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# Depletion of 32-kbp circular plasmids from *Borrelia burgdorferi*

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DEPLETION OF 32-KBP CIRCULAR PLASMIDS FROM *BORRELIA BURGDORFERI*

A Thesis

Submitted to the Graduate Faculty of the  
Louisiana State University and  
Agricultural and Mechanical College  
in partial fulfillment of the  
requirements for the degree of  
Master of Science

in

The Interdepartmental Program in  
Veterinary Medical Sciences  
through the  
Department of Pathobiological Sciences

by  
Amanda Paige DeRouen Polito  
B.S., Louisiana State University, 2005  
December 2007

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## ABSTRACT

The Lyme disease spirochete *Borrelia burgdorferi* has a very unusual genome composed of one linear chromosome and up to 21 linear and circular plasmids. Several plasmids are known to be important either for mammalian infection or tick colonization. A single spirochete harbors up to 7 different cp32 plasmids; however, nothing is known about their role in mammalian infection. The plasmids in this family are well maintained during *in vitro* cultivation, making it difficult to study their functions. To effectively deplete the plasmids, an 8kbp fragment containing essential elements for replication and partitioning in *B. burgdorferi* was amplified from one of the cp32 plasmids, cp32-3, and cloned into the vector pGE22 that carries a gentamycin resistance cassette and essential elements for replication in *Escherichia coli*. The resulting construct, pG22cp32-3plus, was electroporated into borrelial cells. By increasing gentamycin selection pressure, the spirochetes were forced to lose the corresponding cp32 plasmid. This strategy can be used to knock out other members of the cp32 family.

# INTRODUCTION

## LYME DISEASE

### DISCOVERY

Lyme disease was discovered in 1975 in Lyme, Connecticut when children in the area were being diagnosed with juvenile rheumatoid arthritis at 100% higher than normal rate. Dr. Allen Steere along with other doctors traced the symptoms to an unknown bacterial infection transmitted by local deer ticks. In 1977, the newly characterized disease was named Lyme disease (Steere, *et al.* 1977).

The Centers for Disease Control and Prevention (CDC) began conducting national surveillance in 1980 and has continued to compile data since 1982 (Centers for Disease Control and Prevention, 1982). Lyme disease became the most commonly reported arthropod-borne disease in the United States between 1984 and 1985 when an average of 1,500 cases were reported to the CDC (Centers for Disease Control and Prevention, 1985). Since that time, incidence of the disease has steadily increased to over 20,000 cases annually (Figure 1).

According to the most recent statistics published by the CDC, there were 23,305 reported cases of Lyme disease in 2005 (Centers for Disease Control and Prevention, 2007). Historically, Lyme disease has been characterized in every state but is most common in the Northeastern, mid-Atlantic, and North Central states (Figure 2). In the ten states where Lyme disease is most prevalent, the incidence per 100,000 persons increases from the national average of 7.9 cases to 31.9 cases (Centers for Disease Control and Prevention, 2007).

### REPORTED CASES OF LYME DISEASE 1982-2005

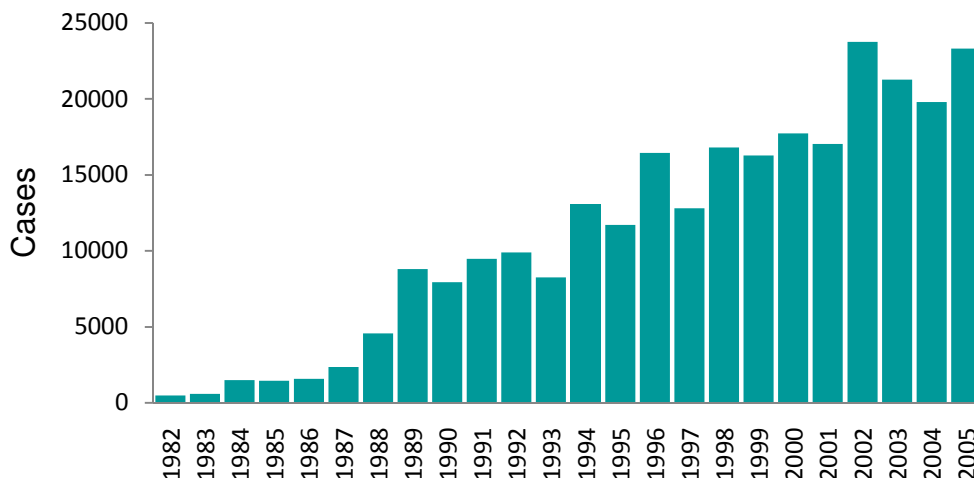


Figure 1: Reported cases of Lyme Disease 1982-2005 (Centers for Disease Control and Prevention, 1982; Centers for Disease Control and Prevention, 1985; Centers for Disease Control and Prevention, 2006; Centers for Disease Control and Prevention, 2001; Centers for Disease Control and Prevention, 2007). Over the last twenty-four years the CDC has tracked Lyme disease in the United States. Since reporting began, the CDC has shown that the number of cases has risen from 491 to 23,305 cases reported in 2005.

### LOCATION OF REPORTED CASES OF LYME DISEASE 2005

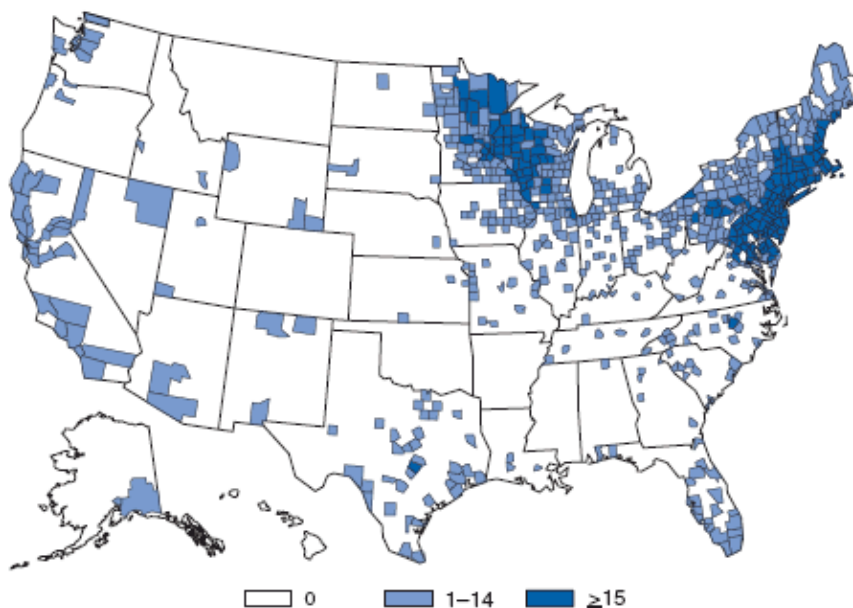


Figure 2: Location of reported cases of Lyme Disease 2005 (Centers for Disease Control and Prevention, 2007). In 2005 the CDC compiled the location data for the 23,305 reported Lyme disease cases in the United States. Higher rates of incidence can be seen in the Northeastern to North-Central United States.

The relative risk of contracting Lyme disease is partly due to the distribution of the disease's known transmission vectors as well as the presence of the disease causing bacteria and availability of hosts for tick survival (LoGiudice *et al.*, 2003). The black-legged tick, *Ixodes scapularis*, previously identified as *Ixodes dammini*, also known as the common deer tick, was originally identified as the key to the bacteria's spread in the original cases by the doctors investigating the then unknown outbreak of Lyme disease in rural Connecticut (Steere *et al.*, 1978; Oliver *et al.*, 1993). *Ixodes scapularis* is primarily responsible for infection in the Eastern United States where the majority of infections takes place; however, *Ixodes pacificus*, the Western black-legged tick, is responsible for infection on the West Coast (Figure 3) (Steere *et al.*, 1978; Burgdorfer, Lane *et al.*, 1985, LoGiudice *et al.*, 2003).

