern blot of genomic DNA	71
Figure 3.12 Multiplex PCR to determine the plasmid content	73
Figure 3.13 ResT assay on isolated genomic DNA	75
Figure 3.14 Determination of the relative DNA content.....	78

Abbreviations

APC – Antigen Presenting Cell

bp – Base pairs

BSA – Bovine Serum Albumin

BSK – Barbour-Stoenner-Kelly

CDC – Center for Disease Control

cp – Circular plasmid

CRASPs – Complement Regulator-Acquiring Surface Proteins

DAPI – 4',6-diamidino-2-phenylindole

DIS – Dulbecco's Ion Solution

DNA – Deoxy-ribonucleic acid

dPBS²⁺ – Dulbecco's Phosphate-Buffered Saline

EDTA – Ethylenediaminetetraacetic acid

ELISA – Enzyme Linked Immunosorbant Assay

EPS – Electroporation Solution

GFP – Green Fluorescent Protein

IPTG – Isopropyl β -D-1-thiogalactopyranoside

kb – Kilo-base

KO – Knock-out

LB – Lauria-Bertani broth

lp – Linear plasmid

LPS – Lipopolysaccharide

Mb – Mega base

MCS – Multiple Cloning Site

NEB – New England Biolabs

NaPPi – Sodium Pyrophosphate

NIH – National Institute of Health

nt – Nucleotides

OD – Optical Density

Osp – Outer surface protein

PCR – Polymerase Chain Reaction

pf – Paralagous Family

PHAC – Public Health Agency of Canada

ResT – Resolvase of Telomeres

SCID – Severe Combined Immuno-deficiency

SDS – Sodium Dodecyl Sulfate

s.l. – Sensu lato

TLR – Toll-Like Receptor

TROSPA – Tick Receptor for OspA

vls – vmp-like Sequence

Chapter One: Introduction

1.1 Lyme Disease

1.1.1 Emergence

In the 1970's, a cluster of cases of what was believed to be juvenile rheumatoid arthritis was described in the area surrounding Lyme Connecticut. Patients presented with a peculiar skin lesion, a bulls-eye rash known as erythema migrans, and approximately four weeks later would demonstrate signs of arthritis; this disease later became known as Lyme disease after the region it was first described (Steere *et al.*, 1977). At the time, the epidemiology suggested that the disease was spread by an arthropod vector, which was confirmed by a later study of 32 patients who developed erythema migrans and subsequently Lyme arthritis after a tick bite (Steere *et al.*, 1977). The etiologic agent of Lyme disease was not described until 1982 when the bacteria, now known as *Borrelia burgdorferi*, were isolated from the mid-gut of hard-bodied ixodes ticks (Burgdorfer *et al.*, 1982).

Lyme disease became a nationally reportable disease in Canada as of 2010 (PHAC, 2010). In the United States approximately 20 000 new cases are reported annually and the number of new cases is increasing every year (CDC, 2011). In Canada the number of reported cases is dramatically lower, however, as the disease only recently became nationally reportable in Canada the number of Canadian cases may be under-represented. As has been mentioned, the

number of cases reported has been increasing since the mid-1990's, which may be in part due to a spread of the territory inhabited by the *Ixodes* ticks, which is predicted to further increase, spreading north further into Canada as the effects of climate change become more pronounced (Ogden *et al.*, 2006).

1.1.2 Etiologic agent

The etiologic agent of Lyme disease was discovered in 1982 through isolation of the bacteria from the tick mid-gut (Burgdorfer *et al.*, 1982). Epidemiologic evidence of the disease in the USA suggested that the disease was spread by ticks. The bacteria were isolated following the collection of live ticks from the field and further examination by immune-reactivity with patient serum and examination of the tick gut contents by electron microscopy. The bacteria were described as loosely coiled spirochetes resembling *Treponema* (Burgdorfer *et al.*, 1982). The bacterium was later classified as a *Borrelia* species based on a high degree of DNA hybridization with the relapsing fever spirochete *Borrelia hermsii* (approximately 30 – 40% similarity) (Schmid *et al.*, 1984).

It is now known that Lyme borreliosis is caused by three main species which are collectively known as *Borrelia burgdorferi* sensu lato including *B. burgdorferi* sensu stricto (hereafter referred to as *B. burgdorferi*), *B. garinii*, and *B. afzelii* (Farlow *et al.*, 2002). The different strains may cause different characteristic long-term symptoms; *B. burgdorferi* tends to cause arthritis, *B. garinii* tends to cause neurological symptoms, and *B. afzelii* can cause a skin condition known as acrodermatitis chronica atrophicans (Farlow *et al.*, 2002). However, all

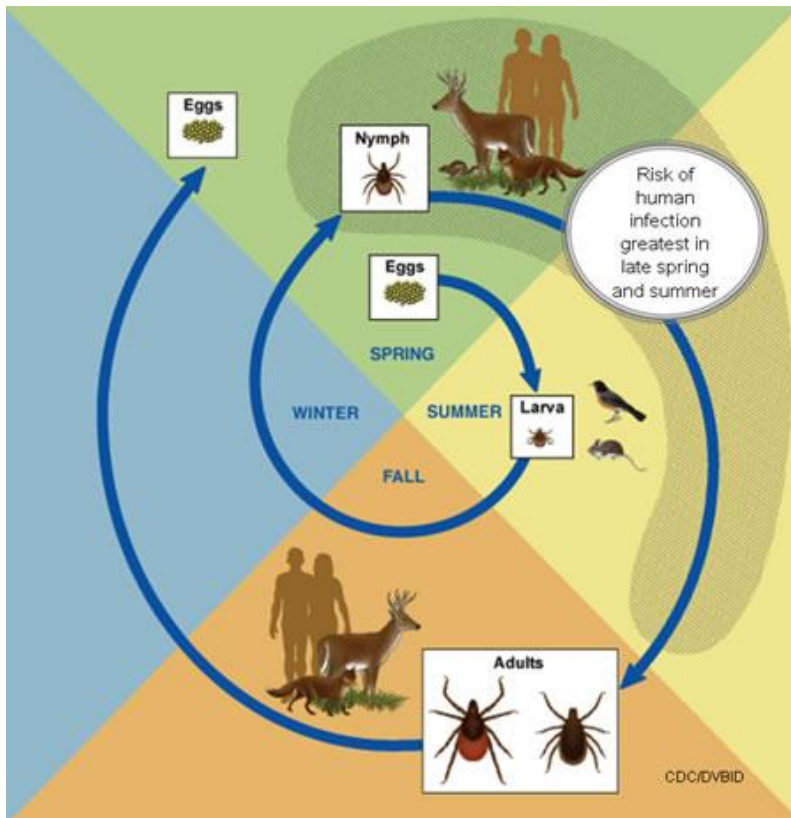
three cause the characteristic bulls-eye rash, erythema migrans, found in roughly half of infected individuals.

1.1.3 Transmission

During the original outbreak of Lyme arthritis, epidemiologic evidence suggested the disease was transmitted by hard bodied *Ixodes* ticks (Steere *et al.*, 1978). A significant number of patients recalled having a tick bite at the initial site of erythema migrans or saw ticks on their pets or tick bites on themselves prior to symptoms; *Ixodes* ticks were then identified as potential vectors for the spread of disease. It has since been confirmed that Lyme disease is indeed spread by various *Ixodes* ticks based on geographic distribution of the tick species; *I. scapularis* is the primary vector in eastern North America while *I. pacificus* is primarily responsible in the west, additionally, *I. ricinus* and *I. persulcatus* are the primary vectors in Europe and Asia respectively (Burgdorfer *et al.*, 1985; Tilly *et al.*, 2008).

Due to transmission by ticks, Lyme disease displays a seasonal occurrence with most new cases occurring in the late spring and summer. This is due to the feeding cycle of the tick as larval and nymphal ticks generally feed during these months (Tilly *et al.*, 2008). As is seen in figure 1.1, larval ticks acquire the bacteria by feeding on an infected host such as a small rodent, bird, or reptile which act as a reservoir for *Borrelia* (Donahue *et al.*, 1987). Following molting to the nymphal stage, the infected tick can pass the bacteria to the human host. Although adult ticks also bite humans, it is less likely that the

Figure 1.1 The enzootic life cycle of *Borrelia burgdorferi*. Spirochetes are acquired by the tick vector during larval feeding on small rodents or birds. Following molting, the bacteria may be passed to larger hosts, such as humans, in the nymphal stage putting the greatest risk of acquiring disease during the late spring and summer (CDC, 2011).



bacteria will be passed on by an adult; due to the adults larger size it is more likely that it will be discovered before adequate time has passed to transmit the bacteria to the human (Tilly *et al.*, 2008).

Several bacterial effectors mediate transmission between the tick vector and mammalian host, notable among these are the outer surface proteins (Osps). After being acquired by the tick, bacteria adhere to the surface of the tick midgut until another blood meal is taken, at this time the spirochetes migrate to the salivary glands and are transmitted to the host through the saliva of the tick (Templeton, 2004). In the mid-gut, *B. burgdorferi* binding is mediated by OspA binding to a specific tick receptor for OspA, TROSPA (Pal *et al.*, 2004). During feeding, OspA is down regulated and OspC is concurrently up regulated and displayed on the surface of the bacteria (Gilmore *et al.*, 2001). OspC is necessary for the bacterial invasion of the tick salivary glands during feeding (Pal *et al.*, 2004), where the bacteria are transmitted to the host through the tick's saliva.

1.1.4 Host Response

Upon entering the blood stream, various host factors can contribute to the initial control of bacterial numbers including phagocytic cells such as antigen presenting cells (APCs) and neutrophils, complement, and ultimately T cell and B cell responses (Tilly *et al.*, 2008). Although the initial responses can control the bacterial load in the bloodstream, they are generally inadequate to completely

clear an infection (Tilly *et al.*, 2008) and infection will progress unless antibiotic intervention is used to clear the bacteria from the host.

Initially upon entering the blood stream, bacteria face the challenges of the innate immune response including complement and innate immune cells. Complement appears to be important in controlling spirochete load during early stages of infection as C3 deficient mice show increased spirochete load during early infection (Lawrenz *et al.*, 2003), complement may act to opsonize bacteria for more efficient phagocytosis, or directly kill spirochetes through the alternative complement pathway (Tilly *et al.*, 2008).

Innate response appears to be mediated through the toll-like receptor TLR-2. Mice deficient in TLR-2 or MyD88 show increased spirochetal load and pathology (Wooten *et al.*, 2002; Bolz and Weis, 2004). During later stages of infection, antibodies are generated to various outer membrane proteins of *B. burgdorferi* (Schwan *et al.*, 1989). However, antibody response is also ineffective in completely clearing the host of bacteria; although the bacteria are largely cleared from the bloodstream, they are able to disseminate into various other tissues avoiding clearance (Steere *et al.*, 2004). The spirochetes are able to continually evade the adaptive immune system due to antigenic variation strategies which allow the spirochetes to avoid antibody mediated clearance (Norris, 2006). T cell response does not appear to play a large role in immunity to *Borrelia* as transfer of exposed T cells to severe combined immuno-deficient (SCID) mice did not confer any protection (Schaible *et al.*, 1994).

1.1.5 Signs and symptoms

Lyme disease may be divided into various stages, with different signs and symptoms displayed at each stage (Steere, 1989). Stage one begins localized in the skin and progresses to a disseminated infection (stage two) within days to weeks, and finally results in a persistent infection (stage three) within months to years (Steere *et al.*, 2004). However, as has been mentioned, the specific set of symptoms may differ from patient to patient geographically due to different species of *Borrelia* responsible for infection (Farlow *et al.*, 2002). Additionally, not all patients manifest the entire set of symptoms, which can be diverse in different patients (Steere *et al.*, 2004).

The hallmark of stage one of infection is the characteristic bulls-eye rash, erythema migrans, forming at the site of the tick bite in 70-80% of patients (Steere, 1989). The rash is also frequently accompanied by general flu-like symptoms such as fever, headache, and malaise (Steere *et al.*, 2004). This stage usually has an incubation period of approximately 3 to 32 days before the onset of symptoms and erythema migrans (Steere *et al.*, 2004). Localized infection can last for days or weeks before disease progresses to disseminated infection.

As mentioned, disseminated infection can develop days, or even weeks after the initial onset of disease. During this stage the bacteria disseminates to various tissues in the body including the blood, cerebro-spinal fluid, bone, heart, spleen, liver, meninges, and brain (Duray and Steere, 1988). During

disseminated infections, various neurological or musculoskeletal symptoms can develop due to bacterial infection (Steere *et al.*, 2004).

After weeks of disseminated infection the bacteria may transition to a persistent infection where the disease may be asymptomatic, however, the bacteria continue to persist in localized niches (Steere *et al.*, 2004). Although *B. burgdorferi*, *B. afzelii*, and *B. garinii* can all infect the joints, skin, and brain, the species show tropisms for specific tissues resulting in different manifestations during persistent infection. *B. burgdorferi* may cause intermittent attacks of arthritis in joints, while *B. afzelii* may cause skin conditions such as acrodermatitis chronica atrophicans, and *B. garinii* may result in a variety of neurological symptoms (Steere *et al.*, 2004; Steere *et al.*, 1987; Oschmann *et al.*, 1998). If left untreated, bacteria may persist in these niches for years.

1.1.6 Diagnosis and Treatment

Diagnosis is generally made by recognition of the characteristic erythema migrans or through a combination of clinical diagnosis and a positive enzyme-linked immunosorbant assay (ELISA) and western blot for those patients not presenting with a rash (Steere *et al.*, 2004); clinical diagnosis is made based on flu-like symptoms of headache with joint and muscle pain without gastrointestinal involvement, occurring during the summer months. If a tick is found, removal of the tick is usually enough to prevent Lyme disease, however, if an engorged tick is found, a one-time treatment of 200 milligrams doxycycline is used to prevent disease development (Nadelman *et al.*, 2001).

A positive diagnosis is confirmed by a positive serum antibody response in an ELISA and a Western Blot of whole cell sonicated *Borrelia* (Steere *et al.*, 2004). In the case of confirmed Lyme disease, generally patients are treated with oral doxycycline or intravenous ceftriaxone or clindamycin. For patients with continued arthritis after spirochetal killing, anti-inflammatory drugs may be prescribed.

1.2 *Borrelia burgdorferi*

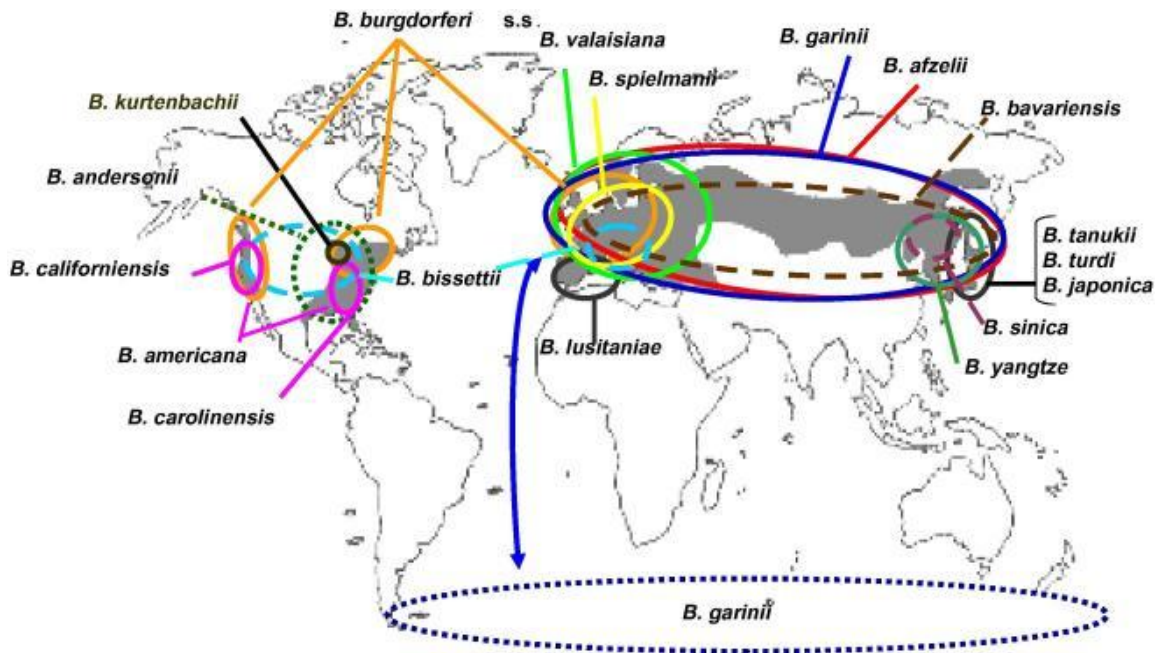
1.2.1 Classification

Borrelia species belong to the family *Spirochaetaceae* which includes several pathogens such as the etiologic agent of syphilis, *Treponema pallidum*, as well as the *Borrelia* species which cause relapsing fever. As has been mentioned, when first discovered, the Lyme borreliosis pathogen was first described as a spirochete resembling *Treponema* (Burgdorfer *et al.*, 1982). The pathogen was later classified as a *Borrelia* species based on sequence homology to the relapsing fever pathogens (Schmid *et al.*, 1984). It is now known that many different *Borrelia* species are capable of causing Lyme disease with different pathogens responsible for disease at different locations in the world (Fig. 1.2) (Margos *et al.*, 2011), and these are collectively known as the *Borrelia burgdorferi* sensu lato (s.l.) complex.

1.2.2 Morphology

Borrelia are unique among spirochetes in displaying a flat-wave morphology and are generally 10-30 μm in length and 0.2-0.3 μm wide (Burgdorfer *et al.*, 1982; Krupka *et al.*, 2007). They are neither gram positive nor gram negative; although they do have a double membrane typical of gram negative species, they are lacking several other features such as LPS that are typically characteristic of gram negative organisms (Takayama *et al.*, 1987). Their shape is conferred through several flagella inserted in the poles of the

Figure 1.2 Global distribution of the *Borrelia burgdorferi sensu lato* complex.
Figure from (Margos *et al.*, 2011).



bacterium that run along the cell wall in the periplasm (Motaleb *et al.*, 2000). The flagella are also responsible for motility of the bacterium as it moves in a corkscrew like manner through its environment.

B. burgdorferi is also capable of adopting non-helical forms, known as round bodies, which can be seen under various stress conditions (Krupka *et al.*, 2007). Such changes in morphology are seen under some circumstances in the tick mid-gut during feeding, and it is suggested that this morphology may be adopted by the spirochetes when conditions for transmission are not favorable (Dunham-Ems *et al.*, 2009). This form of *B. burgdorferi* remains in the midgut lumen, and is not passed from the tick during feeding.

1.2.3 Virulence

As *B. burgdorferi* is transmitted to and maintained in mammalian hosts, it has a variety of mechanisms which aid in its survival. These can largely be divided into two groups: factors which are necessary for establishing colonization of a naïve individual and those which are necessary for evasion of the acquired immune system and maintenance of infection (Tilly *et al.*, 2008). It is notable that *B. burgdorferi* has a surprising amount of outer membrane proteins when compared to other bacterial species (Fraser *et al.*, 1997), many of which are important in the enzootic life cycle of the organism.

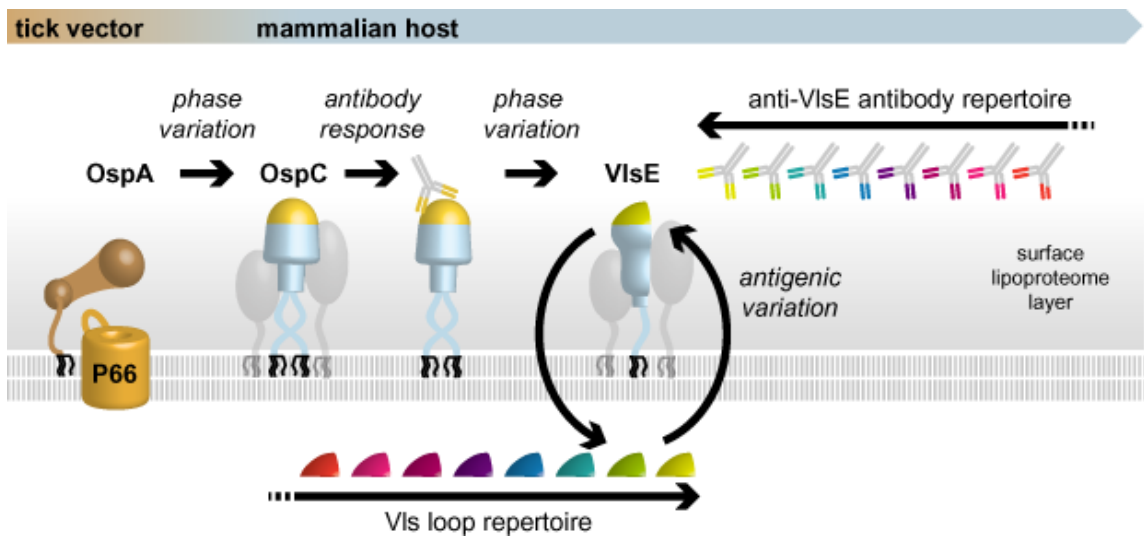
Perhaps somewhat obvious, one component required for mammalian infection with *B. burgdorferi* is the flagellar component *flaB*. Spirochetes deficient for *flaB* show an inability to establish infection in immuno-competent mice (Sultan

et al., 2013). This loss of infectivity is due to the loss of motility resulting in an inability to disseminate in the host tissues. Additionally, the highly motile nature of *B.burgdorferi* may help the bacteria evade phagocytosis by monocytes and macrophages, loss of motility allows the infection to be cleared much more easily (Radolf *et al.*, 2012).

A large portion of the genome of *B. burgdorferi* encodes various lipoproteins that are displayed on the surface of the bacteria; approximately 5% of the genome encodes such proteins, a much greater proportion than in other bacteria (Casjens *et al.*, 2000). Many of these contribute to virulence through acting as adhesins allowing the bacteria to bind endothelial cells and disseminate from the blood stream. These surface exposed proteins bind a variety of molecules normally present at the endothelial surface (Behera *et al.*, 2008;Parveen *et al.*, 2003;Brissette *et al.*, 2009) facilitating invasion into different tissues of the host.

Additionally, some of the surface exposed proteins are involved in direct avoidance of the immune system in the mammalian host. Upon entering the bloodstream spirochetes are confronted with components of the innate immune system such as complement. Tick salivary proteins aid the spirochete initially after feeding (Guo *et al.*, 2009;Kotsyfakis *et al.*, 2006). The bacteria express several complement regulator-acquiring surface proteins (CRASPs) which bind factor H and prevent activation of the complement cascade allowing the spirochete to survive in the blood (Zuckert, 2013).

Figure 1.3 Phase and antigenic variation in *B. burgdorgeri*. In the tick *ospA* is displayed on the spirochete surface allowing the attachment to the tick mid-gut as well as protecting the P66 porin protein from immune detection. In the mammalian host the spirochetes undergo phase variation expressing the OspC protein on its surface which appears to be necessary for establishing early infection. In response to antibody pressure, the spirochetes go through an additional phase variation to express the antigenically variable VlsE. Recombination from the *vls* silent cassettes into the expressed locus results in antigenic variation and continued evasion of the host immune system. Figure taken from (Zuckert, 2013).



Once established in the mammalian host the bacteria are confronted by the cellular effectors of the host immune system. It appears that the bacteria deal with this through a complex system involving both phase and antigenic variation (see Fig. 1.3) (Zuckert, 2013;Tilly *et al.*, 2013). As has been discussed above, *ospA* is expressed during colonization of the tick mid-gut (Fingerle *et al.*, 1995). Shortly after tick feeding the spirochetes down-regulate the expression of *ospA* and increase the expression of *ospC* (Schwan *et al.*, 1995). Although the function of *ospC* is unknown it appears to be essential for immune evasion during early infection of the mammalian host (Tilly *et al.*, 2013).

During later infection the adaptive immune system begins to mount an antibody response to the outer surface proteins of the spirochete (Wilske *et al.*, 1988). In response to immune pressure the spirochete down-regulates the expression of *ospC* and begins to express the antigenically variable protein VlsE (Crother *et al.*, 2004;Zhang *et al.*, 1997). The *vls* locus is present at the right end of lp28-1 as a single expressed locus, *vlsE*, and 15 silent cassettes. Sequence from the silent cassettes recombines into the expressed locus resulting in a mosaic sequence and display of a different antigen (Zhang and Norris, 1998;Coutte *et al.*, 2009). In this way VlsE allows the bacteria to avoid killing by the adaptive immune system and maintain infection in the host.

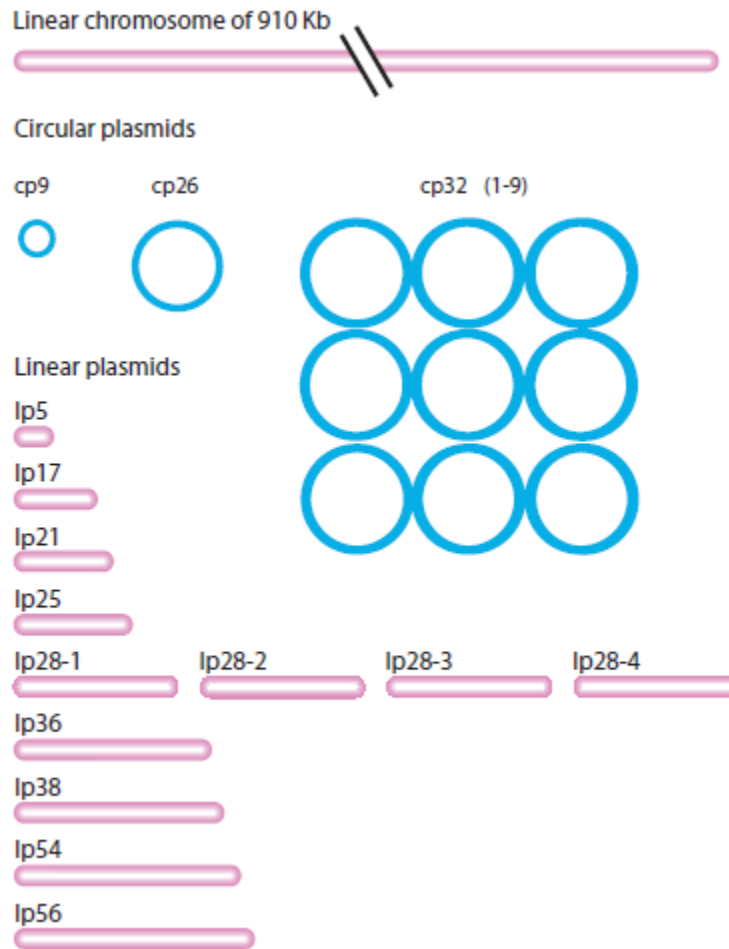
1.3 Genome maintenance and replication in *B. burgdorferi*

1.3.1 Genome of *B. burgdorferi*

Perhaps the most striking feature of *Borrelia* is its unusual, segmented genome. The genome is composed of a single linear chromosome of approximately 1 Mb, and multiple smaller circular and linear plasmids, totaling over 20 different replicons (Fig 1.4) (Chaconas, 2005; Chaconas and Kobryn, 2010). Many of these plasmids carry essential genes, and as such some have been called mini-chromosomes (Barbour and Zückert, 1997). In addition to being highly segmented, the genome of *Borrelia* has a very low %GC content, at just 28.6% for the chromosome (Fraser *et al.*, 1997). Similar to other bacterial species, most of the genome represents coding sequence with very little intergenic sequence.

The genome of *Borrelia burgdorferi* has been sequenced and largely annotated (Fraser *et al.*, 1997). Typical of other obligate pathogens, *B. burgdorferi* is lacking many biosynthetic and metabolic genes and relies on obtaining these products from the host. Also it appears that the genome encodes a large number of lipo-proteins when compared to other bacteria (Fraser *et al.*, 1997), which may be necessary during its lifecycle as an extra-cellular pathogen. Curiously, *B. burgdorferi* does not appear to require iron as iron is apparently not utilized by bacteria grown in culture (Posey and Gherardini, 2000). Additionally, the bacteria only encode one iron acquisition protein which is necessary only in

Figure 1.4 The unusual segmented genome of *Borrelia burgdorferi*. Linear plasmids are covalently closed through hairpin telomeres. Figure taken from (Chaconas, 2005).



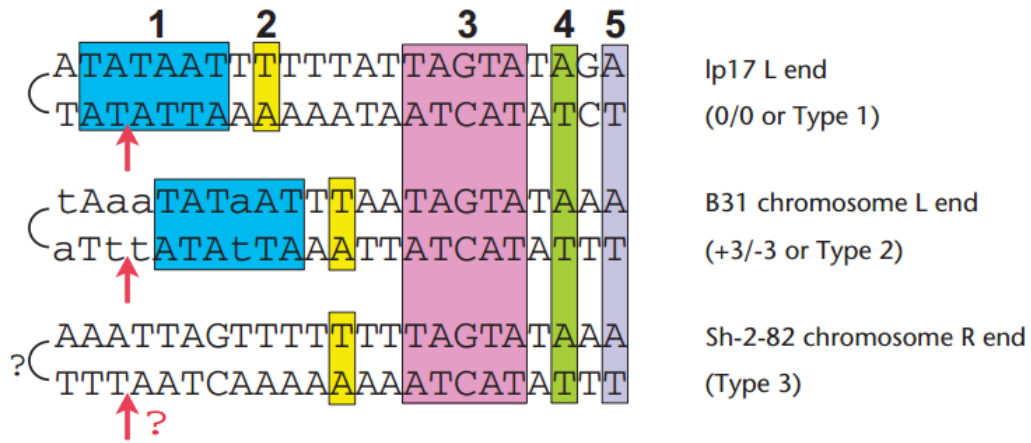
the tick vector (Li *et al.*, 2007). Finally, there are no known toxins encoded by the *B. burgdorferi* genome, all pathology is caused through the actions of the host immune system (Casjens *et al.*, 2000).

1.3.2 Telomeres of linear plasmids in *B. burgdorferi*

The ends of the chromosome and plasmids are covalently closed in a hairpin telomere structure (figure 1.5) (Barbour and Garon, 1987). This structure is rare, the only bacterial examples of such structures are seen in *Agrobacterium tumefaciens* in addition to the various *Borrelia* species (Goodner *et al.*, 2001). The telomeres of *Borrelia burgdorferi* are divided into three types based on the spacing and presence of five conserved boxes (Chaconas, 2005; Tourand *et al.*, 2009; Tourand *et al.*, 2003). There are an extra three base pairs before box one and a 3 bp deletion between box 2 and 3 in type two telomeres (+3/-3), while type three telomeres are missing box one altogether (see Fig. 1.5). Because of this unusual telomere structure, *B. burgdorferi* requires specialized enzymes for the replication and processing of the linear genetic elements.

The hairpin telomeres of *B. burgdorferi* strain B31 have been sequenced and the telomere components characterized (Tourand *et al.*, 2009). Telomeric sequence comprises approximately the last 25 bp of the linear elements prior to the hairpin end (Tourand *et al.*, 2003). As mentioned above, telomeres of *B. burgdorferi* have 5 homology boxes; the different telomere types are differentiated based on the presence and positioning of these boxes

Figure 1.5 Hairpin telomeres of *B. burgdorferi*. There are three telomere types based on the presence and spacing of five conserved telomere boxes. Figure taken from (Chaconas, 2005).



(Tourand *et al.*, 2009; Tourand *et al.*, 2003). Especially important appear to be boxes 3-5; these boxes are never absent from any telomere and are invariably spaced 14 bp from the terminus. Additionally, box 3 always carries the sequence TTAGTATA at positions 14-21 which appear to be important in directing binding of the telomere resolvase (discussed below) at this position (Tourand *et al.*, 2007); mutations in box 3 were not tolerated in telomere resolution assays (Tourand *et al.*, 2003).

Requirements for boxes 1 and 2 appear to be less stringent. There is variant spacing of these boxes between type 1 and type 2 telomeres while type 3 telomeres are missing box 1 altogether (Chaconas, 2005). However, despite different positioning of boxes 1 and 2 in different telomere types the DNA strand is invariably cleaved between nt 3 and 4 of the replicated telomere (Tourand *et al.*, 2003) indicating that proximity to the axis of symmetry and not positioning of box 1 or 2 sequence directs the site of telomere resolution.

1.3.3 DNA replication in *B. burgdorferi*

DNA replication in *B. burgdorferi* is initiated from an internal origin of replication and continues bi-directionally along the replicon (Picardeau *et al.*, 1999, 2000). Each of the plasmids encode their own set of related, but non-identical plasmid maintenance genes (Casjens *et al.*, 2000). Each plasmid contains a complete set of four genes belonging to one of five paralogous families; PF32, PF49, PF50, PF57, and PF62. The gene products of these maintenance genes are thought to be involved in plasmid replication and

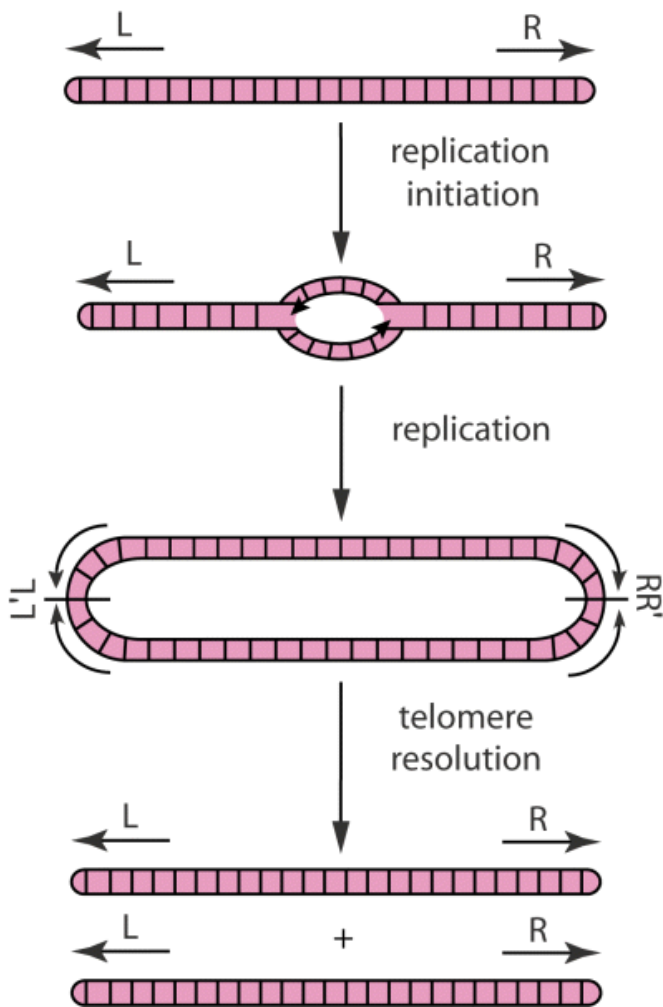
partitioning. If two plasmids have the same set of genes they are incompatible and one plasmid will displace the other (Byram *et al.*, 2004; Austin and Nordstrom, 1990).

The PF57 and PF62 families appear to be distantly related and are thought to encode the replication initiator protein and members of these families are required for autonomous replication in *B. burgdorferi* (Casjens *et al.*, 2000; Chaconas and Norris, 2013; Beaurepaire and Chaconas, 2005). PF49 members appear to play a role in transcriptional regulation in *B. burgdorferi*, however, more evidence is necessary to confirm this. The role of PF32 proteins is currently unknown; although they show some similarity to the plasmid partitioning ParA proteins, there is no data to confirm that they fulfill this role in *B. burgdorferi* (Chaconas and Norris, 2013). Finally, the role of PF50 members is still unknown.

1.3.3 Replication of linear plasmids in *B. burgdorferi*

One of the initial mysteries of *Borrelia burgdorferi* was the mechanism by which the spirochetes dealt with the end replication problem for linear DNA elements. At the time, the two most appealing theories were that the bacteria initiated replication from an internal origin resulting in a circular dimer as an intermediate which is then resolved to two daughter molecules (Fig. 1.6), or that site specific enzymes generated a single stranded nick near to the telomere so the free 3' end could act as a primer for DNA elongation (Chaconas *et al.*, 2001). The first model was proved to be correct, as a replicated telomere placed in the

Figure 1.6 Replication of linear genetic elements in *B. burgdorferi*. DNA replication is initiated at an internal origin of replication and proceeds bi-directionally along the length of the replicon resulting in a circular head-to-head (L'L) tail-to-tail (RR') dimer. The dimer is resolved to two daughter molecules through the process of telomere resolution. Figure from (Chaconas, 2005).



middle of lp-17 was resolved creating a size shift as seen by Southern blot. Resolution of replicated telomere substrates has since been described extensively *in vitro* (Chaconas, 2005;Chaconas and Kobryn, 2010;Kobryn and Chaconas, 2002) and *in vivo* (Beaurepaire and Chaconas, 2005;Chaconas *et al.*, 2001), however, specific observation of a replication intermediate *in vivo* has yet to be reported.