### PRESENCE OF TRANSMISSION VECTORS OF LYME DISEASE

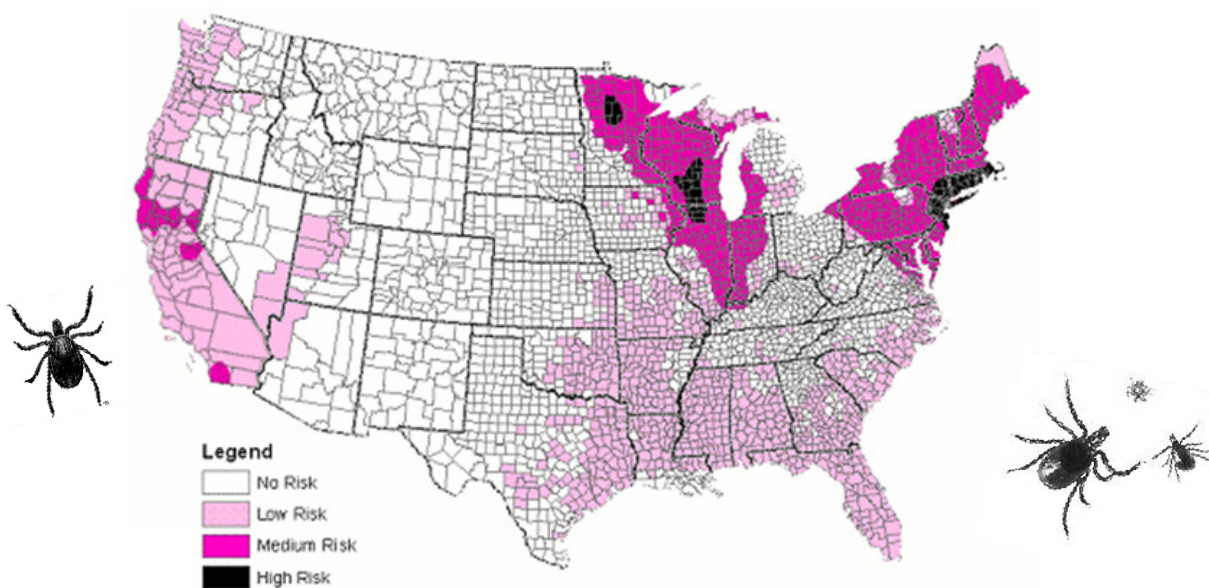


Figure 3: Presence of transmission vectors of Lyme Disease (Centers for Disease Control and Prevention, 1999). The above map shows the relative risk of residents to contract Lyme disease due to the presence of the transmission vector. In the Eastern United States *I. scapularis* ticks are responsible for transmitting infection while *I. pacificus* is the primary vector on the West Coast.

The ticks known to transmit Lyme disease have a two-year life cycle (Figure 4). Peak larval activity occurs in the latter summer months when larvae feed on a wide variety of small mammals, primarily mice and birds. Late the next spring the newly molted tick exits dormancy and the nymph feeds a second time on small mammals. A second molt occurs late in the second summer and the adult tick becomes active and begins to feed again in the fall until temperatures drop below freezing. A tick may pick up the infection at any feeding during its life cycle and has the ability to transmit the disease at any stage of growth; larvae, nymph, or adult in any feeding after the tick has become infected (Klompen, 2004). Mice, specifically the white-footed mouse *Peromyscus leucopus*, are the primary natural reservoirs for the bacterial infection (Levine *et al.*, 1985).

### LIFE CYCLE OF LYME DISEASE VECTORS

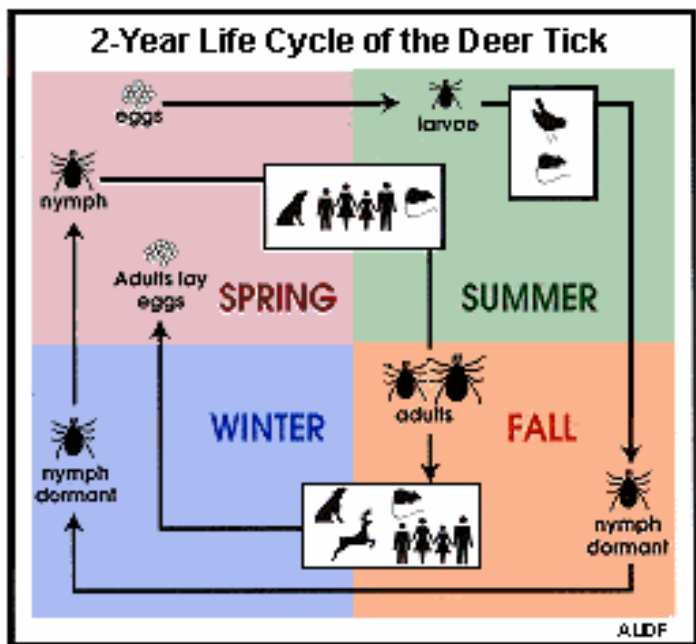


Figure 4: Life cycle of Lyme Disease vectors (American Lyme Disease Foundation, 2006). The vector of Lyme disease has a two-year life cycle. The tick can obtain the bacteria at any feeding and transmit the bacteria to any subsequent host.

Lyme disease patients are most likely to become infected in June, July, or August. This trend is the result of the ticks feeding schedule and the higher prevalence of human outdoor activity during these months (Centers for Disease Control and Prevention, 2006).

## SYMPTOMS

Lyme disease can produce a wide range of symptoms. The most common sign of the disease is the presence of the classic indicator rash, erythema migrans, located at the site of the tick bite (Figure 5) (Steere *et al.*, 1983). According to the CDC's official definition, erythema migrans begins as a focal red lesion that expands over several days to weeks (Centers for Disease Control and Prevention, 1997). As the lesion expands, the rash will often resemble a bull's-eye or target due to partial clearing of the center of the lesion (Centers for Disease Control and Prevention, 1997). The rash, present in 80% percent of infected people, can last up to several weeks and may be accompanied by localized swelling (Steere, 1989; Centers for Disease Control and Prevention, 1997). Around the time the rash presents, other flu-like symptoms such as joint pains, chills, fever, and fatigue may also appear (Steere *et al.*, 1983; Centers for Disease Control and Prevention, 1997).

If the infection is left untreated, the bacteria will continue to spread through the body within several weeks following initial infection. As dissemination occurs, an infected person may experience migratory joint pain, severe fatigue, and a stiff or aching neck. The infected individual may also experience tingling or numbness in the extremities as well as facial palsy (Steere, 2001; Agüero-Rosenfeld *et al.*, 2005).

## ERYTHEMA MIGRANS RASH

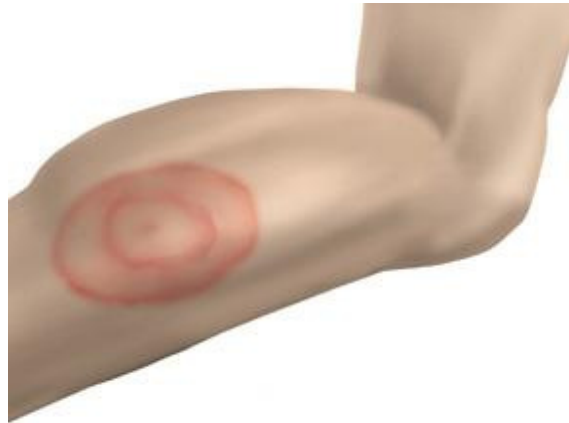


Figure 5: Erythema migrans rash (Siegel *et al.*, 2007). The erythema migrans or bull's eye rash is a hallmark sign of the invading bacterium that causes Lyme disease.

More severe symptoms can be seen in the months to years following initial infection. These symptoms include severe headaches, heart palpitations, and dizziness. The most prominent late stage infection symptom is the progression of severe swelling and pain in the joints known as arthritis. At this stage of infection, the central nervous system may also be affected and cognitive disorders may ensue (Steere, 2001; Agüero-Rosenfeld *et al.*, 2005).

### DIAGNOSIS

In 1996 the CDC published the latest official case definition of Lyme disease in order to standardize the requirements for reporting a disease case. The presence of the hallmark erythema migrans rash satisfies the criteria the CDC sets forth for a positive diagnosis (Centers for Disease Control and Prevention, 1997). However, if the patient does not exhibit evidence of a tick bite or have any known exposure to ticks within the previous 30 days, the CDC does recommend a laboratory confirmation as with late stage diagnoses (Centers for Disease Control and Prevention, 1997). Early diagnosis

and subsequent treatment can prevent further complications and the advancement of the disease (Centers for Disease Control and Prevention, 2005).

If early symptoms went undetected, the CDC criteria for a positive diagnosis requires the presence of at least one late stage infection symptom that is confirmed via laboratory diagnostic testing (Centers for Disease Control and Prevention, 1997). Confirmation of the presence of the bacteria can be obtained via the culture of the bacteria from a clinical specimen removed from the patient (Aguero-Rosenfeld *et al.*, 2005). Diagnostic confirmation may also be obtained with a CDC recommended two-step blood test for the disease causing bacteria (Centers for Disease Control and Prevention, 1997).

First the blood should be tested using an enzyme immunoassay, such as enzyme-linked immunosorbent assay (ELISA) or immunofluorescent assay (IFA), to detect bacterial antibodies present in the patient specimen (Centers for Disease Control and Prevention, 1997). Kits approved by the FDA test for either IgG or IgM antibodies to the bacteria; however, more sensitive test kits screen for both types of antibodies (Aguero-Rosenfeld *et al.*, 2005). Because these tests provide a high rate of false-positive results, if a positive result is obtained a second, more specific test must also be performed (Centers for Disease Control and Prevention, 1997). A FDA approved standardized western immunoblot kit should also be used to detect antibodies to proteins of various sizes produced by the bacteria (Centers for Disease Control and Prevention, 1997; Aguero-Rosenfeld *et al.*, 2005). Positive results on both tests satisfy the CDC criteria for a confirmed disease case. In some chronic cases doctors may



choose to use FDA approved Polymerase Chain Reaction (PCR) kits to test fluid drawn from a joint or spinal tap to detect bacterial DNA (Aguero-Rosenfeld *et al.*, 2005).

## TREATMENT

The Infectious Diseases Society of America (IDSA) has recently updated the clinical practice guidelines for the treatment of Lyme disease. According to the IDSA's 2006 guidelines, treatment of the infection with a 14 day course of oral antibiotics such as doxycycline, amoxicillin, or cefuroxime axetil usually clears the infection and can prevent further complications caused by the dissemination of the bacteria (Wormser *et al.*, 2006). Of the three recommended treatments, doxycycline should not be used for children under 8 or for pregnant or lactating females (Wormser *et al.*, 2006).

The ISDA recommends a 28 day course of oral antibiotics for patients with persistent symptoms after a first course of oral antibiotics and for patients with the later stages of disease (Wormser *et al.*, 2006). The longer course of treatment should significantly improve arthritis and other symptoms of the disease (Wormser *et al.*, 2006). Patients that exhibit the neurologic components of the disease should be administered intravenous  $\beta$ -lactam antibiotics such as ceftriaxone, cefotaxime, or penicillin G (Wormser *et al.*, 2006). Although full resolution of the disease can be obtained, the chance of the treatment providing a cure decreases with disease progression (Centers for Disease Control and Prevention, 2006; Wormser *et al.*, 2006).

## ***BORRELIA BURGDORFERI***

### DISCOVERY

In the fall of 1981, Dr. Jorge Benach collected nymphal *I. scapularis* ticks from Shelter Island, New York (Burgdorfer *et al.*, 1982; Oliver *et al.*, 1993). The ticks were

sent to Dr. William Burgdorfer, a researcher at the NIH Rocky Mountain Laboratories, where they were analyzed (Burgdorfer *et al.*, 1982). During his analysis an unidentified spirochetal bacterium was isolated from the tick midgut (Burgdorfer *et al.*, 1982). In 1982 the spirochete was established as the etiologic agent of Lyme disease (Burgdorfer *et al.*, 1982). The spirochete was later named *Borrelia burgdorferi* after its discoverer (Johnson *et al.*, 1984).

Since discovery, at least three genospecies of *B. burgdorferi* have been found to cause Lyme disease in humans. In the United States *B. burgdorferi sensu stricto* is the only strain of the bacteria that causes Lyme disease (Steere *et al.*, 2004). Two distinctly different genospecies of the bacteria, *Borrelia garinii* and *Borrelia afzelii*, cause infection in Asia (Baranton *et al.*, 1992; Canica *et al.*, 1993). In Europe, all three genospecies are present and cause disease (Baranton *et al.*, 1992; Canica *et al.*, 1993).

## CHARACTERISTICS AND STRUCTURE

Borrelial cells average 0.2 to 0.5  $\mu\text{m}$  in diameter and 4 to 18  $\mu\text{m}$  in length (Barbour and Hayes, 1986; Baron, 1996). It is the spirochete's flagella that are responsible for the motility and corkscrew, helical cell shape (Figure 6). The 7-20 periplasmic flagella, or axial filaments, originating from the ends of the spirochete are anchored into the cytoplasmic membrane. These endoflagella wind lengthwise between the outer membrane and cell wall around the protoplasmic cylinder (Charon and Goldstein, 2002).

Borreliae are microaerophilic organisms that require oxygen at lower than normal, 21%, oxygen to survive (Baron, 1996). The bacterium is an obligate parasite, as it relies on its host's metabolism. *B. burgdorferi* does not synthesize its own essential

nutrients and must obtain amino acids, fatty acids, and other essential elements from its host (Todder, 2005). In addition, unlike many pathogenic organisms, *Borrelia* does not require iron for infection or cellular survival (Posey and Gherardini, 2000).

### INTERNAL FLAGELLA

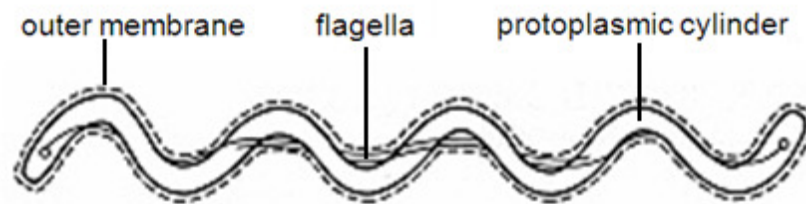


Figure 6: Internal Flagella (Charon and Goldstein 2002). The spirochete is given its unique helical shape by the internal flagella that are anchored in cytoplasmic membrane at the ends of the bacteria. The flagella wind lengthwise around the protoplasmic cylinder. Although the bacteria have many flagella, the graphic depiction is simplified with a single flagellum.

For laboratory culture, *Borrelia* is grown in a nutrient rich tissue culture medium such as variations of Barbour-Stoner-Kelly (BSK) culture medium (Wang *et al.*, 2004). Since 1993, a modified version of BSK called BSK-Harvard (BSK-H), supplemented with mammalian serum is the standard medium used (Pollack *et al.*, 1993). During its life cycle, the bacteria must adapt to survive changing environments that are encountered in the transition from the cold-blooded tick to a warm-blooded mammal. Mammalian conditions are mimicked by culturing *B. burgdorferi* at 35°C/pH 7.0 whereas the tick environment is approximated by growth at 23°C/pH 8.0 (Stevenson *et al.*, 1995; Stewart *et al.*, 2005). Although the media can be manipulated to mimic the host or the vector, experiments performed *in vitro* can not always be extrapolated to *B. burgdorferi*

during the infectious cycle because the bacteria can display different behavior inside vector or vertebrate host (Singh and Girschick, 2004).

## HOST – ORGANISM INTERACTION

The spirochete presents outer surface protein A (OspA) on its surface while it survives inside the resting, non-feeding tick. The protein serves as an anchor for the spirochete to the tick midgut. During the bloodmeal, the bacteria migrate out of the tick midgut into the salivary glands. The bacteria travels along with the saliva excreted into the animal during tick feeding where it moves through the opening in the skin created by the tick. In order to establish mammalian infection, *Borrelia* downregulates the expression of OspA and upregulates expression of OspC when feeding begins (Figure7) (Schwan *et al.*, 1995).

After transmission, *B. burgdorferi* migrates through the extracellular matrix of host tissue by binding to epithelial cells, neural elements, platelets and red blood cells. To disseminate, the bacterium uses special binding or adhesion proteins to bind to

## OUTER SURFACE PROTEIN EXPRESSION FROM TICK TO MAMMALIAN HOST

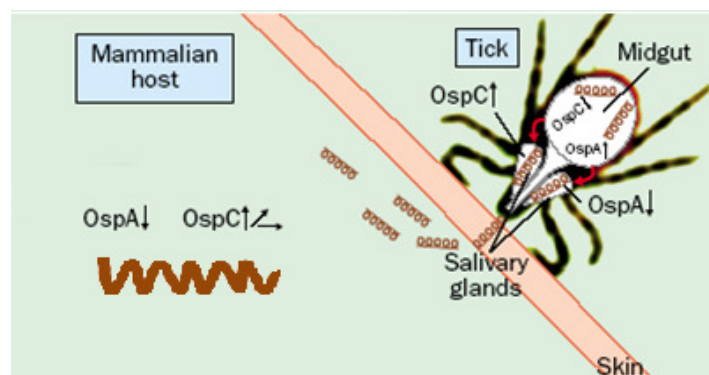


Figure 7: Outer Surface Protein Expression from Tick to Mammalian Host (Singh and Girschick, 2004). The bacterium differentially regulates the expression of OspA and OspC depending upon its location in order to effectively establish infection. The figure has been modified from the original version.

select host proteins such as integrins, proteoglycans and glycoproteins located on the surface of host cells and in tissue matrixes (Guo *et al.*, 1995; Steere *et al.*, 2004,).

## IMMUNOLOGY

It is at the site of the tick bite where the bacterium is presented with the vertebrate host's first line of defense, complement-mediated lysis (Steere *et al.*, 2004). The process of complement activation and subsequent cell lysis as well as borrelial lipoproteins and cell signals recruit more immune cells to the site of infection (Abbas and Lichtman, 2005). Initially primary lymphocytes, macrophages, and plasma cells are localized to the infection site around the erythema migrans rash (Mullegger *et al.*, 2000). Macrophages stimulated by the bacteria will begin to engulf and kill the bacteria (Abbas and Lichtman, 2005). The activated macrophages stimulate the maturation of helper T cells and production of proinflammatory cytokines, particularly interferon- $\gamma$  (IFN $\gamma$ ), interleukin-10 (IL-10), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), and interleukin-6 (IL-6) (Mullegger *et al.*, 2000; Abbas and Lichtman, 2005).

At the same time, borrelial lipoproteins act as B cell mitogens that independently stimulate a B cell response to produce antibodies (Steere *et al.*, 2004; Abbas and Lichtman, 2005). Additionally, T cell mediated responses to nonlipidated proteins use Th1 cells to prime B cell responses (Steere *et al.*, 2004; Abbas and Lichtman, 2005). The antibodies produced by the host immune system begin killing spirochetal cells via complement fixation and opsonization (Steere *et al.*, 2004).