1.3.4 Telomere resolution in *B. burgdorferi*

The enzyme responsible for the resolution of replicated telomeres was later found. The *bbb03* locus was selected as the putative telomere resolvase due to limited homology with the telomere resolvase from the *E. coli* phage N15 (Kobryn and Chaconas, 2002). This protein was able to resolve replicated telomere substrates *in vitro* and was able to generate the hairpin telomere products. *bbb03* has since been named resolvase of telomeres (*resT*).

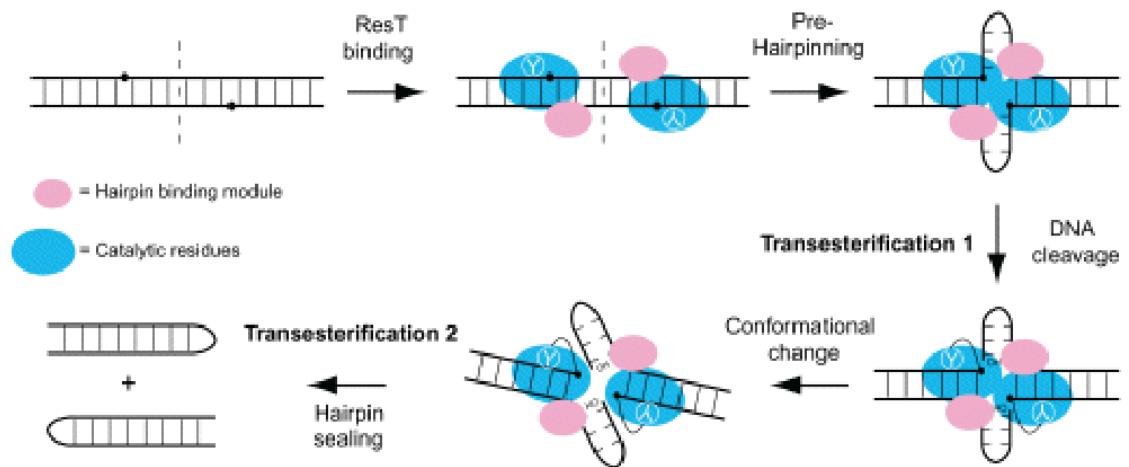
Telomere resolution requires two molecules of ResT bound on either side of the axis of symmetry working in co-operation to form the hairpin telomere (Kobryn and Chaconas, 2002). ResT binds the replicated telomere and forms a cross-axis complex which is a necessary first step for reaction to occur (Kobryn *et al.*, 2005). Positive supercoiling appears to play a role in the telomere resolution process as positive supercoiling of the substrate promotes co-operative binding of the enzyme to the DNA substrate (Bankhead *et al.*, 2006). Normally positive supercoiling only exists in the cell as a by-product of unwinding DNA during replication. However, rotation of the DNA helix allows the monomers

to interact productively and leads to telomere resolution (Bankhead *et al.*, 2006). It appears that it is the productive interaction of the two ResT monomers and not DNA topology itself that facilitates binding; ResT binding to a telomere half-site showed no increase due to positive supercoiling.

ResT is directed to replicated telomeres through sequence recognition of telomeric box 3 sequence, as discussed above, by the C-terminal domain of the protein (Tourand *et al.*, 2003; Tourand *et al.*, 2007). Sequence specific binding of the C-terminal domain to the replicated telomere serves to position the N-terminal domain which displays non-specific DNA binding capabilities. The N-terminal domain also contains a putative hairpin binding module similar to that in the Tn5 transposase (Bankhead and Chaconas, 2004). Mutation of the hairpin binding domain of ResT completely blocked formation of covalent protein-DNA complexes indicating that formation of the hairpin precedes the cleavage of the DNA strands.

Telomere resolution occurs through a two step DNA breakage and re-union event (figure 1.7) (Kobryn and Chaconas, 2002). ResT is bound at the replicated telomere with one molecule on each side of the dimer. Catalytic attack of the DNA backbone is carried out by a tyrosine in the ResT active site (Y335) resulting in breakage of the phosphodiester bond and creating a temporary DNA-protein intermediate (Deneke *et al.*, 2004). Catalytic attack of the protein-DNA intermediate by the free hydroxy group on the opposite strand then generates the hairpin telomere.

Figure 1.7 Mechanism of ResT in *Borrelia burgdorferi*. Telomere resolution occurs through ResT binding on each side of the replicated telomere and pre-forming hairpins prior to catalytic attack of the DNA backbone by the active site tyrosine. Telomeres are formed when the protein-DNA intermediate undergoes another nucleophilic attack performed by the free hydroxy group on the opposite strand. Figure adapted from (Kobryn *et al.*, 2005).



1.4 Study Introduction

1.4.1 General Introduction

In the past, limited molecular tools have hampered the study of *B. burgdorferi* (Rosa *et al.*, 2005). However, tools such as selectable markers (Bono *et al.*, 2000; Elias *et al.*, 2003; Frank *et al.*, 2003) and shuttle plasmids (Stewart *et al.*, 2001; Sartakova *et al.*, 2000) have greatly aided the studies of this organism. Even more recent is the adaptation of inducible expression systems for use in *B. burgdorferi* (Gilbert *et al.*, 2007; Whetstine *et al.*, 2009; Blevins *et al.*, 2007). Tight regulation of expression *in vivo* has allowed the conditional expression of essential genes, something which was not previously possible (Liang *et al.*, 2010; Groshong *et al.*, 2012).

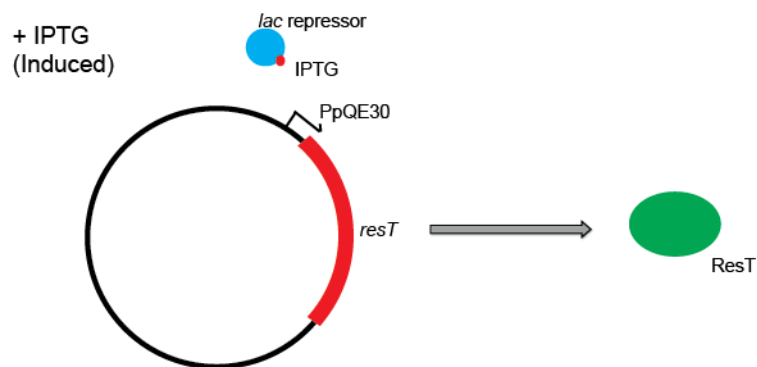
Although multiple inducible systems have been reported in *B. burgdorferi*, knock-out of essential genes has only been reported using one of them (Liang *et al.*, 2010; Groshong *et al.*, 2012). pJSB104 allows tight expression in *B. burgdorferi* from a phage T5 promoter under control of two *lac* operator sequences; the *lac* repressor has been codon optimized for expression in *B. burgdorferi* and is constitutively expressed from the *flaB* promoter (Blevins *et al.*, 2007). Conditional expression is achieved through control of the inducing agent isopropyl β -D-1-thiogalactopyranoside (IPTG) in the media; when no IPTG is present the repressor protein binds the operator sequences and expression of the gene is turned off resulting in gradual depletion of the expressed protein.

resT has been demonstrated as an essential gene in *B. burgdorferi*. Previous attempts at generating a knock-out or displacing the plasmid harboring *resT* have been unsuccessful (Byram *et al.*, 2004; Tourand *et al.*, 2006; Jewett *et al.*, 2007). Use of an inducible expression system should allow conditional expression of *resT in vivo* and investigation into *resT* depletion phenotypes. As seen in figure 1.8, expression will be controlled by controlling the availability of the inducing agent, IPTG, to the bacteria. When IPTG is present ResT should be expressed normally and the cells should display no phenotype. However, when IPTG is not present, the repressor should bind to the operator sequence allowing conditions where ResT is depleted from the spirochetes.

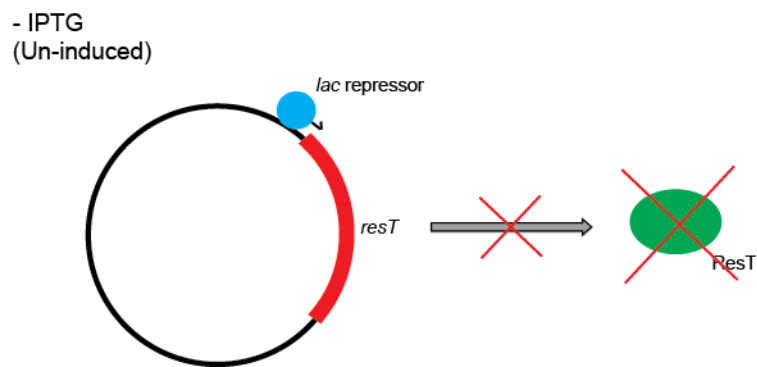
Under ResT depletion conditions we may expect several phenotypes. First, as spirochetes will not be able to resolve dimers of linear molecules we expect that they will not be able to segregate their DNA to daughter cells and thus not be able to divide. This may result in a filamentous phenotype where daughter cells remain linked together as has been seen in other mutants unable to complete cell division (Liang *et al.*, 2010). Additionally, this should allow the accumulation of replication intermediates in the spirochete such that they may be observed and investigated. Finally, it is unknown what effects a *resT* knock-out may have with respect to genome replication; replication may continue in *resT* knock-out conditions resulting in an accumulation of DNA in the spirochete.

Figure 1.8 Control of *resT* expression in *B. burgdorferi*. **(A)** When IPTG is present it will bind to the *lac* repressor allowing expression of *resT* from the PpQE30 promoter. **(B)** When IPTG is washed out of the media, the *lac* repressor will bind operator sequences present in the PpQE30 promoter and block the expression of *resT*.

A



B



1.4.2 Study Aims

The focus of this study is the production and characterization of a *resT* mutant *in vivo*. To this end I have focused on three aims:

1. Construction of a conditionally expressing *resT* strain
2. Characterization of the *resT* mutant with respect to growth and viability
3. Investigation into the state of linear replicons in the *resT* mutant upon ResT depletion

Chapter Two: Materials and Methods

2.1 Growth and transformation of bacterial strains

All plasmids were propagated in the *E. coli* strain DH5 α . Competent cells were prepared according to standard protocols, and incubated on ice with a ligation reaction for 30 minutes. This was followed by a 45 second heat shock at 42°C before placing the bacteria on ice again for 5 minutes. The bacteria were then allowed to recover at 37°C with shaking in 1 mL of fresh LB media with no antibiotic selection. Following recovery, the bacteria were then plated on LB agar plates with the appropriate antibiotic selection and grown overnight at 37°C

Competent *Borrelia burgdorferi* was prepared by first growing the cells to a cell density between 5×10^7 and 1×10^8 in BSK II media as enumerated by a Petroff-Hauser Counting Chamber. The bacteria were grown at 35°C with 1.5% CO₂ until appropriate cell density was reached, then the culture was placed on ice for 30 minutes. The cells were harvested by centrifugation at 6000 g for 15 minutes at 4°C and the cells were resuspended in 30 mL of ice cold dPBS²⁺ then centrifuged again as before. The cells were then resuspended in 20 mL of ice cold EPS and centrifuged a third time as before. The bacteria were then resuspended in 1.8 mL of EPS and transferred to a 2 mL eppendorf tube and centrifuged again at 6000 g for 15 minutes at 4°C. The bacteria were resuspended in 150 μ L of EPS and the optical density was determined by diluting 35 μ L of cells in 750 μ L of EPS to a final volume of 785 μ L then reading the OD

at 600 nm. The cells were adjusted to an optimum OD of 0.36 then aliquoted into 50 μ L aliquots and frozen at -80°C until they were needed.

For transformation, the cells were thawed on ice then 50 ng of plasmid DNA was added to the cell suspension. The cells were allowed to incubate with the DNA for 30 minutes before electroporation in a Bio-Rad cuvette at 200 Ω , 25 μ F and 2.5 kV. The cells were immediately added to 10 mL of pre-warmed BSK II media then aliquoted into 5 separate 2 mL aliquots. Transformations were allowed to recover for 20 hours at 35°C with 1.5% CO₂ with no antibiotic selection then were added to a final volume of 20 mL of warm BSK II media for each 2 mL aliquot, and plated in 250 μ L aliquots on a 96 well plate with appropriate antibiotic selection. For the construction of the *resT* knock-out strains, IPTG was also added to the plates at concentrations of 0.05, 0.1, 0.5, and 1 mM to allow induction of *resT* from pNB12. The cultures were allowed to grow until the culture in individual wells began to turn yellow, approximately 8 to 14 days.

2.2 Extraction of *Borrelia* DNA

Borrelia were grown in 5 mL of BSK II media until the culture media turned yellow and the bacteria reached a density of approximately 1×10^8 spirochetes per mL. The bacteria were harvested by centrifugation at 6000 g at 4°C for 15 minutes, then resuspended in ice cold PBS. The cells were harvested once again by centrifugation then resuspended in 50 μ L of extraction buffer (100 mM Tris pH 8.5, 10 mM EDTA, 30 mM NaCl). The cells were lysed by the addition of 2.5 μ L of 10% SDS and lysis was allowed to carry out for 10 minutes at room

temperature. Following lysis, 10 units of RNase A were added to the suspension and digestion was allowed to proceed for 30 minutes at room temperature. Following this, 10 units of Proteinase K was added to the mixture and digestion was allowed to proceed for at least 4 hours at 37°C.

DNA was extracted from the digestion mixture with phenol:chloroform:isoamyl alcohol; the digested cell lysate was rotated with the phenol:chloroform:isoamyl alcohol for 20 minutes at room temperature, then the phases were separated by centrifugation at 13,000 rpm for 5 minutes. The aqueous phase was recovered, and phenol:chloroform:isoamyl alcohol treatment was repeated. The DNA was dialyzed on a Millipore VSWP microdialysis membrane for 2 hours at 4°C into 10 mM Tris and 0.1 mM EDTA. All genomic DNA was stored at 4°C.

2.3 Plasmid construction

A reporter construct was built which contains *gfp* in the expression site of the pJSB104 backbone (Blevins *et al.*, 2007). The inducible *PpQE30* promoter was amplified from pJSB104 using the primers B2145 and B2146. At the same time *gfp* was amplified from pTM61 (Moriarty *et al.*, 2008) using the primers B2147 and B2148 by polymerase chain reaction (PCR)(for primer sequence see Table 2.1). Both PCR reactions were carried out with an initial denaturation of 3 minutes at 98°C followed by 25 cycles of 98°C for 10 s, 57°C for 30 s, and 68°C for 75 s then a final elongation of 10 minutes at 68°C then holding the sample at 4°C. The bands resulting from these reactions were purified using a PCR

purification kit (QIAGEN) and 2.5 μ L of each of the purified reactions were used as template for the following reaction. The PCR was repeated as above using the primers B2145 and B2148 to produce *PpQE30-gfp*, this was ligated into the pJet 1.2 blunt vector (Fermentas) and used in transformation of *E. coli* DH5 α . The intermediate plasmid was recovered using the CloneJet Miniprep kit (Fermentas); the *PpQE30-gfp* insert was removed from the backbone by a double digest using both BamHI and HindIII for 1 hour at 37°C, the insert was recovered through electrophoresis on a 1% agarose gel and extraction using a gel extraction kit (Qiagen). Likewise, pJSB104 was digested with both BamHI and HindIII and the backbone recovered as described above. Ligation was carried out with T4 ligase (NEB) at 16°C for 3 hours then transformed into competent DH5 α as described above. The resulting plasmid, pNB11, was transformed into *B. burgdorferi* as described above and used for the induction and wash-out assays below.

The construction of the inducible *resT* plasmid, pNB12, was completed in a two step process. First, the IPTG inducible *PpQE30* was linked to *resT* using overlap extension PCR. The *PpQE30* promoter was amplified from the plasmid pJSB104 (Blevins *et al.*, 2007) using the primers B2145 and B2149. At the same time *resT* was amplified from B31-A (GCB908) genomic DNA using the primers B2150 and B2169. Both reactions were carried out with an initial denaturation of 3 minutes at 98°C followed by 25 cycles of 98°C for 10 s, 57°C for 30 s, and 68°C for 75 s then a final elongation of 10 minutes at 68°C then holding the sample at 4°C. The bands resulting from these reactions were purified using a PCR purification kit (QIAGEN) and 2.5 μ L of each of the purified reactions were

used as template for the following reaction. PCR was repeated as above using the primers B2145 and B2160 to generate a product where *resT* is under control of the *PpQE30* promoter. This product was purified from a 1% agarose gel and ligated into pJet 1.2 blunt (Fermentas) as per the manufacturers directions. The resulting ligation was transformed in DH5 α as described above.

As the HindIII cut site at the 3' end of the expression site in pJSB104 was not conducive to cloning with *resT*, a construct was made which included the multiple cloning site (MCS) from pJLB12g (Bono *et al.*, 2000) downstream of the expression site of pJSB104. The MCS was amplified by PCR using the primers B2155 and B2156 using the PCR conditions above. This product was cloned into the pJet 1.2 blunt cloning system then removed from pJet through digestion with HindIII. The pJet construct and pJSB104 were digested with 10 units of HindIII at 37°C for 1 hour. The resulting digestion products were cut out of a 1% agarose gel and purified using a gel purification kit (QIAGEN). The pJSB104 was de-phosphorylated with 1 unit of calf intestinal phosphatase (NEB) for 15 minutes at 37°C to avoid re-ligation of the backbone. Ligation was carried out with T4 ligase (NEB) at 16°C for 3 hours then transformed into competent DH5 α as described above to give the pJSB104MCS vector.

Next, the *PpQE30-resT* insert was cut out of the pJet 1.2 blunt backbone using the enzymes BamHI and XhoI, similarly pJSB104MCS was digested with the same enzymes to generate the compatible sticky ends. Digest was set up as a double digest and allowed to complete for 1 hour at 37°C. The products were gel purified and ligated as described above before transformation into competent

DH5 α . *E. coli* colonies were screened for the presence of the insert through PCR with the primers B2145 and B2169 as described above, as well as digestion with BamHI and XhoI to check the size of the inserts.

Similarly, the *resT* knock-out plasmid, pNB13, was also built through overlap extension PCR. First, 500 bp flanking *resT* on either side in cp26 were amplified using the primers B2209 and B2210 for sequence 3' of *resT* as well as B2213 and B2214 for the 5' flanking region and PCR was carried out as described above; a kanamycin resistance cassette was amplified using the primers B2211 and B2212. Similar to the construction of pNB12, the kanamycin cassette was first joined to the 3' flanking region as described above using the primers B2209 and B2212, this fragment was then joined to the 5' flanking region using primers B2209 and B2214. The final PCR product was purified and cloned into pJet 1.2 blunt as described above.

2.4 GFP induction and washout assays

In order to assess GFP induction, *Borrelia* were grown to approximately 1×10^8 spirochetes per mL without IPTG. The bacteria were then diluted to 1×10^6 spirochetes per mL in fresh BSK II media and supplemented with 0, 0.05, 0.1, 0.5 or 1 mM IPTG and allowed to grow for 48 hours. The number of fluorescent spirochetes was enumerated through fluorescence microscopy by counting 30 spirochetes by phase contrast microscopy then determining how many of those were fluorescent under GFP excitation conditions.