Although an active immune response is present, the borrelial cells survive by minimizing or changing expression of surface proteins or inhibiting certain responses (Steere *et al.*, 2004). In order to escape selective immunological pressure caused by the

host, the bacterial cells can downregulate lipoproteins like OspC (Liang *et al.*, 2002). The variation of protein expression renders the OspC antibody useless as the antibody selects for cells with OspC-negative phenotypes or actually induces the protein's downregulation (Liang *et al.*, 2002). The bacterial cell also evades the host immune system by varying the antigenic portions of a expressed protein, namely, the *vmp*-like sequence protein E (VisE) (Zhang *et al.*, 1997). The variation of the VisE protein is essential to the survival of the bacteria because the antibody will not recognize the protein variant (Zhang *et al.*, 1997).

### COMPLEMENT REGULATOR-ACQUIRING SURFACE PROTEINS

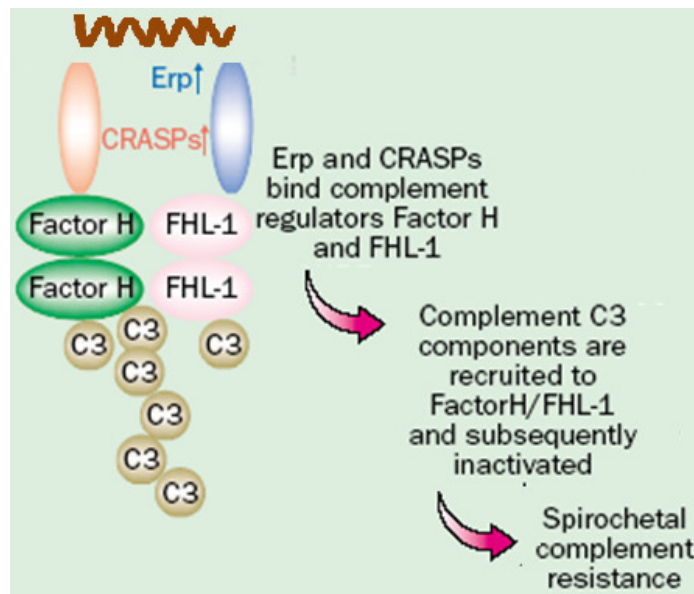


Figure 8: Complement Regulator-Acquiring Surface Proteins (Singh and Girschick, 2004). Inside the mammalian host the bacterial cells express Erp and CRASP proteins on its surface. These proteins confer alternative pathway complement resistance by binding Factor H and FHL-1 proteins. The figure has been modified from the original version.

Complement activation via the classic or alternative pathways destroys pathogens by coating them with opsonising molecules to form the complement membrane attack complex after entry in the human host; formation of the complex usually leads to cell lysis (Abbas and Lichtman, 2005). In this instance, *Borrelia* has evolved to provide an effective method of avoiding some complement mediated killing (Singh and Girschick, 2004). *B. burgdorferi* can express five distinct complement regulator-acquiring surface proteins (CRASPs) and several OspE/F related proteins, Erps, on their surfaces that bind the host complement regulators of the alternative pathway. CRASPs bind Factor H and Factor H-like Protein 1 (FHL-1) which inactivate C3b and protect the cell from complement mediated killing (Figure 8) (Stevenson and Akins, 2000; Kraiczy *et al.*, 2001; Kraiczy *et al.*, 2004).

The host's innate and adaptive responses must work together to control disseminating bacteria (Steere *et al.*, 2004). While in the later stages of infection the infected host has high antibody responses towards several borrelial proteins, the host may still be unable to clear infection. The host's hyperimmunization is caused by waves of bacterial growth following antigenic shifting (Akin *et al.*, 1999).

## GENETICS

The genome of *B. burgdorferi* cells consists of about 1,521,419 base pairs (bp) of nucleic acid. This information is contained on one linear chromosome and up to 21 linear and circular plasmids, the largest plasmid complement of any bacteria (Figure 9) (Casjens *et al.*, 2000). The single linear chromosome is approximately 910 kilobase pairs (kbp) in length and contains 853 predicted genes that provide mainly housekeeping functions for the cell (Baril, *et al.* 1989, Ferdows and Barbour 1989,

Fraser, et al. 1997). A significant amount of the bacterium's genetic material is held in the extrachromosomal plasmids (Casjens, Palmer *et al.*, 2000; Fraser *et al.*, 1997). In total, the cell holds 40% of its genetic information in the extrachromosomal DNA; 361,364 bp in 12 distinct linear plasmids as well as 249,330 bp in nine circular plasmids (Casjens *et al.*, 2000).

The genetic information contained in the cell is highly redundant and dispersed throughout the chromosome and plasmids (Zuckert and Meyer, 1996; Fraser *et al.*, 1997; Casjens *et al.*, 1997). The complete genome contains 161 families of paralogous genes that are similar in function. Members of these gene families can be found on both the chromosome and on extrachromosomal plasmids. The paralogous gene families range in size from 2 to 41 members (Casjens, et al., 2000).

### DIVERSITY OF GENETIC COMPOSITION

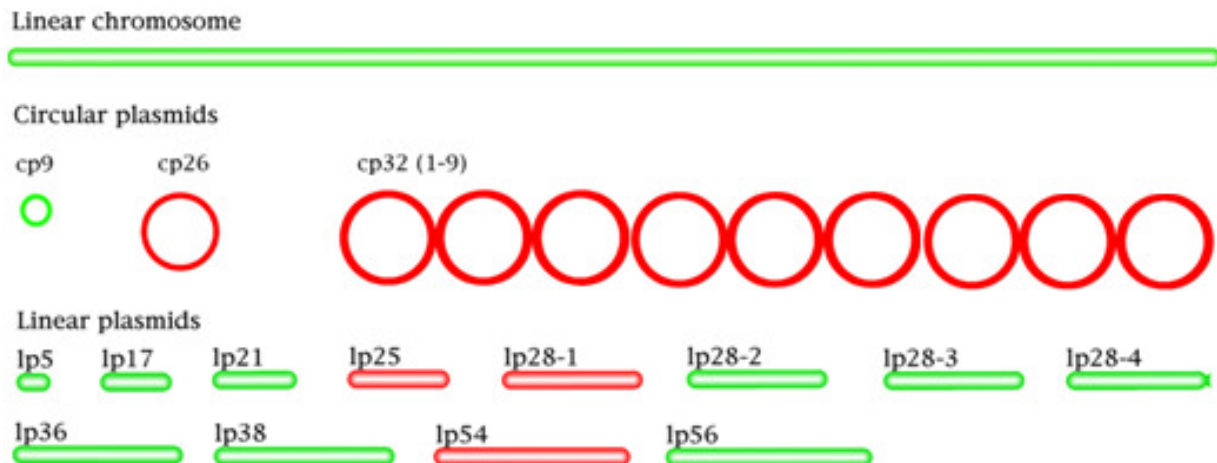


Figure 9: Diversity of Genetic Composition (Stewart *et al.*, 2005). *Borrelia* is a unique bacterium that contains one linear chromosome and up to 21 extrachromosomal elements. The figure has been modified from the original version.



*Borrelia* extrachromosomal plasmids can coexist in an individual bacterium (Hinnebusch and Barbour, 1992). However, *Borrelia* strains have often been found to lose one or more plasmids during laboratory propagation and cloning procedures (Schwan *et al.*, 1988). Among the plasmid-encoded products that have characterized or proposed functions are proteins required for nutrient acquisition, metabolism, and outer surface proteins (Fraser *et al.*, 1997; Casjens *et al.*, 2000).

## LINEAR PLASMIDS

*B. burgdorferi* strains have been known to spontaneously lose lp28-1. Bacterial cells that lose lp28-1 are capable of infecting mice; however, the infection does not persist (Purser and Norris, 2000; Labandeira-Rey and Skare, 2001). The lack of a persistent infection may be due to the loss of the *vlsE* coding region of the plasmid. The region is comprised of a set of 15 silent cassettes that switch into an expression locus to give the bacteria antigenic variation of the surface exposed protein (Zhang *et al.*, 1997).

Similarly to lp28-1, borrelia cells can spontaneously lose lp25. This plasmid is essential for survival in both tick and mammalian hosts, however; lp25 is not required for *in vitro* growth (Purser and Norris, 2000). The plasmid harbors *bbe22*, the gene that encodes a nicotinamidase. Nicotinamidase is absolutely essential in that it provides the critical step in converting nicotinamide to nicotinic acid in the exogenous pathway to synthesizing NAD as an energy source (Purser *et al.*, 2003).

Linear plasmid lp54 also contains many loci hypothesized to be required for survival in the mammalian host and the tick vector. Lp54 contains the highest percentage of temperature-regulated gene products, including OspA, OspB and the decorin binding proteins A and B (DbpA and DbpB) (Ojaimi *et al.*, 2003; Fischer *et al.*,

2003). Disruption of the *ospA/B* operon is linked to reduced tick colonization (Yang *et al.*, 2004). DbpA and DbpB mediate attachment to host tissue, which aids in initial colonization and dissemination of the bacteria (Guo *et al.*, 1995; Fischer *et al.*, 2003). CRASP-1, a factor H-binding protein of *B. burgdorferi* that is presumably needed to aid the cell in avoiding complement-mediated killing, is also encoded on lp54 (Kraiczy *et al.*, 2004).