For assessing the control of the IPTG induction in *Borrelia*, IPTG wash-out assays were performed in order to assess the time needed for loss of GFP expression in *Borrelia*. *Borrelia* were grown to 1×10^8 spirochetes per mL and diluted back to 1×10^6 spirochetes per mL in fresh BSK II media supplemented with 1 mM IPTG. The spirochetes were allowed to grow for 48 hours then were harvested by centrifugation at 6000 g at 4°C for 15 minutes. The spirochetes were resuspended in PBS before centrifugation again, and then finally resuspended in 5 mL of fresh BSK media either with or without IPTG. The cells were counted every 2 hours for 24 hours on a Petroff-Hauser Counting chamber and 1 mL of spirochetes were collected through centrifugation and resuspended in 100 μ L PBS. The fluorescence was read on a Victor II Wallac plate reader and fluorescence of the washed out strain was compared to that of the induced strain.

2.5 Screening *Borrelia* transformants

Potential *Borrelia* transformants were identified by wells which had turned yellow following incubation after electroporation on the 96 well plates. Yellow wells result from a decrease in pH of the growth media due to spirochete growth and indicate the presence of viable spirochetes in the well. Spirochetes from these wells were diluted to approximately 1×10^6 spirochetes per mL in fresh BSK II with appropriate antibiotic selection then the DNA was extracted as described above. PCR was performed on the spirochete DNA to screen for the presence of insert. In the case of pNB12 the primers B2180 and B2181 were used to screen for the presence of the insert in *Borrelia* DNA. Clones which were positive for the

insert were verified by DNA sequencing. Potential *resT* knock-out clones were screened using the primers B2251 and B2252 which anneal in the homologous recombination target; the native *resT* gene is larger than the kanamycin cassette which is inserted in this site and gives a larger band than that observed in the case of insertion. Again, recombinants were confirmed by DNA sequencing.

2.6 Displacement of pBSV2BhresT from GCB51

The plasmid containing the *Borrelia hermsii resT* gene, pBSV2BhresT, was displaced from the *Borrelia burgdorferi* strain GCB51. GCB51 was made competent and transformed with pNB12 which encodes streptomycin resistance. The resulting clones were isolated through serial passage in increasing concentrations of streptomycin at 100 µg/ml, 2.5 mg/ml, 4 mg/ml, and 6 mg/ml. After each passage the cultures were plated in 96 well liquid plates at the same concentration of streptomycin and allowed to grow to late log phase. To ensure that positive wells were the result of a single *B. burgdorferi* clone, the bacteria were plated at a concentration of approximately 2.8 spirochetes/ml in 250 µL aliquots and allowed to grow until the wells turned yellow. These were then replica plated in 96 well liquid plates in fresh BSK-II in 200 µg/ml kanamycin to screen for clones that were resistant to streptomycin but sensitive to kanamycin. Clones which grew under the selection of streptomycin but not under the selection of kanamycin were identified as potential displacement clones and screened by PCR for the loss of the *Borrelia hermsii resT* gene using the primers B322 and B323 which are unique to the *Borrelia hermsii resT* gene.

2.7 Growth of conditional *resT* knock-outs

Borrelia was grown in BSK II media supplemented with 1 mM IPTG and 100 µg/mL streptomycin at 35°C until it reached a density of approximately 1×10^8 spirochetes per mL. The spirochetes were collected by centrifugation at 6000 g at 4°C and resuspended in 5 mL sterile PBS then centrifuged again. The spirochetes were resuspended in sterile PBS and used to seed fresh cultures at 1×10^6 spirochetes per mL with or without IPTG.

Growth curves were constructed by growing the conditional knock-out strains as outlined above. Spirochetes were enumerated at 24 hour intervals by counting on a Petroff-Hauser Counting Chamber. Spirochetes were diluted by $\frac{1}{2}$ in fresh BSK II after 48 hours to ensure that cells remained in the log phase so that any deficiencies in growth could be observed.

2.8 Western blotting

B. burgdorferi were grown as described for conditional *resT* knock-out above. At 0, 8, 16, 24, 32, and 40 hours 5 ml aliquots were taken from the culture and harvested by centrifugation at 6000 g for 15 minutes. The cells were resuspended at 5×10^9 spirochetes/ml in a direct lysis SDS loading dye (125 mM Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 1.4 M 2-mercaptoethanol and 0.2% bromophenol blue) (Knight and Samuels, 1999). The bacteria were boiled for 5 minutes at 95°C and loaded on a 7% SDS polyacrylamide gel with a 5% stacking gel. The gel was run at 80 V until the dye front reached the bottom of the gel,

then transferred to an Amersham Hybond-ECL nitrocellulose membrane (GE Healthcare) for blotting.

Blotting was performed by blocking in 5% non-fat dry milk in TBS (50 mM Tris-HCL pH 7.4). The membrane was blotted for 1 hour with a 1:10,000 dilution of an antiserum raised against ResT in 5% milk followed by three washes in 1x TBS at room temperature. A secondary anti-rabbit antibody conjugated to horseradish peroxidase (Cedarlane) was used in a 1:5,000 dilution to probe the membrane for another hour before three more washes in 1x TBS. Protein signal was detected by application of 3,3',5,5'-tetramethylbenzidine (Sigma) to the membrane until bands became visible.

2.9 Field inversion and southern blotting of *Borrelia* DNA

Total genomic *Borrelia* DNA was run on field inversion gel electrophoresis on a 0.65% agarose gel to visualize genomic DNA. Genomic DNA was extracted as is described above and 200 ng of total genomic DNA was loaded on a 0.65% agarose gel and run for 40 minutes at 80 V. After 40 minutes field inversion was turned on to the manufacturer preset Program 0 on a MJ Research PPI-200 programmable power inverter with buffer circulation and the gel was run under field inversion conditions for an additional 21 hours. The gel was then stained with ethidium bromide and visualized in a FluorChem 8900 imager.

For southern blotting, the gel was then treated in a 1% HCl depurination solution for 45 minutes then washed for 2 minutes in distilled water. The gel was

then treated twice with 500 mL of denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 15 minutes each, followed by another 2 minute rinse in distilled water. Finally, the gel was treated twice with 500 mL of renaturation solution (1 M Tris, 1.5 M NaCl, pH 7.4) for 15 minutes each.

DNA was transferred to the nitrocellulose membrane through an overnight transfer. First, two 3M Whatman papers and nylon membrane cut to the size of the gel were pre-soaked in 6x SSC buffer (3 M NaCl, 5.7 M sodium citrate, pH 7.0). The treated agarose gel was transferred to cling wrap on the bench top and covered with the pre-soaked nylon membrane ensuring no air bubbles between the gel and the membrane. This was then covered by the pre-soaked filter paper then approximately 2 inches of dry paper towel. The transfer was allowed to sit on the bench top over night so that the transfer of DNA could take place. DNA was fixed to the membrane using a UV Stratalink 1800 using the auto-crosslink feature.

Probes were generated to base pairs 12,980 to 13,727 in Ip17 using the primers B2275 and B2276 through PCR (98°C for 3 minutes followed by 25 cycles of 98°C for 10 s, 57°C for 30 s, and 68°C for 75 s then a final elongation of 10 minutes at 68°C then holding the sample at 4°C). Similarly, a probe was generated to base pairs 18,725 to 19,374 of Ip28-2 through PCR as above. For ResT assay on the ends of the linear plasmids (see below) additional probes were needed for the left end of Ip17 and the right end of Ip28-2. These were made by PCR as above using the primers B2317 and B2318 for Ip17 and B2331

and B2332 for Ip28-2. In all cases, the PCR product was generated directly from extracted *Borrelia* DNA.

Probes were purified using a QIAquick PCR purification kit (Qiagen) before radioactive labeling with the Random Primers DNA Labeling System (Invitrogen). Labeling was performed as per the manufacturer's instructions, using α -³²P dCTP to label the probes. This was allowed to incubate for an hour at 25°C before being stopped with the manufacturer's Stop Buffer. At this point 300 μ L of BLOTTO was added to the reaction to prepare it for use in hybridization.

For hybridization, the membrane was first pre-incubated at 55°C for 2 hours in BLOTTO (SSC, 0.1% SDS, 0.5% non-fat dairy milk, 1 mM Sodium Pyrophosphate (NaPPi)). After pre-incubation the BLOTTO was poured off and approximately 15 mL of fresh BLOTTO was added to the hybridization tube. The labeled probe was heated to 95°C followed by a pulse spin before adding directly to the hybridization tube. The hybridization was allowed to go overnight at 55°C with rotation.

Following overnight hybridization, the blot was washed with 2x SSC with 0.1% SDS for twenty minutes at 55°C with rotation, this was repeated for a total of 3 washes. Following this, the blot was washed with 0.2x SSC with 0.1% SDS at 55°C with rotation for twenty minutes, again this was repeated for another 3 washes. Finally, the blot was removed from the hybridization chamber and placed on dry 3M Whatman paper and covered with cling wrap. The radioactive signals of the bands were detected by exposure using phosphor screens (Perkin

Elmer) for exposures between one hour and up to overnight. The screens were imaged using a Packard Cyclone imager (Packard) and using the OptiQuant imaging software.

2.10 Multiplex PCR for analysis of genomic content

Multiplex PCR was performed for analysis of genomic content as described by Bunikis *et al.* (Bunikis *et al.*, 2011). Briefly, *B. burgdorferi* DNA was purified as described above and 100 ng total genomic DNA was used as template for the detection of linear and circular plasmids. PCR reactions were run on a 3 % metaphor agarose gel and stained with Gel Red (Cedar Lane) for visualization.

2.11 ResT assays on *Borrelia* DNA

Total *B. burgdorferi* genomic DNA was purified from spirochetes grown in non-inducing conditions after 72 hours as described above. The DNA was digested with 10 units of either EcoRI for detection of the left-end lp17 telomere or BamHI for the detection of the right-end lp28-2 telomere. The reactions were carried out at 37°C for 2 hours in the appropriate reaction buffer as recommended by the manufacturer (NEB). The buffers were adjusted to 100 mM NaCl, 10 mM EDTA, 100 µg/ml bovine serum albumin (BSA), and 5 mM spermidine prior to incubation with 200 ng ResT. The reaction was allowed to continue for 2 hours at 30°C then immediately loaded on a 1 % agarose gel. The

bands were detected through southern blotting for the telomeres as described above.

2.12 Microscopy for length measurements and genomic content

Wild-type *B. burgdorferia* (GCB908) as well as the conditionally expressing *resT* strain (GCB2127) were grown to late log phase and IPTG wash-out was performed as described above. Cultures were sampled every 24 hours and culture was stained with DAPI to a final concentration of 100 mM DAPI (Invitrogen) in a total of 10 μ L. The culture was mixed with 1 μ L of Fluoromount (Sigma-Aldrich) in preparation for microscopy.

Slides were imaged on a Leica DMIRE2 fluorescent microscope with a Hamamatsu ORCA-ER camera. An exposure time of 500 ms was used for capture of all images. Fluorescence was measured using the ImageJ software (NIH), individual spirochetes were selected and the background was subtracted from the raw integrated fluorescence to give the corrected total fluorescence. Length was measured by tracing the length of the spirochete in ImageJ (NIH) and comparison with a calibrated scale bar generated by the microscope.

2.12 qPCR for quantitation of chromosomal origins and telomeres

A qPCR was performed on the chromosome to quantify the ratio of telomeres to origins. The qPCR was carried out using the primers B2439 and B2440 for the telomere and B2441 and B2442 for the origin. Reactions were made in a final volume of 25 μ l containing 1x iQ SYBR Green Supermix (Bio-

Rad), 200 nM of each primer, and 100 ng of template DNA. Additionally, a 10-fold serial dilution of the wild-type DNA was used to calculate primer efficiencies and allow quantification for each primer set. PCR was performed on a Bio-Rad iQ5 Multicolor Real-Time PCR Detection System as follows: 98°C for three minutes followed by 45 cycles of 98°C for 10 s, 57°C for 30 s and 68°C for 30 s. Threshold values were determined using the Bio-Rad iQ5 software.

Table 2.1 Primers used in this study

Primers used in construction of an inducible <i>resT</i> expression vector			
Product	Primer s	Target	Sequence ¹
<i>PpQE30-resT</i>	B2145	<i>PpQE30</i>	BamHI - ggatcct ctagaaaatcataaaaaatttattgc
	B2149	<i>PpQE30</i> + <i>resT</i>	CTTCACTTTTGGAGGCATatgtaatttctcctcttaat gaattc
	B2150	<i>PpQE30</i> + <i>resT</i>	gaattcattaaagaggagaaattacatATGCCTCCAAA GTGAAG
	B2169	<i>resT</i>	XhoI - ctcgag CTATAGCTTATAATTA AAAATTATTGA TAAGTATTCTG
MCS	B2155	pJLB12g	HindIII - aagctt CTCGAGATCTATCG
	B2156	MCS	CGAATTCGAGCTCCC
¹ Lower case text indicates <i>PpQE30</i> , bold text represents added restriction sites.			
Primers used in knock-out of wild-type <i>resT</i>			
Product	Primer s	Target	Sequence ¹
Δ <i>resT::kan</i>	B2209	<i>bbb04</i>	GTTTAACAAGTACCTTAACCTTATTTTTTG
	B2210	<i>bbb04</i> + <i>kan</i>	gatgctcgatgagttttctaaTTTTTGTTTTTTAAATT CAGGC
	B2211	<i>bbb04</i> + <i>kan</i>	GCCTGAATTTAAAAAACAAAAAttagaaaaact catcgagcatc
	B2212	<i>kan</i> + <i>bbb02</i>	CTCTACTTTTGCAGGAAAAAGAATAGtaccgga gctcaagga
	B2213	<i>kan</i> + <i>bbb02</i>	tcctgaagctcgggtaCTATTCTTTTCTGCAAAA GTAGAG
	B2214	<i>bbb02</i>	CAAATCTTGATTCTAATATTAGGGC
¹ Lower case text represents <i>kan</i> sequence			

Table 2.1 cont.

Primers used for Southern blotting			
Target	¹ Probe Generated	Primer	Sequence
lp17	12,980-13,727	B2275	GCTAAGACAGGAATTTTCCG
		B2276	GCAAATTCAAATCCCAATTTTC
	4,854-5,431	B2317	CAATTATTGTATAGCTATCCAG AATAAAGG
		B2318	GTAAAATATATTTTTATTGATGA AAATTTGG
lp28-2	18,725-19,374	B2295	GGTGCATTTTTAGTAATTTTCAT ACTC
		B2296	GATACTAAAAAATTTTGTATTC CAGATGG
	26,461-26,960	B2331	GGAGGTAAGGGGAGCAAG
		B2332	AGTGTGGAGATACAAAAACAC AC
¹ The coordinates noted are from the complete linear plasmid sequences, including telomeres (Tourand <i>et al.</i> , 2009).			
Primers used for qPCR of chromosome			
Primer	Target	Sequence	
B2439	Telomere	GCATTAATCAATCCCTTTGTC	
B2440		GAGCAAATAACGTGGGAAG	
B2441	Origin	GTATAGTGCTAAAACCAACAAGG	
B2442		CAAGCTGGCATTGTAAGC	
Primers used for screening			
Primer	Sequence	Target	
B2177	ATACGAATATGATTTTTATCTGCG	Middle of <i>resT</i> , reverse	
B2178	ATACCTATATTGAAATAATTAAGCTTCTA CTG	Middle of <i>resT</i> , forward	
B2180	CAAATAATGCCCGTG	pNB12, flanking the expression locus	
B2181	CTTTCATGCGCTTAACG		
B2251	GAAATGTAAATCGTTCTATTGTCATTAG	cp26, flanking the <i>resT</i> locus	
B2252	GGTTGTCCATGGAACCAG		
B322	CGACGTCGACGATAAGGATATACAAGTT TCTAC	<i>Borrelia hermsii resT</i>	
B323	CTCCGTCGACGGATATCGAACATAAAAG CA		

Table 2.2 Strains used in this study

<i>B. burgdorferi</i> strains		
Strain number (GCB)	Description	Reference
908	B31-A, a high-passage B31 derivative	(Bono <i>et al.</i> , 2000)
2083	IPTG inducible <i>gfp</i> carried on pNB11 in GCB51	This Study
2093	IPTG inducible <i>resT</i> carried on pNB12 in B31-A	
2127	Complete <i>bbb03</i> deletion in B31-A, complemented by <i>resT</i> on pNB12	
51	Partial <i>resT</i> deletion, complemented by <i>Borrelia hermsii resT</i>	(Tourand <i>et al.</i> , 2006)
2103	GCB51 with pBSV2 <i>BhresT</i> displaced by pNB12	This Study
<i>E. coli</i> strains		
Strain number (GCE)	Description	Reference
2753	DH5 α carrying the IPTG inducible cloning vector pJSB104	(Blevins <i>et al.</i> , 2007)
2755	DH5a carrying pNB11 (pJSB104 with MCS from pJLB12g inserted in HindIII site)	This Study
2773	DH5a carrying pNB12 (<i>resT</i> in the expression site of pNB11)	
2769	DH5a carrying pNB13 (KO plasmid with 500 bp <i>B. burgdorferi</i> DNA flanking both sides of deleted <i>resT</i> gene, which has been replaced with <i>kan</i> cassette)	

Table 2.3 Plasmids used in this study

Plasmid	Selectable Marker	Description	Reference
pJSB104	Strep	IPTG inducible expression vector	(Blevins <i>et al.</i> , 2007)
pTM61	Kan	Plasmid encoding a highly fluorescent <i>gfp</i> allele	(Moriarty <i>et al.</i> , 2008)
pNB11	Strep	pJSB104 with <i>gfp</i> inserted in the expression site	This Study
pJLB12g	Kan	Plasmid for targeted mutagenesis of <i>B. burgdorferi</i>	(Bono <i>et al.</i> , 2000)
pJSB104MCS	Strep	pJSB104 with the MCS from pJLB12g inserted at the HindIII site	This Study
pNB12	Strep	pJSB104MCS containing <i>resT</i> in the expression site	
pNB13	Strep	Plasmid for targeted knock-out of the <i>bbb03</i> locus through homologous recombination	
pBSV2BhresT	Kan	Plasmid constitutively expressing <i>resT</i> from <i>Borrelia hermsii</i>	(Tourand <i>et al.</i> , 2006)

Chapter Three: Results

3.1 Optimizing IPTG induction in *B. burgdorferi*

Until recently creating mutations in essential genes in *B. burgdorferi* has not been possible. Targeted inactivation of essential genes resulted in merodiploids, where both the native gene and the inactivated gene were retained after allelic exchange (Byram *et al.*, 2004; Jewett *et al.*, 2007). Now systems for the inducible expression of genetic elements in *B. burgdorferi* have been created (Gilbert *et al.*, 2007; Whetstine *et al.*, 2009; Blevins *et al.*, 2007) and have since been used to create conditional lethal mutations for an essential cell division protein (*bb0250*) (Liang *et al.*, 2010) and the gene for the essential response regulator Rrp2 (Groshong *et al.*). Creating a construct which allows conditional expression of the telomere resolvase, *resT*, may similarly allow conditional knock-out of this essential protein *in vivo*.