Unlike lp28-1, lp25, and lp54, linear plasmids lp21, lp28-2, lp28-4, and lp56 do not appear to be required for mammalian infection or growth *in vitro* (Purser and Norris, 2000). It is speculated that the genes carried on the plasmids provide different capacities of selective advantages. The plasmids' genes may aid the borrelial cells by in adapting to different hosts and establishing a lasting infection (Purser and Norris, 2000).

#### CIRCULAR PLASMIDS

The unique circular plasmid cp26 has been shown to be essential and present in all natural isolates of the bacteria (Byram *et al.*, 2004). Housekeeping genes and virulence factors are found to be encoded on the plasmid. The very important OspC protein is encoded by a gene located on cp26 (Grimm *et al.*, 2004; Pal *et al.*, 2004). The plasmid also encodes a telomere resolvase (*resT*), which is required to resolve circular dimers of linear plasmids after replication (Kobryn and Chaconas, 2002).

#### 32 KILOBASE CIRCULAR PLASMIDS

The 32 kilobase circular plasmid family contains almost 20% of the genetic information of *Borrelia*. These nine plasmids are each about 30,000 bp in length (Casjens *et al.*, 2000). Although the plasmids have relatively the same amount of coding

DNA, the plasmids encode for 33 to 44 functional genes and between 0 to 9 pseudogenes (Table 1) (Casjens *et al.*, 1997; Casjens *et al.*, 2000; Eggers *et al.*, 2002).

### 32 KILOBASE CIRCULAR PLASMIDS COMPOSITION

Table 1: 32 Kilobase Circular Plasmids Composition (Casjens *et al.*, 2000). The cp32 family of plasmids are highly similar in composition as they are derivatives of each other. The plasmids are roughly the same size and contain similar numbers of genes. Table was adapted from original version for conciseness. The figure has been modified from the original version.

Plasmid	Size	Coding %	Genes	Pseudogenes
cp32-1	30750	92	42	0
cp32-3	30223	92	44	0
cp32-4	30229	92	39	4
cp32-6	29838	92	41	0
cp32-7	30800	93	42	0
cp32-8	30885	92	43	0
cp32-9	30651	92	33	9

No borrelial isolate has ever lost all members of the cp32 family (Casjens *et al.*, 1997; Purser and Norris, 2000). This important fact suggests that they are essential to *B. burgdorferi*, as they may provide critical virulence genes, aid in the bacterium's survival, or increase the range of possible hosts. The cp32 family contains at least nine unique members that are about 80% homologous throughout their sequences (Casjens *et al.*, 2000). Despite this homology, the cp32 plasmids can all stably coexist within the same cell (Casjens *et al.*, 1997, Casjens *et al.*, 2000).

The explanation for the ability of such similar plasmids to replicate, segregate, and coexist in the same cell is found in the plasmids' first of three hypervariable regions (Stevenson and Akins, 2000). The first region contains the paralogous gene families, PF57, PF50, PF32, and PF49 that are responsible for the cp32 family's replication and segregation into daughter cells (Figure 10) (Casjens *et al.*, 2000; Eggers *et al.*, 2002;

Stevenson and Akins, 2000). The region responsible for the plasmids replication is PF57, as well as the intergenic sequence immediately upstream. The intergenic sequence is theorized to contain approximately six DnaA boxes, the sites at which dnaA, a DNA replication initiation protein, bind. The PF57 gene serves as the plasmids origin of replication. Based on sequence and location, PF32 and PF49 are proposed partitioning proteins, ParA and ParB, respectively. The ParA and ParB proteins ensure that the plasmid segregates into each daughter cell (Casjens *et al.*, 2000; Eggers *et al.*, 2002). Through deletion experiments, PF50 is also deemed essential for as of yet undetermined reasons (Eggers *et al.*, 2002). It is the higher variability among these genes, as much as 69% compared to the plasmids' overall 20% variability, that ensures all the cp32 plasmids are compatible and can coexist within the same cell (Casjens *et al.*, 2000; Eggers *et al.*, 2002,).

*B. burgdorferi* has two other variable regions of DNA on their cp32 plasmids. The

## REPLICATION, MAINTENANCE, AND SEGREGATION HYPERVARIABLE REGION

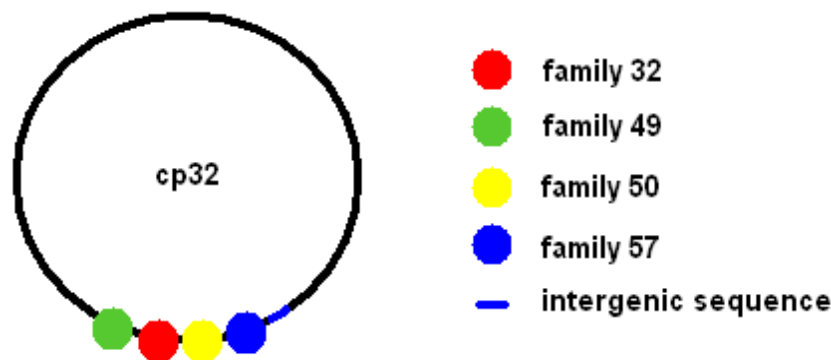


Figure 10: Replication, Maintenance, and Segregation Hypervariable Region (Casjens *et al.*, 2000). The region found in every cp32 plasmids is responsible for the family's replication, maintenance, and segregation. The four genes in the region, PF32, PF49, PF50, and PF57, and the intergenic sequence upstream of PF57 are clustered together in each of the cp32s. The figure has been modified from the original version.

second region contains the *erp* locus. The Erp proteins OspE and OspF are surface exposed lipoproteins that are upregulated in the bacteria once the spirochete has entered the mammalian host (Stevenson *et al.*, 1995). Later experiments have linked several of these proteins to complement evasion mediated by binding Factor H and FHL-1 proteins (Kraiczy *et al.*, 2001; Stevenson *et al.*, 2002; Kraiczy *et al.*, 2004).

The final hypervariable region has been named the 2.9 locus (Porcella *et al.*, 1996). This region contains genes from the multicopy lipoprotein (*mlp*) paralogous gene family. The *mlp* genes encode for a borrelial surfaced exposed protein to which infected hosts will develop antibodies (Yang *et al.*, 1999). It is hypothesized that this protein product may assist in interacting with host tissues (Theisen, 1996; Yang *et al.*, 1999). Most 2.9 loci also contains a gene from the *Borrelia* direct repeat (*bdr*) gene family that possibly play a role in cell regulation, sensing, or signaling (Zückert *et al.*, 1999; Roberts *et al.*, 2000; Stevenson and Akins, 2000). The two cp32 plasmids, cp32-1 and cp32-6, that do not contain a *bdr* contain a *rev* gene, a gene is a reverse orientation, that is surface expressed and can act as an antigen while giving the bacteria survival advantages in diverse environments (Gilmore and Mbow, 1998; Stevenson and Akins, 2000).

Other than these three hypervariable regions, the cp32 plasmids are between 99-80% similar (Stevenson and Akins, 2000; Casjens *et al.*, 2000). These nearly indistinguishable regions of DNA have not been extensively studied; therefore, little is known about the regions and their potential protein products. Some of these proteins are speculated to be additional surface expressed lipoproteins as well as secreted proteins (Stevenson and Akins, 2000).

Some of the known genes outside of the hypervariable region encode for borrelia haemolysin proteins A and B (BlyA and BlyB) (Stevenson and Akins, 2000). The two open reading frames upstream of the 2.9 locus work together to create pore-forming toxins (Guina and Oliver, 1997). Further experiments have determined that these genes are actually involved in cell lysis for the release of *B. burgdorferi* bacteriophage particles (Eggers and Samuels, 1999). *Borrelia* uses the bacteriophage  $\Phi$ BB-1 as a natural means of laterally transferring genetic material to other cells by packaging the genetic information from the members of the cp32 family and transducing them into other cells (Figure 11) (Eggers *et al.*, 2001).

### PHIBB-1 BACTERIOPHAGE



Figure 11: PhiBB-1 Bacteriophage (Eggers *et al.*, 2001). *Borrelia* can laterally transfer genetic information by utilizing the bacteriophage particle  $\Phi$ BB-1.

## RESEARCH GOALS

Many experiments have been conducted to determine the function of the different genes on the plasmids of *B. burgdorferi*. If the genes on a plasmid encode for essential proteins, the plasmids on which the gene is located must be faithfully maintained by *Borrelia* (Purser and Norris, 2000). The required plasmids of *Borrelia*, lp28-1, lp25, lp54, and cp26, have genes that encode for proteins that confer infectivity and persistence of the bacteria (Zhang *et al.*, 1997; Labandeira-Rey and Skare, 2001; Purser *et al.*, 2003; Ojaimi *et al.*, 2003; Fischer *et al.*, 2003; Kraiczy *et al.*, 2004; Byram *et al.*, 2004).