In order to assess induction conditions *in vivo*, a construct was built which included *gfp* in the expression site. This construct was transformed into both wild-type B31-A as well as a strain which the native *resT* has been partially knocked out and complemented by the orthologous *resT* from the relapsing fever spirochete *Borrelia hermsii* (GCB51) (Tourand *et al.*, 2006). No clones were recovered in B31-A, the lack of recovered clones was likely due to an inefficient batch of competent cells. However, six clones were recovered in GCB51 which were fluorescent and these were used for analysis of induction and conditional loss of *gfp* expression. Although GCB51 differs slightly from B31-A in that it relies

on the constitutively expressed *resT* from *B. hermsii*, no phenotype is observed in the clone, GCB51 grows at the same rate as wild type and appears to have no difficulty resolving telomeres *in vivo*, thus there should be no impact on the expression of *gfp* that would prevent the use of these clones as a reporter strain.

One transformant was selected (GCB2083) and was assessed for induction of *gfp* at different concentrations of IPTG to determine the optimal conditions for induction of *resT* (Fig 3.1). Briefly, the bacteria were grown to late log phase in non-inducing conditions (medium containing no IPTG), the spirochetes were then collected by centrifugation and resuspended in fresh BSK-II medium at different concentrations of IPTG. Maximum expression of *gfp* is observed at very low concentrations of IPTG; medium supplemented with 100 μ M IPTG was enough to achieve maximum expression and expression levels did not increase beyond this with increasing concentrations of IPTG.

The GFP reporter strain, GCB2083, was also used to assess loss of *gfp* expression following wash-out of IPTG from the medium. Spirochetes were grown for 48 hours in the presence of 1 mM IPTG then collected through centrifugation and resuspended in fresh BSK-II medium lacking IPTG; the culture was sampled for 24 hours and assessed for GFP fluorescence at each time point using a 96 well plate reader (Fig. 3.2). GFP fluorescence fell very quickly after IPTG wash-out and continued to gradually decrease until it was no longer detectable by the plate reader after 24 hours.

Figure 3.1 - Induction of *B. burgdorferi* (GCB2083) containing an inducible construct with the gene for GFP in the expression site (pNB11). Briefly, *B. burgdorferi* were inoculated at 1×10^6 spirochetes per mL in medium containing 0, 0.05, 0.1, 0.5, and 1 mM IPTG and allowed to grow for 48 hours. After 48 hours, slides were prepared and 30 spirochetes were examined by phase contrast microscopy and by GFP fluorescence at each concentration of IPTG to determine the percentage of cells expressing GFP.

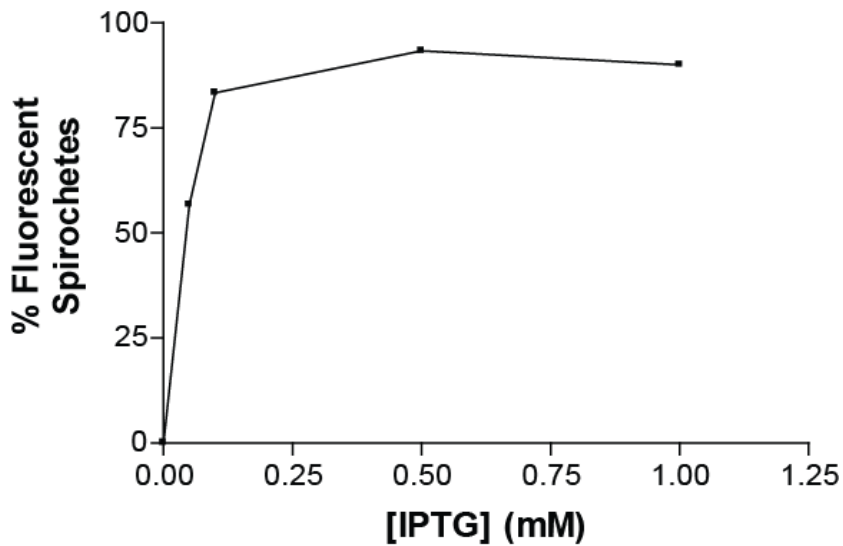
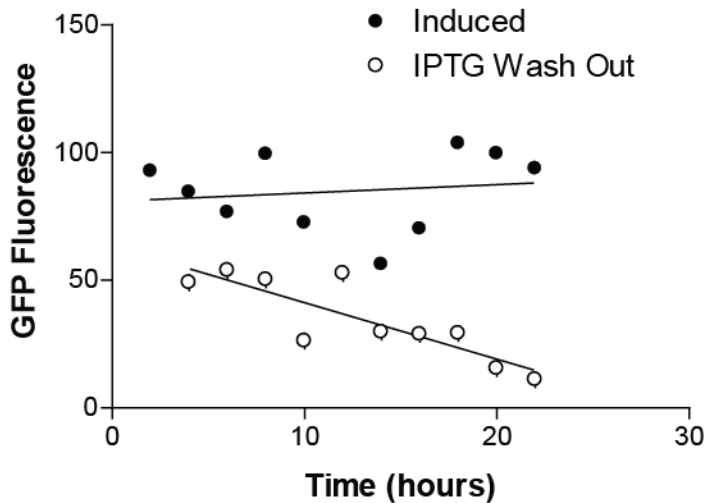


Figure 3.2 – Loss of GFP fluorescence after washing IPTG out of a *B. burgdorferi* culture. *B. burgdorferi* containing the inducible GFP plasmid pNB11 was grown for 48 hours in the presence of 1 mM IPTG, then harvested by centrifugation and resuspended in fresh BSK-II with or without 1 mM IPTG. Cultures were analyzed for fluorescence on a Wallac 1420 Victor 2 plate reader for total fluorescence, expressed above as a percentage of the fluorescence of the induced culture. Data is normalized by arbitrary selection of the average fluorescence of the induced culture at all time points as 100% fluorescence.



3.2 Construction of a conditional lethal *resT* mutant

Prior studies have shown that the *resT* gene (*bbb03*) cannot be knocked out in *B. burgdorferi*, demonstrating the essential role of this gene (Byram *et al.*, 2004; Tourand *et al.*, 2006; Jewett *et al.*, 2007). An inducible *resT* plasmid construct was generated by insertion of the wild-type *resT* gene from *B. burgdorferi* B31-A into the inducible expression vector pJSB104 (Blevins *et al.*, 2007) to generate pNB12 (Fig. 3.3) as described in Materials and Methods. The resulting construct has the *resT* gene under control of the *lac* inducible *PpQE30* promoter along with a constitutively expressed *lacI* gene under the control of the *B. burgdorferi flaB* promoter.

Construction of a strain in which the native *resT* gene was inactivated was accomplished using two different approaches, as shown in Fig. 3.4. First, the high passage *B. burgdorferi* strain B31-A was transformed with pNB12 (Fig. 3.4A). This strain (GCB2091) was then transformed with the plasmid pNB13 which deleted the entire *resT* locus through allelic exchange, replacing it with a kanamycin resistance cassette. Additionally, a conditional lethal mutant was independently recovered through transformation of the previously reported strain GCB51 (Tourand *et al.*, 2006) with the conditionally expressing plasmid pNB12 (Fig. 3.4B). GCB51 carries an inactivated *resT* gene on cp26 but is viable because it carries the orthologous *resT* gene from *Borrelia hermsii* on a shuttle vector (pBSV2*BhresT*). The recovered transformants were analyzed for the loss of pBSV2*BhresT* by PCR for the *B. hermsii resT* and sensitivity to kanamycin. As both plasmids have the same replication origin they should be incompatible,

Figure 3.3 Plasmid map of pNB12. Wild type *resT* was amplified from isolated B31-A genomic DNA and inserted into the inducible plasmid pJSB104 (Blevins *et al.* 2007) to make the inducible *resT* plasmid pNB12 (see Materials and Methods for plasmid construction). Briefly, the *resT* gene is under control of the PQE30 promoter, which is controlled through two *lac* operators upstream of the *resT* gene. The plasmid is selectable with streptomycin due to the *aadA* gene encoded on the plasmid.

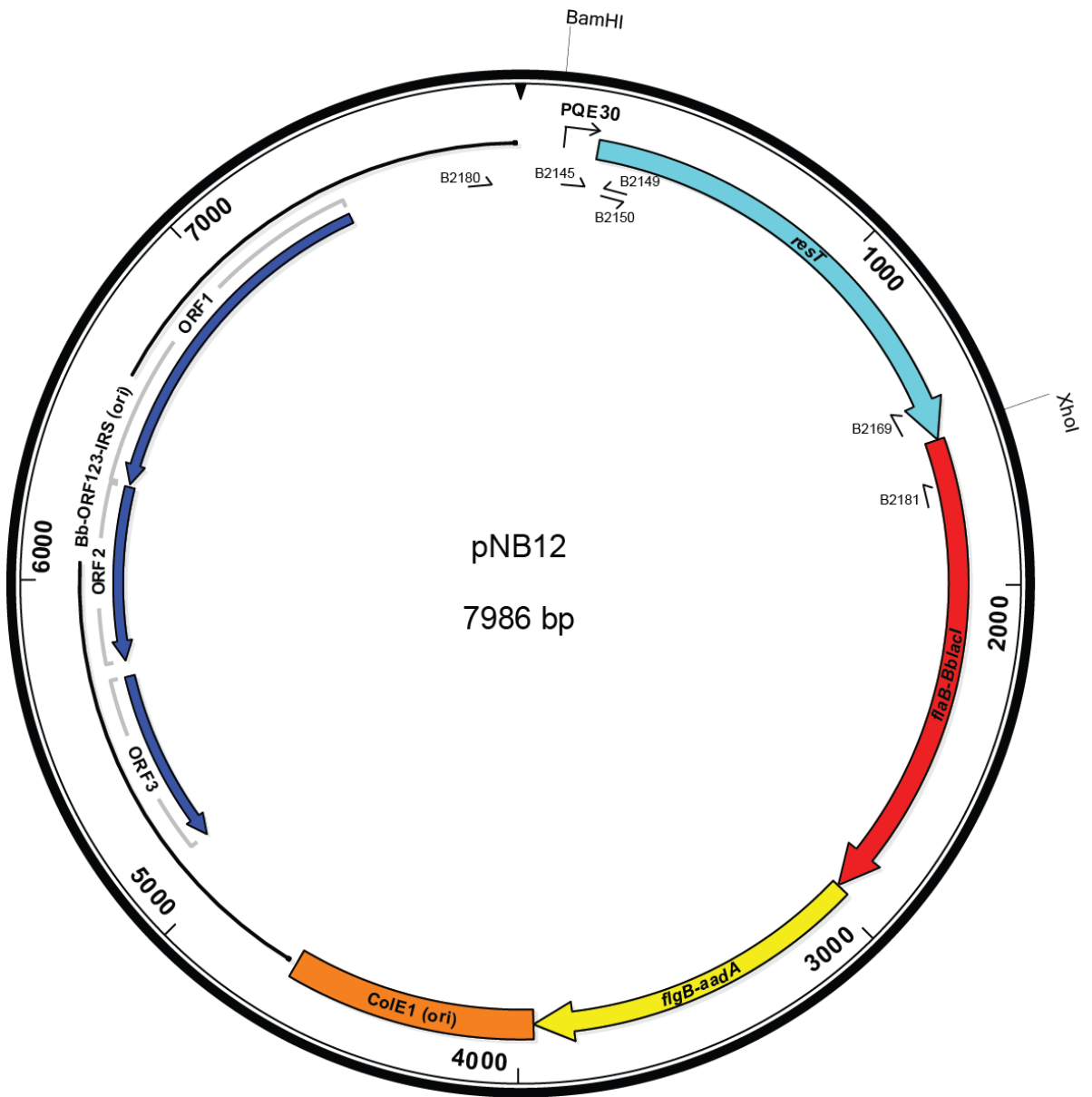
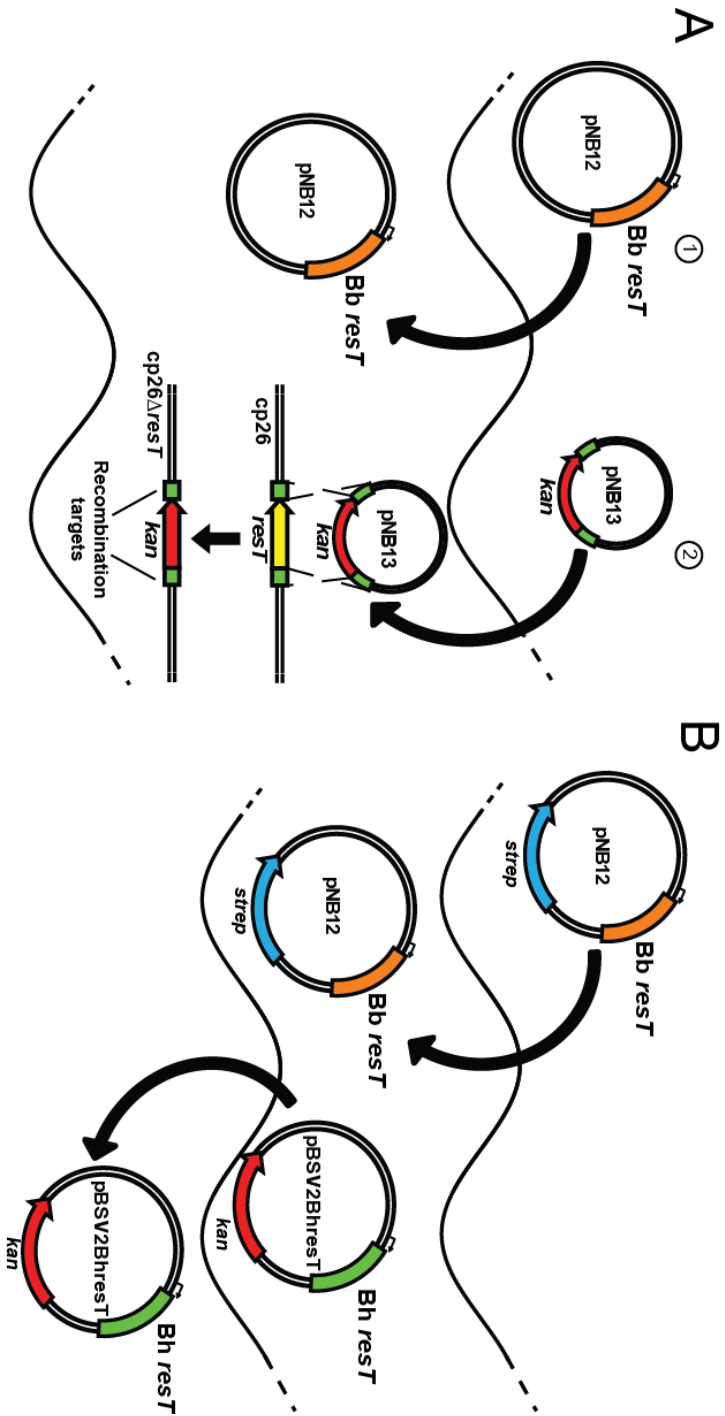


Figure 3.4 Strategies for generating a *resT* knock-out in *Borrelia burgdorferi*. **A)** In B31-A *resT* was knocked-out in a two step process. First *B. burgdorferi* was transformed with the plasmid pNB12 which contains a *resT* gene under the IPTG inducible PQE30 promoter. Next, the strain was transformed with the recombination plasmid pNB13 which targets the native *resT* on cp26, completely deleting the gene through allelic exchange, replacing *resT* with a kanamycin resistance cassette. **B)** pNB12 was also used to transform the strain GCB51, which contains a deletion of the native *resT* on cp26, but which is complemented by the plasmid pBSV2BhresT which expresses the related *Borrelia hermsii resT* (Tourand *et al.*, 2006). As the two plasmids have the same backbone, the drug-selected plasmid will displace the other and the correct strain can be selected for based on the different antibiotic resistance cassettes on the two plasmids.



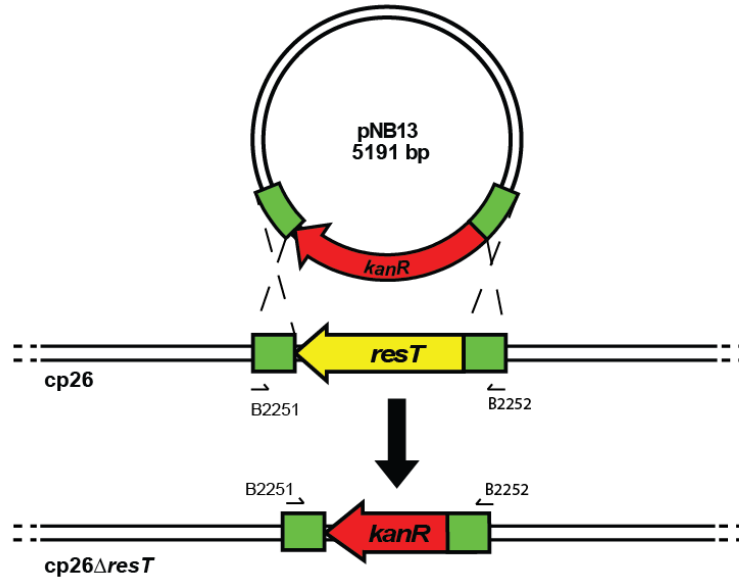
transformation with pNB12 will displace pBSV2*BhresT* giving the conditional knock-out

For the case outlined in Fig. 3.4A transformants were recovered in as little as 50 μ M IPTG, however, as expected, no transformants were recovered when IPTG was not present. The recovered clones were analyzed for the presence of the kanamycin cassette, as well as for the complete deletion of the native *resT* gene by PCR and confirmed through sequencing. The possibility existed that clones obtained after allelic exchange of the native *resT* with the kanamycin cassette contained both the kanamycin cassette while still retaining a functional copy of *resT*, otherwise known as merodiploid. To screen for this, primers were used outside of the *resT* locus (B2251, B2252) which would give a 2,295 bp product if the native *resT* is present and a 1,874 bp product if it had been replaced by the kanamycin resistance cassette (Fig 3.5). All recovered clones were positive for the smaller PCR product and not the larger indicating that these clones represented true transformants and did not represent merodiploids. Sequencing of the *resT* locus confirmed the presence of the kanamycin cassette in place of *resT*.