The cp32 family of plasmids are well maintained inside the *B. burgdorferi* cell. Recent studies have characterized many natural isolates of the bacteria obtained from several sources; no isolate of *Borrelia* has ever been found to exist with all of the cp32 plasmids missing (Purser and Norris, 2000). When these isolates were examined, plasmids cp32-1, cp32-2, cp32-7, cp32-4, cp32-6, cp32-8, cp32-9 were almost always present, suggesting that at least some of the cp32 plasmids provide genetic information that is necessary for spirochetal survival in nature (Casjens *et al.*, 1997; Purser and Norris, 2000).

Little is known about the true function of the majority of the cp32 plasmids' genes, which comprise almost 20% of the borrelial genome (Casjens *et al.*, 2000). Experiments to determine their function must be performed. To get a broad understanding of their purpose, plasmid knockout mutants of *B. burgdorferi* can be produced. These knockout mutants will provide information on the cp32 plasmids role in infectivity and pathogenesis and determine if they are required by the bacterium.

## MATERIALS AND METHODS

### REAGENTS

#### LOADING DYE

24.0 mL	Glycerol (EMD Chemicals, Incorporated, Gibbstown, NJ)
4.8 mL	Ethylenediamine Tetraacetic acid (EDTA) (Amresco, Solon, OH)
0.036 g	Xylene Cyanol (Amresco)
11.2 mL	ddH <sub>2</sub> O

Stored at Room Temperature

#### 50X TRIS-ACETATE-EDTA (TAE) BUFFER

242 g	Tris Base (Amresco)
57.1 mL	Glacial Acetic Acid (Mallinckrodt Baker, Incorporated, Phillipsburg, NJ)
100 mL	0.5 M EDTA (pH8.0) (Amersco)

Dissolved to 1 L with ddH<sub>2</sub>O

Stored at Room Temperature

#### 1X TAE BUFFER

10 mL	50xTAE
900 mL	ddH <sub>2</sub> O

Stored at Room Temperature

#### ELECTROPORATION SOLUTION (EPS) BUFFER

46.5 g	Sucrose (Fisher Scientific Company, Pittsburgh, PA)
75 mL	100% Glycerol (EMD Chemicals, Incorporated)

Dissolved to 500 mL with ddH<sub>2</sub>O

Adjusted to pH to 7.3 and filtered



Stored at 4°C

#### 0.8% ETHIDIUM BROMIDE INCORPORATED AGAROSE GEL

0.26 g UltraPure™ Agarose (Sigma-Aldrich Corporation, St. Louis, MO)

30.0 mL 1x TAE

3 µL Ethidium Bromide (Sigma-Aldrich Corporation)

#### 1.0% ETHIDIUM BROMIDE INCORPORATED AGAROSE GEL

0.30 g UltraPure™ Agarose (Sigma-Aldrich Corporation)

30.0 mL 1x TAE

3 µL Ethidium Bromide (Sigma-Aldrich Corporation)

#### LURIA-BERTANI (LB) BROTH

10 g Tryptone (Acros Organics, Geel, Belgium)

10 g Sodium Chloride (Fisher Scientific Company)

5 g Yeast Extract (Becton, Dickinson and Company, Sparks, MD)

Dissolved to 2000 mL with ddH<sub>2</sub>O

Adjusted to pH to 8.0

Stored at Room Temperature

\*Containing ampicillin (Fisher Scientific Company) at 100 µg/mL (LB-amp)

Stored at 4°C

\*Containing gentamycin (Invitrogen Corporation, Carlsbad, CA ) at 5 µg/mL (LB-gent)

Stored at 4°C

#### LURIA-BERTANI (LB) PLATE

10 g Tryptone (Acros Organics)

10 g Sodium Chloride (Fisher Scientific Company)

5 g Yeast Extract (Becton, Dickinson and Company)

Dissolved to 2000 mL with ddH<sub>2</sub>O

Adjusted to pH to 8.0

32 g BatoAgar (Becton, Dickinson and Company)

Autoclaved in 500 mL increments

Stored at 4°C

\*Containing ampicillin (Fisher Scientific Company) at 100 µg/mL (LB-amp)

\*Containing gentamycin (Invitrogen Corporation) at 5 µg/mL (LB-gent)

## **BACTERIAL STRAINS AND PLASMIDS**

*Borrelia burgdorferi* strain 5A4 and 13A (Table 2) were cultured from the laboratory stock in BSK-H complete media (Sigma-Aldrich Corporation) supplemented with 6% rabbit serum (Sigma-Aldrich Corporation) and incubated inside a Forma Series II water jacketed CO<sub>2</sub> Incubator (Thermo Electron Corporation) at 33°C and 5% CO<sub>2</sub>. The cells were grown to an approximate density of 100 spirochetes per field of view for further use or flash frozen in liquid nitrogen and stored at -80°C until needed.

DH5α chemically competent *Escherichia coli* cells (Invitrogen Corporation) were transformed using the *E. coli* cell transformation procedure (Table 2). During the course of the study several constructed plasmids were introduced into the cells for amplification for further use in the study (Table 2).

## **GENOMIC DNA PREPARATION**

Genomic DNA was isolated from *B. burgdorferi* 5A4 and used for Polymerase Chain Reaction (PCR) amplification procedures. Cells were harvested in 1 mL volumes by centrifugation at 16,100 x g for 12 minutes at 4°C in an Eppendorf 5415R Table Top Micro-Centrifuge (Eppendorf Scientific, Incorporated, Madison, WI). The DNA from

## BACTERIAL STRAINS AND PLASMIDS USED IN STUDY

Table 2: Bacterial strains and plasmids used in study. The bacterial strains and plasmids listed above were used to perform the outlined experiments.

Strain or Plasmid	Description	Source
<i>Borrelia burgdorferi</i>		
5A4	<i>B. burgdorferi</i> high-infectivity, low passage, strain B31 with all plasmids present	Steven Norris (Purser and Norris, 2000)
13A	<i>B. burgdorferi</i> highly transformable, low-infectivity, high passage strain 5A13 (Purser and Norris, 2000) lacking plasmids lp25 <sup>-</sup> lp56 <sup>-</sup>	Liang Lab
pG22cp32-3plus	<i>B. burgdorferi</i> strain 5A13 with pG22cp32-3plus plasmid	This Study
<i>Escherichia coli</i>		
DH5 $\alpha$	F <sup>-</sup> , $\phi$ 80dlacZ $\Delta$ M15, $\Delta$ (lacZYA-argF)U169, <i>deoR</i> , <i>recA1</i> , <i>endA1</i> , <i>hsdR17</i> (rk <sup>-</sup> , mk <sup>+</sup> ), <i>phoA</i> , <i>supE44</i> , $\lambda^-$ , <i>thi-1</i> , <i>gyrA96</i> , <i>relA1</i>	Invitrogen Corporation
Plasmids		
pNCO1T	TA cloning vector	Liang Lab (Downie <i>et al.</i> , 2004)
pNCO1Tcp32-3	pNCO1T with cp32-3 putative maintenance region obtained from <i>B. burgdorferi</i> 5A4 inserted into multiple cloning region at <i>NcoI</i> site	This Study
pBSVGE22	pBSV2G cloning vector inserted with borrelial <i>bbe22</i> gene	Liang Lab
pG22	pBSVGE22 shortened with GenF/GenR primers	This Study
pG22cp32-3plus	pG22 inserted with cp32-3plus region obtained from pNCO1Tcp32-3	This Study

pelleted cells was extracted using the Sigma GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich Corporation).

The pellet of cells was resuspended in 200 µL Resuspension Buffer (Sigma-Aldrich Corporation) and 20 µL of Proteinase K (Sigma-Aldrich Corporation) was added. The mixture was incubated in a 50°C water bath for 1 hour. After incubation, 200 µL of Lysis Solution (Sigma-Aldrich Corporation) was added to the centrifuge tube and mixed via inversion. The tube was then incubated for 10 minutes at 70°C.

A Nucleic Acid Binding Column (Sigma-Aldrich Corporation) was prepared by adding 500 µL of Column Preparing Solution (Sigma-Aldrich Corporation) to the column and spun at 3,900 x g for 1 minute. The collection tube was discarded. Ethanol at a volume of 200 µL was added to the lysed cell suspension. The tube was then vortexed and the contents added to the prepared binding column with new collection tube attached. The apparatus was spun at 3,900 x g for 1 minute.

The collection tube was replaced again and the bound DNA was washed by with 500 µL Wash Solution (Sigma-Aldrich Corporation) with ethanol and spun again for 1 minute at 3,900 x g. The wash was repeated and the apparatus was spun at 16,100 x g for 3 minutes to allow the DNA to dry onto the column.

Once the final collection tube was added to the column, the DNA was eluted by adding 200 µL sterile autoclaved ddH<sub>2</sub>O and spinning the apparatus at 3,900 x g for 1.5 minutes. The resulting collection of DNA in ddH<sub>2</sub>O was either used immediately or stored for later use at -20°C.