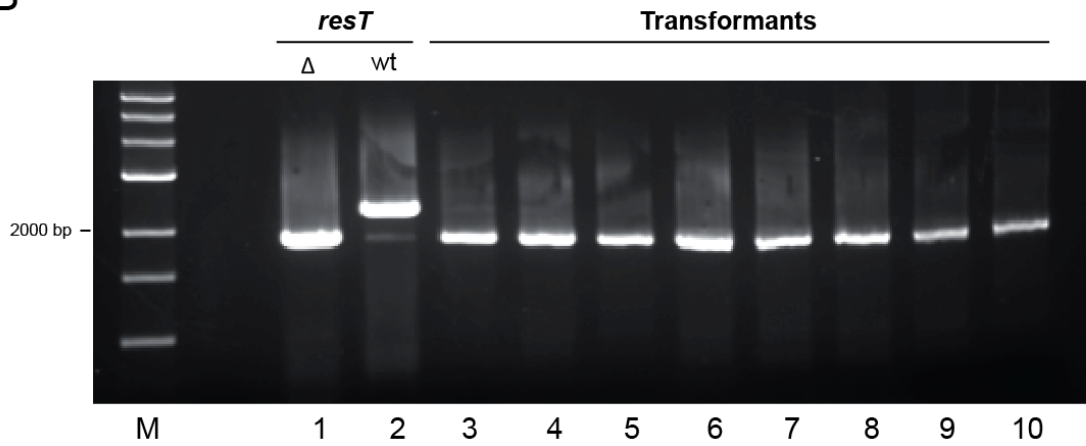
Although GCB51 transformants were recovered which contained the inducible pNB12 (as outlined in 3.4B), this plasmid, which carries the same replication region as pBSV2, co-existed in the bacteria with pBSV2*BhresT*. Both plasmids were maintained even after passage in the absence of kanamycin, the selective marker for pBSV2*BhresT*. Loss of pBSV2*BhresT* was finally achieved by increasing the concentration of streptomycin in the media until clones were

Figure 3.5 Generation and demonstration of *resT* knock out in pNB12 containing *Borrelia*. **A)** Complete deletion of *resT* was achieved through allelic exchange resulting in a kanamycin resistance cassette inserted in place of *resT*. PCR with primers B2251 and B2252 on cp26 gives a smaller product in the knock out (1875 bp) than the wild-type (2295 bp). **B)** Potential *resT* knock-out clones (lanes 3-10) were grown to late log phase and total genomic DNA was extracted and tested by PCR for the size of the insert at the *resT* locus as compared to wild type (lane 2) and pNB13 (lane 1).

A



B



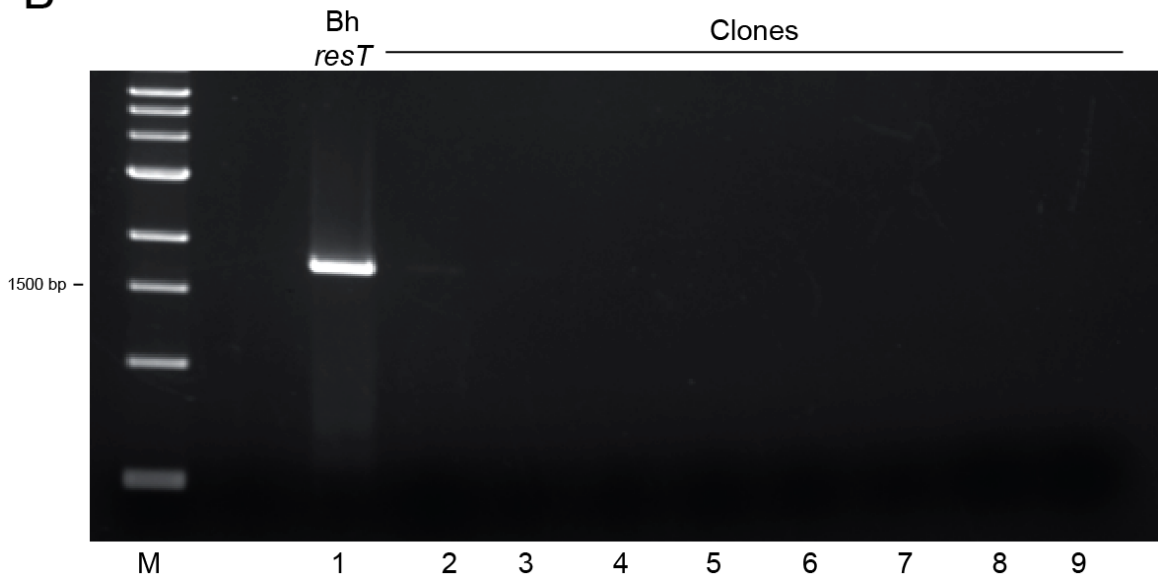
recovered that were resistant to streptomycin but sensitive to kanamycin. The logic for this approach was that the selective pressure would drive up the copy number of pNB12, resulting in a decrease in copy number and eventual loss of pBSV2*bhresT*. 12 clones were recovered after several passages under the selection of streptomycin—bacteria harboring both pNB12 and pBSV2*BhresT* were passaged at 2.5 mg/ml, 4 mg/ml, and 6 mg/ml streptomycin. Clones were screened for the loss of pBSV2*BhresT* by kanamycin sensitivity followed by PCR with primers for *B. hermsii resT* (B322 and B323) (Fig. 3.6). All of the clones sensitive to kanamycin that were recovered were negative for the presence of *B. hermsii resT*.

Figure 3.6 Confirmation of pBSV2BhresT displacement in *B. burgdorferi* expressing *resT* from the plasmid bNB12. **A)** Primers B322 and B323 are specific for *resT* from *Borrelia hermsii* and do not amplify DNA from *B. burgdorferi*. **B)** GCB51, which had been transformed with pNB12 was screened for kanamycin susceptibility. Susceptible clones were grown to late log phase and their DNA extracted for analysis by PCR (lanes 2-9) as compared to DNA from GCB51 (lane 1).

A



B



3.3 Characterization of the conditional *resT* mutant

To assess the effect of IPTG wash-out on ResT levels in the cell a Western blot was performed using antiserum to purified ResT (Fig. 3.7). The conditional lethal strain (GCB2127) was grown to late log phase with IPTG, then harvested by centrifugation and resuspended in fresh BSK-II without IPTG. The bacteria were sampled every eight hours and whole cell lysates were assessed for ResT content by Western blotting. Under inducing conditions, ResT was produced at approximately the same level in the conditional mutant as in the wild-type parental strain B31-A (lanes 1 and 2). However, after IPTG wash-out ResT levels gradually decreased at 8, 16, and 24 hours (lanes 3, 4, and 5) and was not visible at the 32 and 40 hour time points (lanes 6 and 7). Also notable is the dense band immediately above the ResT band in the Western blot. This band arises due to non-specific labeling with the ResT antibody; a similar band was observed in blots using a pre-immune serum (data not shown).

To assess the effects of loss of ResT, *B. burgdorferi* was grown with or without IPTG and characterized with respect to growth. The bacteria were grown in BSK-II medium under inducing conditions (plus IPTG) to late log phase prior to collecting the bacteria through centrifugation, the bacteria were resuspended at 1×10^6 spirochetes/ml in fresh BSK-II with or without IPTG. The bacteria were assessed for growth through daily counting using dark field microscopy. After 48 hours the bacteria were diluted with an equal volume of fresh media with or without IPTG to avoid entering stationary phase. For the first 48 hours the mutant (plus or minus IPTG) grew at the same rate as wild-type B31-A (Fig. 3.8A).

Figure 3.7 Western blot of ResT from IPTG washed-out knock out cultures. A *B. burgdorferi* culture was grown for 48 hours with IPTG followed by IPTG wash-out and dilution of the culture to 1×10^6 spirochetes/ml. Levels of ResT from the wild-type control B31-A (lane 1) and the induced mutant (lane 2) are compared with the mutant strain sampled at 8, 16, 24, 32 and 40 hours after IPTG wash out (lanes 3-7). The band immediately above the ResT band represents non-specific binding by the antibody as a similar band was observed using the pre-immune serum.

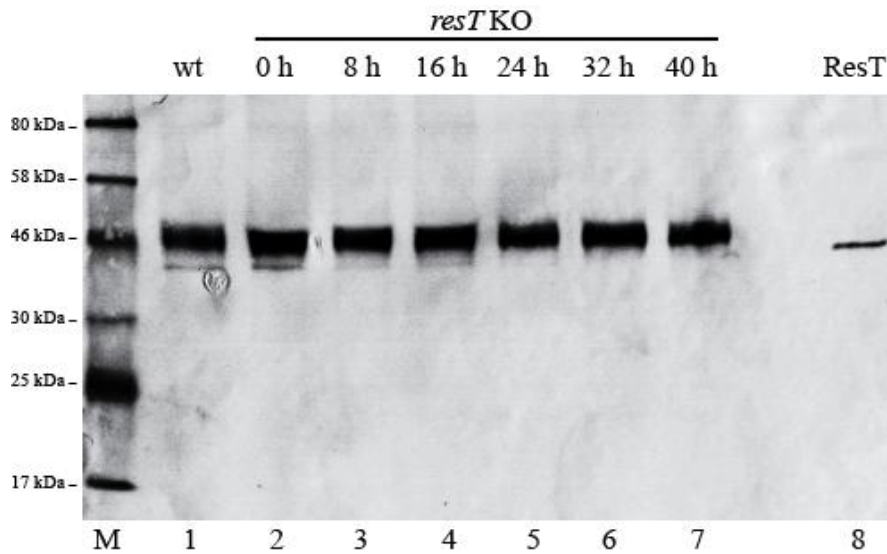
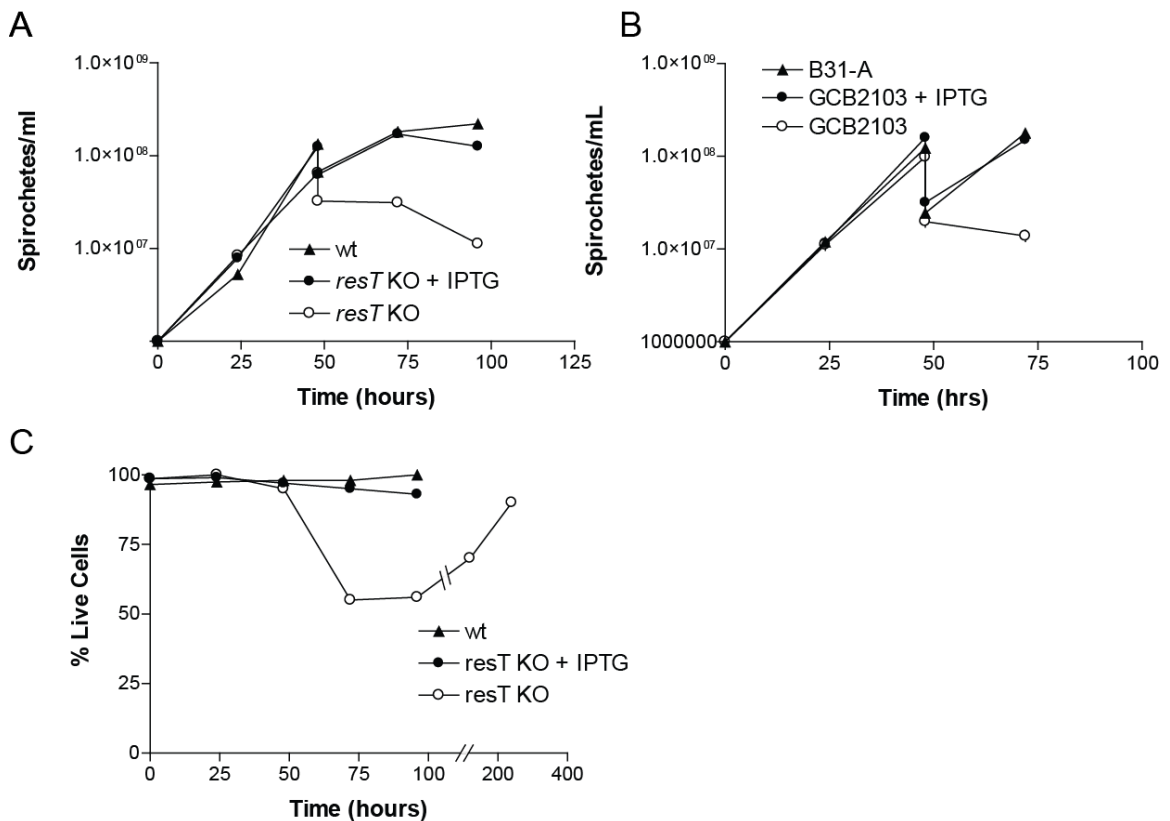


Figure 3.8 (A) Growth curves of the *resT* KO mutant. *B. burgdorferi* cultures were grown in BSK-II medium with and without IPTG at 35° C, and spirochetes were counted every 24 hours. After 48 hours the cultures were diluted with an equal volume of medium to avoid stationary phase. Growth of the wild type B31-A (GCB908) was compared to the growth of the *resT* KO strain (GCB2127). **(B)** The pBSV2*BhresT* displacement strain (GCB2103) was grown under the same conditions as for GCB2127 and enumerated every 24 hours. **(C)** Cultures were stained with LIVE/DEAD *BacLight* staining kit (Invitrogen) to determine if cells were alive or dead. Cultures were then analyzed by fluorescence microscopy for staining with syto-9 (live) or propidium iodide (dead). *B. burgdorferi* strains used were GCB908 (B31-A) and GCB2127.



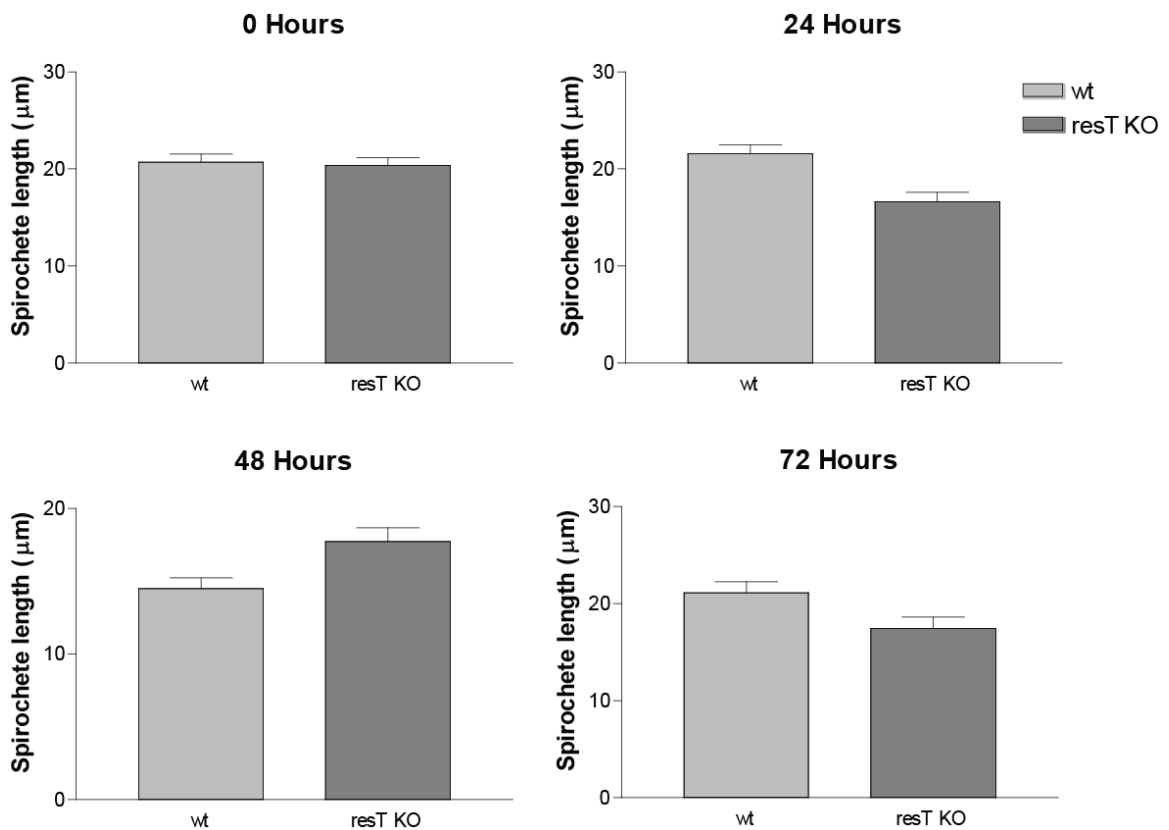
However, 48 hours after IPTG wash-out the conditional *resT* mutant was unable to continue growing and remained at the same cell density at 72 hours, decreasing slightly by 96 hours. Growth was not affected in the wild-type B31-A, nor in the induced culture. This was also observed in strains where pBSV2*BhresT* had been displaced by pNB12 (data not shown). As the displacement clones displayed the same growth phenotype as the complete *resT* knock-out, the complete knock-out strain (GCB2127) was used for the remainder of experiments. Additionally, as GCB2127 was originally recovered in 1 mM IPTG after transformation, this concentration of IPTG was used for induction for the remainder of experiments. The growth of the strain in which pBSV2*BhresT* was displaced in GCB51 (see Fig. 3.4B) was also compared to see if it displayed the same phenotype (Fig 3.8B). Similar to GCB2127 the pBSV2*BhresT* displaced clone (GCB2103) grew normally for the first 48 hours and ceased to divide further after this time point. As this strain displayed the same phenotype as GCB2127 it was assumed that the two strains should be equivalent and as such the latter was used for the remainder of the experiments.

Cultures were also assessed for the viability of spirochetes through live dead staining and fluorescence microscopy (Fig. 3.8C). ResT-depleted spirochetes showed no difference to the wild-type B31-A or to the induced culture for the first 48 hours. However, after 48 hours they decreased in viability, falling to about fifty percent but remained at this level, in fact they seem to increase slightly to 70 percent by 8 days after wash-out and 90 percent viability by 16 days post wash-out. When this culture was diluted into fresh BSK-II with IPTG, at the

same concentration of living spirochetes as the wild-type control, both cultures grew at the same rate. Hence, the living *resT* mutant cells were rescued by restoration of ResT synthesis (data not shown).

Finally, to assess the possibility of a cell division phenotype resulting from a depletion of ResT, spirochete length was measured as described in Materials and Methods. The conditional *resT* mutant was grown in non-inducing conditions as described for Fig. 3.8 above, then stained with DAPI and examined by fluorescence microscopy at each time point (Fig. 3.9). ResT depletion did not result in any increase in spirochete length relative to the wild-type strain; all fell within the 10 to 30 μm range as expected for *Borrelia burgdorferi* (Burgdorfer *et al.*, 1982). Therefore, a specific defect in cell division, expected to result in longer spirochetes, does not appear to occur upon ResT depletion.