## PRIMER DESIGN

Primers for this experiment were designed using Clone Manager Suite version 7.1 (Scientific and Educational Software, Cary, NC). Sequences used in the design process were obtained via the Entrez Nucleotide database (Table 3). All primers were synthesized by Integrated DNA Technologies, Incorporated (Coralville, IA) (Table 4). The primers were used at a working concentration of 12 pM.

## GENETIC SEQUENCE ACCESS NUMBERS

Table 3: Genetic Sequence Access Numbers. cp32 genetic sequences were accessed via the Entrez Nucleotide database. Accession numbers for each cp32 are listed above.

Plasmid	GenBank Accession Numbers
cp32-1	NC 000948
cp32-3	NC 000949
cp32-4	NC 000950
cp32-6	NC 000951
cp32-7	NC 000952
cp32-8	NC 000953
cp32-9	NC 000954

Primers to amplify the putative maintenance required for autonomous replication and segregation region of cp32-3 were designed to include PF57, PF50, PF32, and PF49 genes along with 5.5 kbp of DNA flanking the region. Although the regions surrounding this area are highly homologous between cp32 plasmids, the extra DNA was included in the amplification to ensure amplification of only the single cp32-3. The forward primer cp32-3F1NcoI was used in combination with either reverse primer cp32F1Nco1 or cp32R2 at an annealing temperature of 60°C in amplification PCR

procedures. The amplification produced the approximately 8.4 kbp fragment of DNA used in the experiments. All three primers introduced an *Nco*I restriction site onto the ends of the DNA.

Primers to confirm the presence of the 8.4 kbp region of cp32-3 DNA were developed to amplify the 500 bp region of DNA located toward the end of the PF161 gene extending toward PF57. The concp32F and concp32R primers used in confirmation PCR procedures were annealed to denatured DNA at 60°C.

Primers to amplify and shorten the pBSVGE22 plasmid were designed to amplify only the necessary components of the plasmid. For amplification purposes the Gen32F and Gen32R plasmids were used in combination at an annealing temperature of 60°C. The approximately 2.8 kbp product with newly introduced *Nco*I sites was used in the construction of the pG22cp32-3plus plasmid.

To confirm the presence of the gentamycin acetyltransferase gene, primers were developed to amplify a 519 bp portion of the gene. The GenF and GenR primers were used in PCR confirmation procedures with annealing temperatures of 50°C.

#### PRIMERS USED IN STUDY

Table 4: Primers used in study. The primers above were used throughout the various procedures described. The primers were synthesized by Integrated DNA Technologies, Incorporated.

Primer Name	Sequence (5' to 3')
cp32-3F1 <i>Nco</i> I	ATCCATGGGTTTTGGAGTTATTGTAGT
cp32R1 <i>Nco</i> I	AACCATGGCTAGCTGCATTTGTGCTTGTA
cp32R2	GACCATGGTACTCAGCAATAGCACTACT
concp32F	CCAGTACTTGTACTGTAGAATGTA
concp32R	TAGGCTATACTACAAACAGTGTAG
Gen32F	AACCATGGAATAAGCAGTCAAGTCTAGTCT
Gen32R	AACCATGGAAACCCTAAGGATGAACTT
GenF	TCACGGTGTTATGGAAATAG
GenR	GACTGCGAGATCATAGATATAG
BBS41F	CTGTTTAATCCACCACCAGTA
BBS41R	GGAGAAAGTACTGAGAAAGT

Technologies) made to 50  $\mu\text{L}$  volume with sterile autoclaved ddH<sub>2</sub>O. The PCR reaction mixture was subjected to the following conditions inside the MyCycler Thermal Cycler (Bio-Rad Laboratories, Incorporated, Hercules, CA). The DNA was initially denatured at 98°C for 6 seconds. Next, the reaction mixture was subjected to 35 cycles of amplification during which the DNA was denatured at 98°C for 6 seconds, primers were annealed at  $T_m+3^\circ\text{C}$  for 23 seconds, and the primer was extended at 72°C for 30 seconds per 1 kbp DNA. Finally, the reaction mixture was allowed a final extension at 72°C for 7 minutes and cooled to 4°C.

Confirmation reactions contained approximately 1.0  $\mu\text{L}$  DNA template or 1  $\mu\text{L}$  cultured cells, 2  $\mu\text{L}$  10x PCR Buffer (Takara Bio Incorporated) with Mg<sup>2+</sup>, 1.6  $\mu\text{L}$  dNTP (Takara Bio Incorporated), 0.1  $\mu\text{L}$  rTaq polymerase (Takara Bio Incorporated), and 0.5  $\mu\text{L}$  reaction specific primers (Integrated DNA Technologies, made to 20  $\mu\text{L}$  volume with sterile autoclaved ddH<sub>2</sub>O). To begin, the DNA was denatured at 94°C for 60 seconds. Next, the reaction mixture was put through 35 cycles of amplification. In the amplification process, the DNA was denatured again at 94°C for 60 seconds, primers were annealed at primer specific temperatures for 30 seconds, and the primers were extended at 72°C for 30 seconds per 1kbp DNA. At the end of these cycles, the reaction mixture was finally extended for 7 minutes at 72°C and subsequently cooled to 4°C. The DNA resulting from PCR reactions was either used immediately or stored at -20°C for future use.

## **AGAROSE GEL ELECTROPHORESIS**

PCR amplification and confirmation products as well as DNA fragments generated via endonuclease restriction digestions were all visualized by agarose gel

electrophoresis. DNA samples were mixed with 2  $\mu$ L loading dye and run on 0.8% or 1.0% ethidium bromide incorporated agarose gels depending on the size of the fragment of interest. Gels were run at 90 volts with a BioRad 3000Xi (Bio-Rad Laboratories, Incorporated) in a Mini-Sub Cell GT (Bio-Rad Laboratories, Incorporated) for 20 minutes. The separated DNA fragments were compared to 1KbPlus DNA Ladder (Invitrogen Corporation) to confirm approximate size. Bands within the gels were photographed inside a BioRad Universal Hood (Bio-Rad Laboratories, Incorporated) equipped with a CFW-1312M grayscale digital camera and viewed with The Discovery Series QualityOne 1-D Analysis Software version 4.6.1 (Bio-Rad Laboratories, Incorporated).

#### **AMPLIFIED DNA PURIFICATION**

DNA fragments generated from PCR or from digestion reactions were purified using the PureLink™ PCR Purification Kit (Invitrogen Corporation). Binding Buffer B3 (Invitrogen Corporation) at a volume of 400  $\mu$ L was added to the DNA sample and pipetted into a PureLink™ PCR Spin Column (Invitrogen Corporation) snapped into a collection tube. The column in the collection tube was spun at 16,100 x g for 1 minute and the flow through was discarded.

The column was washed by adding 650  $\mu$ L Wash Buffer (Invitrogen Corporation) to column and spun at 16,100 x g for 1 minute. The flow through was discarded and centrifugation continued for 3 additional minutes to remove excess buffer.

The column was placed into a PureLink™ Elution Tube (Invitrogen Corporation) and DNA was incubated with 32  $\mu$ L of sterile autoclaved ddH<sub>2</sub>O for 2 minutes. The DNA



was eluted by spinning column at 16,100 x g for 2 minutes. The purified DNA was used immediately or stored at -20°C for future use.

### ***E. COLI* CELL TRANSFORMATIONS**

Aliquots of 50 µL DH5α competent *E. coli* cells (Invitrogen Corporation) were incubated with 3 µL of ligation product DNA on ice for 35 minutes. Cells were heat-shocked in a 42°C waterbath for 45 seconds then immediately incubated on ice for 2 minutes.

To the tube, 180 µL of LB broth was added to the transformed cells and incubated at 37°C for 1 hour in a Forma Orbital Shaker incubator (Thermo Electron Corporation). The cells were removed from the incubator and 100 µL to 180 µL of cells were plated on LB plates containing the appropriate antibiotics. When needed, 4 µL Isopropyl β-D-1-thiogalactopyranoside ,IPTG, (Applied Biosystems, Foster City, CA) and 40 µL X-gal (Invitrogen Corporation) was also added for blue versus white screening.

The plates were incubated at 37°C overnight and observed for growth. Transformation colonies were inoculated into 500 µL of LB broth containing the appropriate antibiotic and incubated for 4 hours in a shaking incubator. Confirmed transformant cultures were then brought up to 5 mL cultures with the addition of LB broth containing the appropriate antibiotic and returned to the incubator for overnight growth in preparation for plasmid recovery.

### **PLASMID RECOVERY**

Plasmid mini preps were performed on 5 mL overnight *E. coli* cultures using the Qiagen QIAprep Miniprep Kit (Valencia, Ca). Overnight cultures of 5 mL were pelleted

at 3711 x g for 15 minutes in a Sorvall Legend RT tabletop centrifuge (Kendro Laboratory Products, Newtown, CT).

The supernatant was discarded and the pellet was re-suspended by pipetting in 250  $\mu$ L of chilled Buffer P1 with LyseBlue (Qiagen Incorporated) and added to a microcentrifuge tube. Buffer P2 (Qiagen Incorporated) was added to the tube at a volume of 250  $\mu$ L and mixed by inversion until consistent blue color was reached. A 300  $\mu$ L volume of Buffer P3 (Qiagen Incorporated) was added and mixed in immediately by inversion. The resulting solution was centrifuged at 16,100 x g for 10 minutes. The supernatant was removed to a QIAprep spin column (Qiagen Incorporated) and centrifuged at 16,100 x g for 1 minute.