Fig. 3.9 Determination of the average spirochete length of the wild-type (GCB908) and *resT* knock out (GCB2127) strains. The length of individual spirochetes was determined using ImageJ (NIH) as described in Materials and methods. The average length +/- the standard error is plotted below for each time-point post IPTG washout.



3.4 The effect of ResT depletion on the state of linear plasmids

DNA was purified from the mutant strain 72 hours after IPTG washout and run on a 0.65 % agarose field inversion gel (Fig. 3.10). In the absence of ResT some plasmid bands were missing from the mutant (lane 1) as compared to the induced mutant or to the wild-type strain (lanes 2, 3, and 4 respectively). Especially evident were lp17 and bands in the 28 kb range which are present in the wild-type as well as induced mutant but absent in the ResT-depleted (white arrows).

To determine the state of the linear plasmids, genomic DNA was run on a 0.65 % field inversion agarose gel and transferred to a nylon membrane for Southern blotting hybridization with probes for lp17 and lp28-2 (Fig. 3.11). The linear plasmids were gradually converted to higher molecular weight forms. After 24 hours a band in the higher molecular weight region appeared for both lp17 (Fig. 3.11A) and lp28-2 (Fig 3.11B), which is roughly double the size of each of the plasmids (arrows). By 72 hours post-IPTG washout both lp17 and lp28-2 had disappeared. The band intensities in the ResT-depleted samples at 72 and 96 hours became depleted suggesting either plasmid loss or conversion to heterogeneous forms which smeared during the gel run. Even higher weight molecular forms are observable for lp-17 beyond this time point (arrows) while the bands for lp28-2 seem to disappear altogether.

Figure 3.10 Field inversion gel of isolated *B. burgdorferi* genomic DNA. The *resT* KO mutant (lanes 1 and 2) was grown for 48 hours with or without IPTG and the genomic content was compared to that of wild-type B31-A (lanes 3 and 4). M denotes a lambda monocut size marker (NEB). The *B. burgdorferi* strains used were GCB2127 (*resT* KO) and GCB908 (wild-type B31-A). The gel was stained with ethidium bromide.

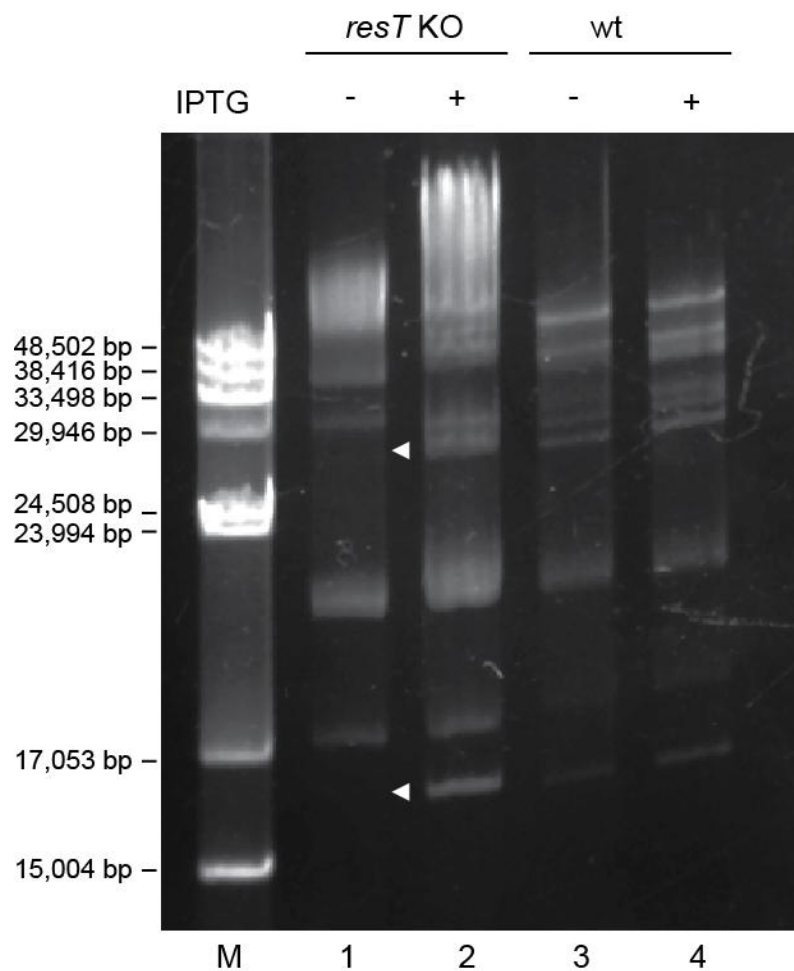
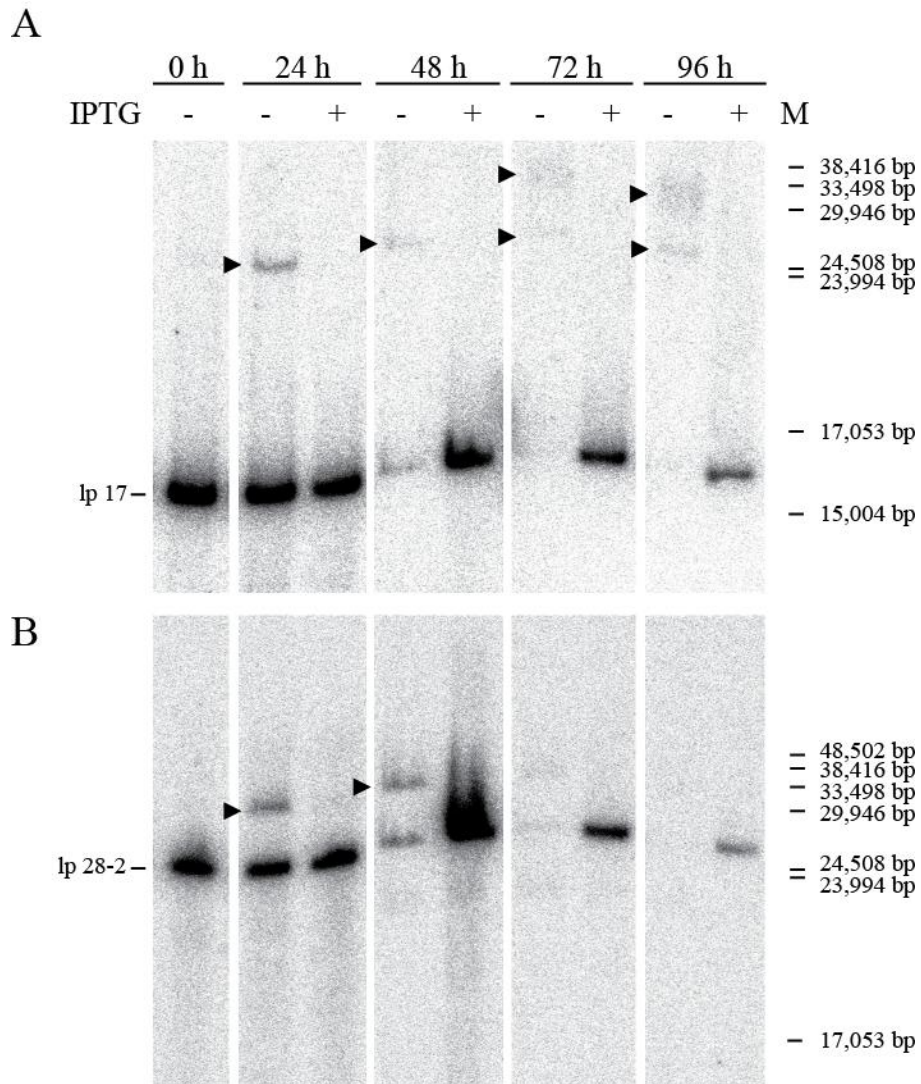


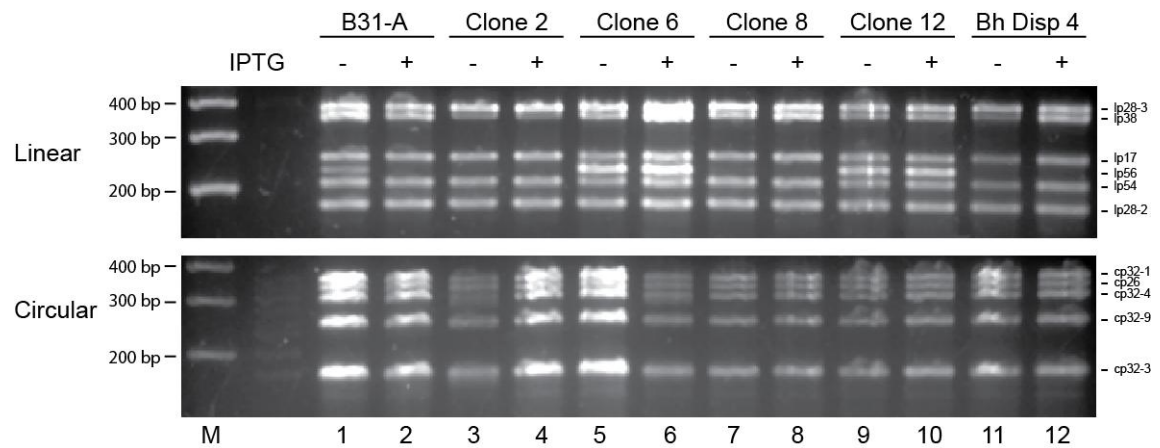
Figure 3.11 Southern blot of genomic DNA from a *resT* knock out strain (GCB2127). Spirochetes were grown either with or without IPTG and the culture was sampled at 0, 24, 48, 72 and 96 hours. DNA was extracted and run on a 0.65% agarose field inversion gel and transferred to a nylon membrane for Southern blotting. Probes were generated against the left end lp17 (Panel A) as well as the right end of lp28-2 (bottom panels) as described in Material and Methods using PCR primers B2275 and B2276 for lp17 and B2295 and B2296 for lp28-2.



To determine whether plasmids were being lost upon ResT-depletion, a multiplex PCR assay (Bunikis *et al.*, 2011) was performed to determine plasmid content. It was observed that the ResT-depleted mutant strain maintained the full plasmid complement as seen in the wild-type and none of the linear plasmids were lost (Fig. 3.12). Although the band for lp-56 seems to disappear in some samples, this band is typically difficult to PCR. This plasmid was detected in a repeat PCR using the same DNA (data not shown).

To determine if the linear plasmids had been converted into the expected replication intermediates (Fig. 1.6) in the ResT-depleted strains, purified ResT was added to genomic DNA, with the expectation that replicative intermediates would be resolved to regenerate linear plasmids. A Southern blot was performed for lp17, however, no linear plasmid was generated by treatment with ResT *in vitro* (data not shown). ResT reactions were performed either alone, or after treatment of the DNA with topoisomerase I, as ResT does not function on negatively supercoiled DNA (Kobryn and Chaconas, 2002; Bankhead *et al.*, 2006). One possible explanation for both the lack of linear plasmid generation by ResT as well as the large loss in lp17 and lp28-2 signal upon ResT depletion (Fig. 3.11) was that multiple replication initiation events were occurring to give rise to complex branched DNA structures that displayed variable non-specific gel migration. It would therefore be expected that DNA close to the replication origin might be present in a higher copy number than DNA far from the origin. PCR reactions were performed with primer annealing near to the origin of lp28-2 as

Figure 3.12 Multiplex PCR to determine the plasmid content of *resT* knock out clones. *B. burgdorferi* was grown with or without IPTG for 48 hours and the DNA was extracted and analyzed by multiplex PCR for the presence of both the circular (bottom panel) and the linear plasmids (top panel) as compared to wild type B31-A. M denotes a ladder of size markers (100 bp ladder, NEB).

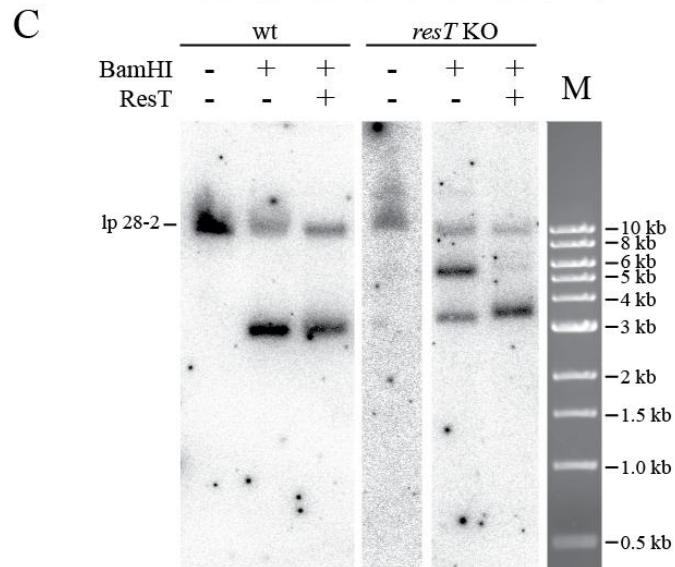
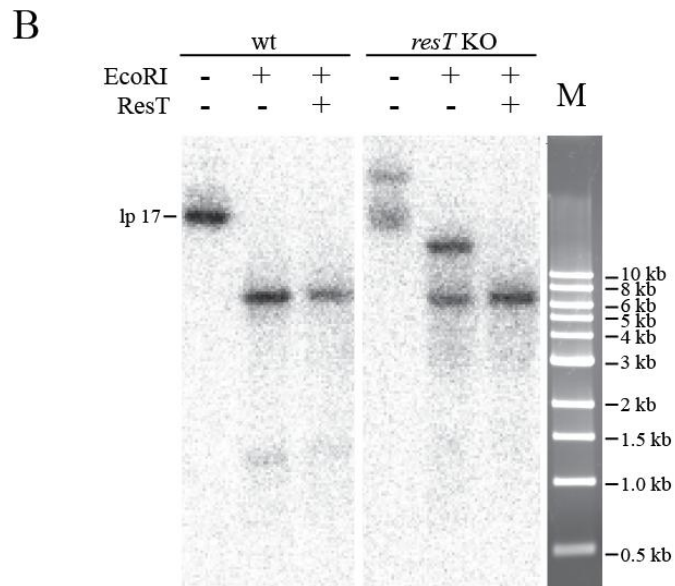
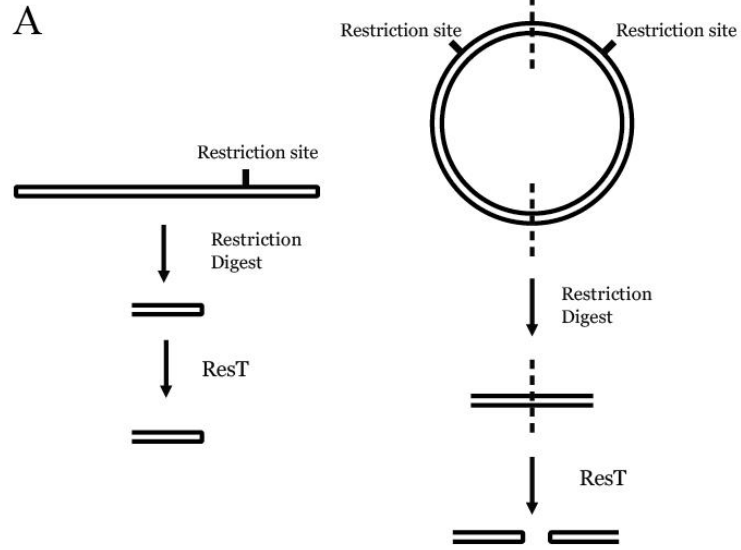


well as at the right telomere to see if this was the case. There was no indication that multiple initiation events had occurred (data not shown).

To simplify detection of replicated telomeres (L'L and RR', see Fig. 1.6), smaller DNA fragments were generated by digesting the DNA with restriction enzymes which cut near the telomeres. These fragments were then treated with ResT to determine if a replicated telomere was present in the DNA. Southern blots from such an experiment for lp17 and lp28-2 is shown in Fig. 3.13. Cleavage of DNA from the wild-type control with EcoRI generated the expected 5,684 bp fragment for the left end of lp17 (lane 2, Fig. 3.13A). Additional treatment with ResT (lane 3) did not result in any changes. In contrast, treatment of the ResT-depleted mutant resulted in two bands: one the 5,684 bp fragment and a second fragment of about twice the size (lane 5), expected for the left end when present as a replicated telomere fragment. Treatment with ResT after EcoRI resulted in the disappearance of the larger left end fragment (lane 6) and intensification of the lower fragment, as expected for telomere resolution of the left end replicated telomere.

Similar results were obtained with lp28-2. For this plasmid BamHI was used to liberate the left end 3,274 bp fragment (lane 2, Fig. 3.13B). BamHI cleavage was not complete and could not be driven to completion with more enzyme. No change resulted from ResT treatment (lane 3). However, as for lp17, treatment of the ResT-depleted mutant resulted in two bands: the 3,274 bp fragment and a second fragment of twice the size (lane 5), expected for the left end when present as a replicated telomere fragment. Treatment with ResT after

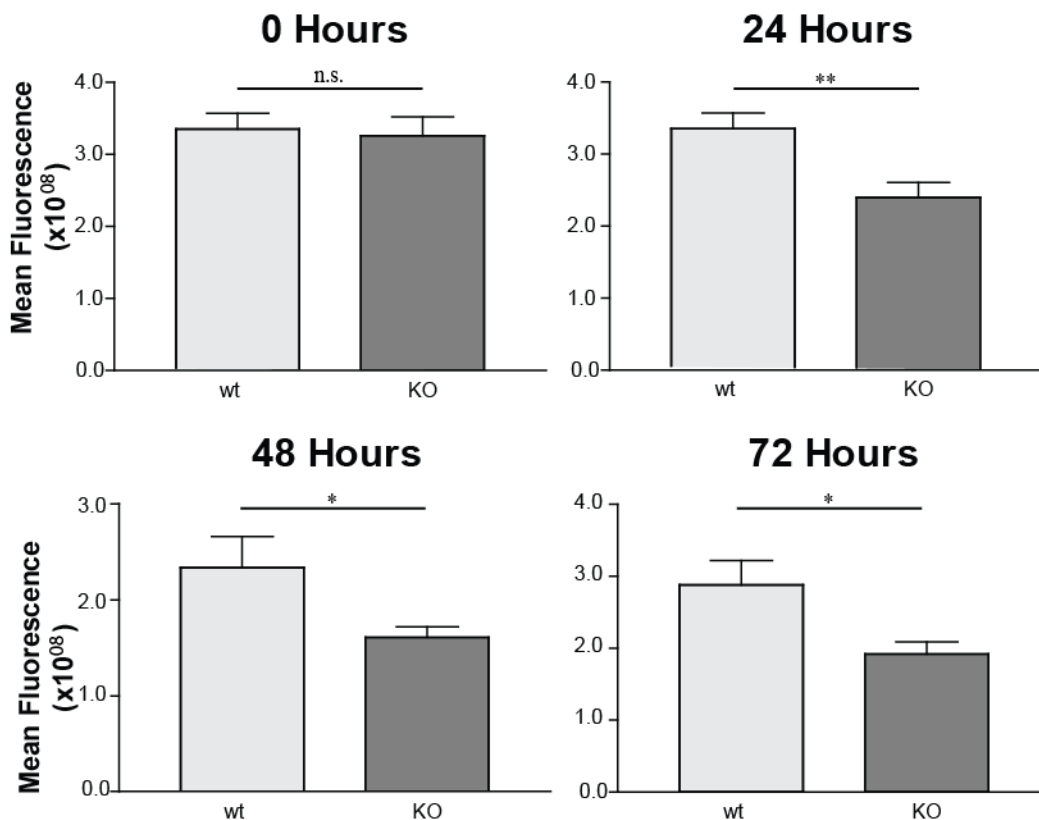
Figure 3.13 Treatment of DNA from the *resT* KO strain (GCB2127) and a wild-type control (GCB908) with the telomere resolvase ResT. **(A)** Schematic of the experimental design. DNA is digested with a restriction enzyme near to the telomere in a DNA monomer or near the axis of symmetry (dashed line) in a dimer generating a fragment containing the telomere or replicated telomere respectively. ResT should only act on the replicated telomere generating the expected hairpin product. **(B,C)** DNA was isolated 72 hours after IPTG washout and digested with EcoRI or BamHI prior to being treated with 200 nM ResT. DNA was run on a 1% agarose gel and transferred to a nylon membrane for Southern blotting hybridization with probes for the lp17 (Panel B) or lp28-2 (Panel C) left ends.



BamHI resulted in the disappearance of the larger left end fragment (lane 6) and intensification of the lower fragment, as expected for telomere resolution of the left end replicated telomere.

Although the spirochetes cease to double after 48 hours and accumulate unresolved telomeres on their linear DNA, it was unclear whether DNA replication continues in the mutant strain or whether a loss of ResT activity causes DNA replication to halt. To analyze DNA levels, wild-type controls or spirochetes at various times after IPTG washout were stained with DAPI. Individual spirochetes were assessed for fluorescence at each time point (Fig. 3.14). To ensure that an increase in DNA content could be observed, spirochetes were stained and examined by fluorescence microscopy with increasing exposure time to ensure that exposure was in the linear range. Additionally, spirochetes were stained at five times the typical cell density used to demonstrate that the DAPI was in excess and that increased DNA content would be detectable with the conditions used. Indeed this was the case as a five fold increase in cell density did not result in a decrease of fluorescent signal of individual spirochetes (data not shown). Examining the *resT* mutant strain using these conditions revealed that DNA synthesis did not continue in ResT-depleted spirochetes after the cessation of cell division. In fact, the total fluorescence of the spirochetes decreased slightly relative to the wild-type.

Figure 3.14 Determination of the relative DNA content of wild type (GCB908) versus *resT* KO spirochetes by quantification of fluorescence intensity of DAPI stained spirochetes in fluorescence micrographs. Spirochetes were grown in BSK-II media in the presence of IPTG to late log phase before harvesting through centrifugation and resuspending in fresh media lacking IPTG. The *resT* KO strain as well as the wild type were stained with DAPI at the times indicated and photographed as described in Materials and Methods. Images of fluorescent spirochetes were analyzed for total fluorescence intensity by collecting the integrated fluorescence density using ImageJ (NIH) and correcting for the fluorescence background. The mean fluorescence intensity \pm the standard error was plotted. Statistical significance was determined by a two-tailed unpaired t-test and is indicated above the graphs (* $p < 0.05$, ** $p < 0.01$).



Chapter Four: Discussion and Conclusions

4.1 Construction of a conditional lethal *resT* mutant

In the past, genetic experiments in the Lyme disease have been limited by the approaches available for such experiments. However, the tools available for genetic manipulation *in vivo* are steadily growing and manipulations that were not previously possible are now becoming possible. One of the previous limitations was the inability to make conditional knock-outs of essential genes in *B. burgdorferi*. However, plasmids are now available that allow conditional expression of proteins in *B. burgdorferi* based upon either a *lac*- or *tet* inducible system (Gilbert *et al.*, 2007; Blevins *et al.*, 2007) (Whetstine *et al.*, 2009). To date only two other conditional disruptions of essential genes have been reported in *B. burgdorferi*; those for the *dedA* orthologue *bb0250* (Liang *et al.*, 2010) and the response regulator protein *rrp2* (Groshong *et al.*, 2012). Here we have demonstrated the conditional disruption of a third essential gene in *B. burgdorferi*: the telomere resolvase, *resT*. Previous attempts to disrupt *resT* have resulted in the recovery of merodiploids where the bacteria maintained both the disrupted gene, as well as a functional copy of *resT* (Byram *et al.*, 2004). Similarly, disruption of *resT* in *B. burgdorferi* was achieved only when the ortholog to *resT* from the relapsing fever spirochete *B. hermsii* was provided *in trans* prior to gene disruption (Tourand *et al.*, 2006).

Both of the previously reported conditional lethal knock-outs in *B. burgdorferi* were constructed based on IPTG inducible expression of the target

gene from the plasmid pJSB104, originally reported by Blevins *et al.* (Blevins *et al.*, 2007; Liang *et al.*, 2010; Groshong *et al.*, 2012). This plasmid provides IPTG-inducible expression of the cloned gene of interest under the control of the *PpQE30* promoter providing for tight regulation of expression from a robust promoter carried on the backbone of a *B. burgdorferi* shuttle vector (Stewart *et al.*, 2001) that is maintained at about 10 copies per cell relative to the chromosome (Beaurepaire and Chaconas, 2007). Protein expression is driven from a T5 promoter controlled by two *lac* operators (Blevins *et al.*, 2007). These operators are bound by the *lac*-repressor protein which has been codon optimized for *B. burgdorferi* and whose expression is driven by the constitutive *flaB* promoter allowing for tight control of *resT* expression under non-inducing conditions.

Expression levels of ResT on pNB12 in the conditional mutant (GCB2127) were estimated from a Western blot (~14,000 monomers per cell) and found to be similar to ResT levels (~22,600) estimated in the wild-type strain B31-A (GCB908) when growth was performed in the presence on 1 mM IPTG. *resT* is normally expressed from its native promoter at approximately the same level as *flaB* in the cell (Salman-Dilgimen, manuscript in progress).

In addition to disruption of the endogenous *resT* gene when the inducible pNB12 was present, we sought to create a conditional lethal mutant by displacing the plasmid pBSV2*BhresT* from the previously reported strain GCB51 that carried a *resT* disruption (Tourand *et al.*, 2006). This strain has a partial deletion of the native *resT* on cp26 which is complemented by the orthologous *resT* from the related *B. hermsii*. As pNB12 contains the same replication origin as

pBSV2*BhresT* it was thought that these two plasmids should be incompatible and under selection for streptomycin, the selectable marker on pNB12, and that pBSV2*BhresT* should be displaced from the cell (Stewart *et al.*, 2001;Eggers *et al.*, 2002;Stewart *et al.*, 2003). However, it has also been reported that the plasmid pKFSS1, from which pJSB104 is built, is co-maintained with pBSV2 despite the fact that the two plasmids share the same replication origin and should be incompatible (Frank *et al.*, 2003). Indeed, this is what was observed when GCB51 was transformed with pNB12. All clones recovered maintained both pNB12 and pBSV2*BhresT* even in the absence of kanamycin. This may be due to the copy number at which the shuttle plasmid is maintained (~10), which may provide a permissive environment for co-maintenance of the two plasmids, even under selective pressure for only one. By increasing the amount of streptomycin our goal was to increase the number of copies of pNB12 relative to pBSV2*BhresT* until the latter was ultimately displaced from the cell. Indeed that was what was observed. After several passages in increasing concentrations of streptomycin, clones were recovered which no longer contained pBSV2*BhresT*; this represents a new approach for plasmid displacement in *B. burgdorferi*.

4.2 Characterization of the *resT* mutant

For the first 48 hours after wash-out of IPTG the *resT* mutant grew at the same rate as the wild-type control, B31-A. However, it was observed that most of the ResT was depleted from the bacteria 24 hours after washout (Fig. 3.7). One explanation for this apparent disconnect between the depletion of ResT and

observation of a phenotype is that ResT may still be present in the cell at a high enough concentration to perform telomere resolution between 24 and 48 hours, although not at high enough levels to be detectable by a Western blot. This contention is supported by the observation that there is still some linear lp17 and lp28-2 observable at 48 hours post IPTG-washout (Fig. 3.11). If all the ResT were depleted by 24 hours, linear plasmids would be completely converted to higher molecular weight replicative intermediates.

A growth phenotype was, however, observed starting at 48 hours. From this point forward, no additional growth was observed. At 48 hours all spirochetes were still alive as determined by live/dead staining, but at 72 hours and thereafter only 50% of the spirochetes were alive. This number remained constant for up to 8 days, at 8 days a slightly higher viability of 70% was observed which increased again to 90% by 16 days. However, although viability of the culture seemed to increase over time it is not due to proliferation of live cells; culture remained at the same cell density over the course of the experiment. Instead, it is likely that the dead cells have begun to lyse as increased cellular debris was observed on the slide during quantitation at 8 and 16 days. Interestingly, the living but non-dividing spirochetes could be rescued by inoculation into fresh media containing IPTG. The presence of ResT is, therefore, required for active growth. However, a substantial fraction of ResT-depleted spirochetes can survive for lengthy periods and be revived by renewed ResT synthesis. The reason for partial survival is unknown.

It was initially expected that bacteria deficient in ResT activity might display a cell division phenotype. Before DNA segregation and cell division can occur, sister DNA molecules must be completely unlinked from one another so that each sister can be partitioned to a new daughter cell (Reyes-Lamothe *et al.*, 2012). It was expected that ResT-depleted *B. burgdorferi* would be unable to complete chromosomal dimer resolution and hence, cell division. Such spirochetes would be expected to become long and filamentous, similar to a *bb0250* (inner membrane protein) knock-out that was unable to complete cell division (Liang *et al.*, 2010). Surprisingly, ResT-depleted spirochetes did not display a filamentous phenotype, suggesting that there may not be an intimate spatial and temporal coupling between cell division and telomere resolution in *B. burgdorferi*.

We also investigated whether ResT-depleted spirochetes would continue to accrue DNA once they had stopped dividing. It was thought that cells would continue to accrue DNA as the bacteria were no longer dividing but appeared to still be viable. As telomere resolution occurs at the end of replication, it is reasonable that ResT may have no bearing on the initiation of replication in *B. burgdorferi* and DNA replication may continue in the spirochetes despite the block in cell division. As seen by DAPI staining of the spirochetes (Fig. 3.14) the DNA content did not increase and in fact decreased relative to the wild-type control, starting as early as 24 hours after IPTG-washout. At this time cell division was still occurring. These results suggest that ResT may interact directly with

components of the replication machinery involved with the initiation and/or elongation process. Further studies will be required to elucidate such interactions.

4.3 State of the linear DNA in ResT depleted *B. burgdorferi*

Although it has been previously shown that ResT can bind and resolve replicated telomere substrates *in vivo* (Beaurepaire and Chaconas, 2005;Chaconas *et al.*, 2001) and *in vitro* (Chaconas, 2005;Chaconas and Kobryn, 2010;Kobryn and Chaconas, 2002) a *resT* mutant and its effect upon the segmented genome has not been previously reported. By depleting the cell of ResT in our conditional mutant we were able to promote the accumulation of replication intermediates so they may be observed.

Evident by southern blotting (Fig. 3.11), bands for both lp17 and lp28-2 were seen which were roughly double the size of the monomeric plasmid bands observed in wild-type B31-A. At early time points (24 and 48 hours) only a single, higher dimer-sized band was observed along with the linear monomer band. It is likely that the upper band represents a true inverted repeat dimer intermediate. At the later time points (72 and 96 hours) additional bands were observed at even higher molecular weights for lp17 while the bands disappeared altogether for lp28-2. The additional higher bands likely represent more complex unresolved forms of the linear plasmids. The disappearance of signal is assumed to result from both heterogeneity of products as well as decreased efficiency of transfer of high molecular weight DNA.

In many microorganisms there are multiple replication initiation events per piece of DNA per generation. In *E. coli*, DNA replicates at a rate slower than cell division so multiple initiation events are a necessity to ensure sufficient DNA content to segregate into daughter cells each generation (Skarstad *et al.*, 1983). This appears to also be the case in *B. burgdorferi*; it has been reported that the copy number of the center of the chromosome is four times that of the right end (Beaurepaire and Chaconas, 2007) indicating that multiple initiation events have occurred. A simple explanation for the observed higher weight bands may be that the higher bands represent a situation where more than one replication bubble is present. However, when the center of lp28-2 was probed relative to its right end there was no obvious difference in copy number observed for the ResT depleted strain relative to wild-type.

Another possibility is that the higher bands observed in the southern blot (Fig. 8) represent cellular attempts to resume DNA replication at stalled replication forks. In *E. coli*, stalled replication forks can lead to fork regression and Holliday junction formation or fork collapse and double strand breakage (Cox *et al.*, 2000; Kowalczykowski, 2000). The progressive decrease in DNA content per spirochete upon IPTG removal is consistent with possible stalling of replication forks. Isolation and further analysis of the altered forms of lp17, lp28-2 or other linear plasmids is not possible because of the large number of plasmids present in *B. burgdorferi*.

Nonetheless, free telomere ends were liberated from lp17 and lp28-2 after digestion with restriction endonucleases (Fig. 3.13). These bands were the same

size as the telomeres from the linear forms of these plasmids. Some high molecular weight plasmid forms, therefore, contain unreplicated telomeres, indicating either that DNA replication in these molecules did not progress to completion or that these telomeres had been resolved with the residual ResT remaining in the cell. Telomere resolution can occur independently at the ends of replicating N15 prophage DNA (Ravin, 2003) and such a scenario could exist in *B. burgdorferi* to generate higher molecular weight replicative intermediates with only one resolved telomere.

4.4 Future Directions

Although the ResT deficient spirochetes ceased to divide, the majority of the culture remained viable for up to 16 days after IPTG wash-out. Additionally, DNA did not accrue in the spirochete indicating that DNA replication had ceased. This may point to ResT playing a larger role in the temporal control of DNA replication than previously thought. It may be possible that ResT is localized to the cell membrane similar to XerCD in *E. coli* (Blakely *et al.*, 2000); depletion of ResT results in the loss of telomere resolution and the inability to partition linear molecules prior to cell division. If this is the case, we may expect to see ResT specifically localized to the septum during cell division. Imaging studies such as this have been difficult in *B. burgdorferi* due to the fact that the spirochetes only measure 0.18 to 0.25 μm in diameter (Burgdorfer *et al.*, 1982). However, recently techniques have been described which allow the visualization of single molecules within the cell in real time (Lee *et al.*, 2012). Use of such a technique in *B.*

burgdorferi would allow visualization of the spatial and temporal control of ResT *in vivo* and would shed light on this subject.

Termination of DNA replication of linear plasmids is thought to occur at the hairpin telomeres of *B. burgdorferi* (Picardeau *et al.*, 2000). Therefore, it is an attractive hypothesis that ResT may play a role in termination for the linear molecules. As replicated telomere intermediates have not been previously observed *in vivo*, telomere resolution may be tightly temporally linked to termination; it has been previously proposed that the build-up of positive supercoiling during DNA replication may act to temporally restrict ResT activity to termination events (Bankhead *et al.*, 2006). The conditional *resT* mutant described here may be useful in future studies of termination events *in vivo* to specifically delineate the temporal control of telomere resolution and termination. Additionally, investigations into interactions between ResT and the replication machinery or other partners would help to shed light on this subject

Although the spirochetes ceased to divide up to 70% of the culture remained viable 16 days after IPTG wash-out. This may be similar to a dormant state of spirochetes observed in the tick mid-gut as availability of nutrients acts as a stressor to limit the metabolic activities of the spirochetes in the tick (Dunham-Ems *et al.*, 2009). In the case of a ResT depletion mutant, inability to resolve telomeres may act as a stressor to limit the growth and division of the spirochetes. Not much is currently known about DNA repair in *B. burgdorferi*; DNA repair genes orthologous to those found in *E. coli* do not necessarily behave the same way in *B. burgdorferi* (Hardy and Chaconas, 2013). A ResT

depletion condition may present an opportunity to investigate specific DNA repair pathways which may be activated to deal with this stress.

4.5 Summary and Conclusions

It has long been proposed that ResT function is essential for growth of *B. burgdorferi* (Byram *et al.*, 2004; Tourand *et al.*, 2006; Jewett *et al.*, 2007). For the first time we report conditional expression of *resT* in *B. burgdorferi* resulting in growth defects in the spirochete when in non-inducing conditions. Although the spirochete ceased to grow after ResT was depleted from the cells, this phenotype could be rescued by the addition of IPTG demonstrating that loss of ResT function is responsible for the observed phenotype. Although the spirochetes cease to divide, they do not take on a filamentous form as reported for other *B. burgdorferi* mutants deficient in cell division (Liang *et al.*, 2010), however, approximately half of the culture appears to remain viable as demonstrated through live-dead staining. This may represent an arrested state where the spirochetes are attempting to deal with their inability to complete DNA replication. As stated above, an attractive hypothesis is that ResT acts at the cell membrane similar to XerCD in *E. coli* (Sherratt *et al.*, 2004). Demonstration of specific ResT localization using super resolution microscopy may help to add credence to this hypothesis.

Additionally, DNA replication does not continue in the ResT depleted spirochetes. This suggests that resolution of telomeres may be more important in DNA replication as a whole than previously thought. As is seen by DAPI staining

(Fig. 10), DNA replication does not continue in ResT depletion conditions. However, ResT depleted spirochetes may be rescued through induction with IPTG indicating that a loss of ResT is also causing a block in DNA replication. Further studies are needed in order to determine exactly how a loss of ResT results in a block of replication.

In summary, we have demonstrated the essential nature of *resT* through by creation of a conditional *resT* mutant and growth in non-inducing conditions. Although roughly half of the spirochetes remained viable, they did not continue to grow nor did they continue to replicate their DNA. Analysis of DNA content in un-induced clones showed replicative intermediates which were double in size of the wild-type plasmid and contained a replicated telomere substrate. We propose that ResT may play a larger role in DNA replication than previously thought, however, further studies are needed to confirm this.

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