The flow through was discarded and the column was washed with 0.5 mL of Buffer PB (Qiagen Incorporated). The buffer was removed via centrifugation and the flow through discarded. The column was washed again with 0.75 mL Buffer PE (Qiagen Incorporated). The column was spun for an additional 1 minute to remove excess buffer then placed into a clean micro-centrifuge tube.

The DNA was eluted after the column was incubated for 1 minute with 30  $\mu$ L sterile autoclaved ddH<sub>2</sub>O by centrifugation at 16,100 x g for 1 minute. A second elution was collected by incubating the column with 20  $\mu$ L sterile autoclaved ddH<sub>2</sub>O for 1 minute and centrifuging again for 1 minute at 16,100 x g. The unused portion of plasmid DNA was stored at -20°C for future use.

## **PLASMID CONSTRUCTION**

DNA from strain 5A4 *Borrelia burgdorferi* was PCR amplified using the primers

cp32-3F1Nco1 and cp32R2 to produce a DNA fragment that represents the putative maintenance region of cp32-3. This 8kbp DNA fragment compared to the DNA ladder after gel electrophoresis to confirm size and then purified. The fragment was then digested with *NcoI* and purified again.

pNCO1T plasmid DNA was also digested with *NcoI* then purified. The digested cp32-3 fragment was then ligated into pNCO1T to produce pNCO1Tcp32-3. The fragment was transformed into *E. coli* and grown on LB-amp plates incorporated with IPTG and X-gal. The white colonies were selected for growth in LB-amp broth. Plasmid construction was confirmed via PCR using the concp32F and concp32R primers and confirmed transformants were allowed to grow overnight before plasmid DNA was recovered.

The resulting DNA was PCR amplified using cp32-3F1Nco1 and cp32-3R1Nco1 to produce the cp32-3plus fragment. The DNA resulting from the amplification was again compared to the DNA ladder to confirm appropriate fragment size. The DNA was then purified and digested with *NcoI*. The DNA fragment was then purified once more.

The plasmid DNA from pBSVGE22 was PCR amplified with Gen32F and Gen32R primers to produce pG22. The plasmid fragment size was confirmed via gel electrophoresis and subsequently purified. The fragment was digested with *NcoI*. The ends of the fragments were dephosphorylated and the DNA fragment was purified once more.

The cp32-3plus fragment was then ligated into the pG22 plasmid. The ligation product, pG22cp32-3plus was transformed into *E. coli* and grown on LB-gent plates. Transformants were confirmed via PCR using concp32F and concp32R and GenF and

GenR primers. Confirmed transformants were allowed to grow overnight in LB-gent broth. Plasmid DNA was consequently extracted for use in electroporation procedures.

## **BORRELIAL ELECTROPORATION**

*Borrelia burgdorferi* strain A13 was resurrected from frozen stock into 2 mL BSK-H complete media (Sigma-Aldrich Corporation) supplemented with 6% rabbit serum (Sigma-Aldrich Corporation) and incubated inside a Forma Series II water jacketed CO<sub>2</sub> Incubator (Thermo Electron Corporation) at 33°C and 5% CO<sub>2</sub>. Once the culture was grown to an approximate density of 100 spirochetes per field of view, the cells were transferred into 50 mL of media and allowed to return to 100 spirochetes per field of view before collection.

To collect the cells, the culture was split into two 25 mL aliquots. The aliquots were pelleted at 3711 x g for 22 minutes in a Sorvall Legend RT tabletop centrifuge. The cells were rinsed with a total of 15 mL EPS and combined into one tube. The tube was spun at 3711 x g for 22 minutes. Again the cells were rinsed using 5 mL EPS and transferred into 20 mL falcon tube. The cells were spun once more at 3711 x g for 22 minutes.

After the final rinse, the cells were resuspended in 50 µL EPS. The suspension of cells was then added to the pG22cp32-3plus DNA to be inserted into the cells and incubated on ice for 1 minute. The chilled suspension was transferred to a pre-chilled 0.1 cm GenePulser™ electroporation cuvette (Bio-Rad Laboratories, Incorporated) for transformation. The cuvette was placed inside the GenePulser Xcell™ (Bio-Rad Laboratories, Incorporated) set to 1.25 kV, 25 µF, 200 e for a constant time of 4-6 ms for use. Immediately after electroporation, 1 mL of prewarmed (37°C) media was added

to the cuvette. Working quickly, the contents of the cuvette was transferred into 35 mL of media and incubated for 24 hours at 34°C.

After overnight incubation at 34°C, the media was supplemented with gentamycin to a final concentration of 50 µg/mL. The culture was then aliquoted into PCR tubes at a volume of 220 µL/tube to produce cultures with single clones. One week later the cultures were monitored for positive transformants. All steps in the transformation process were performed in a class II biosafety cabinet.

### **TRANSFORMANT IDENTIFICATION**

Between 7 and 12 days after a transformation procedure, monitoring for positive transformants began. As *Borrelia* grows, the bacteria lower the pH of the culture media, changing it from a red-orange to a yellow-orange color. The color change is used as an indicator of growth and is produced by the phenol-red dye present in the media. Phenol red is a dye used to indicate the change in pH from 8.4 to 6.6, as the pH gradually decreases the dye will change color from red to yellow.

All cultures were viewed under a Zeiss Axiostar plus microscope to visualize growth. All positive cultures were confirmed via confirmation PCR using primers specific to DNA inserted into transformed cells. All true positive cultures were transferred to 1 mL new media for further growth and use.

### **SELECTION PRESSURE INCREASE**

In order to increase selection pressure, 1 mL cultures of positive transformants were subjected to increasing concentrations of gentamycin. The media was originally increased from 50 µg/mL of gentamycin to 100 µg/mL and then again increased to 150 µg/mL eight hours later.

After twelve hours of growth, the culture was aliquoted out in 50  $\mu\text{L}$  volumes into media supplemented with varying concentrations of gentamycin ranging from 300  $\mu\text{g}/\text{mL}$  to 1400  $\mu\text{g}/\text{mL}$  in 100  $\mu\text{g}$  increments. The new 1 mL cultures were allowed to grow over several generations. Transformants grown in 300  $\mu\text{g}/\text{mL}$ , 500  $\mu\text{g}/\text{mL}$ , 700  $\mu\text{g}/\text{mL}$ , and 900  $\mu\text{g}/\text{mL}$  had 50  $\mu\text{L}$  of culture transferred into new media supplemented with the same concentration of gentamycin.

As marked drop-off in growth was noted at 700  $\mu\text{g}/\text{mL}$  of gentamycin, the 500  $\mu\text{g}/\text{mL}$  culture was recultured at 550  $\mu\text{g}/\text{mL}$  and allowed twelve generations of growth. At that time, 50  $\mu\text{L}$  of the culture was removed to 1 mL new media with the same concentration of gentamycin and allowed to grow for three more days. Following this period of growth, 50  $\mu\text{L}$  of culture was transferred into two tubes of 950  $\mu\text{L}$  culture media supplemented with 750  $\mu\text{g}/\text{mL}$  or 950  $\mu\text{g}/\text{mL}$  of gentamycin.

## RESULTS

### SELECTION OF MAINTENANCE REGION

A previous study by Eggars and colleagues indicates that the PF57, PF50, PF32, and PF49 genes serve as the putative maintenance region of the cp32 plasmids in the *B. burgdorferi* strain 297 (2002). As the regions surrounding this area are extremely similar between cp32 plasmids, a longer segment of DNA was amplified to ensure amplification of only the single cp32-3 plasmid. It is by this reasoning that the 3 kbp required region was amplified along with 5.5 kbp of DNA flanking the region (Figure 12).

The PCR amplification with the primers cp32-3F1NcoI and cp32R2 (Table 4) produced the 8430 bp region of DNA used in the construction of plasmids in latter experiments. After amplification, the DNA fragment size was confirmed via agarose gel electrophoresis (Figure 13). After digestion with *NcoI*, the restriction site introduced with both primers, the DNA fragment size was again confirmed using agarose gel electrophoresis.

### CREATION OF pNCO1Tcp32-3

The pNCO1T vector, which is used extensively in our laboratory, can replicate in *E. coli* but does not have the necessary genes to do so in *B. burgdorferi* (Dowie *et al.*, 2004, Xu *et al.*, 2007a, 2007b). The 8.4 kbp fragment of DNA amplified from cp32-3 was inserted into the vector pNCO1T at the *NcoI* restriction site, disrupting the *lacZ* gene (Figures 14,15). The resulting pNCO1Tcp32-3 plasmid was used to transform *E. coli* cells that were plated onto LB-amp plates supplemented with IPTG and X-gal. Potential successful transformants grew white colonies, as opposed to non-transformed blue colonies (Figure 16), greatly simplifying the selection process.

## cp32-3 PUTATIVE MAINTENANCE REGION

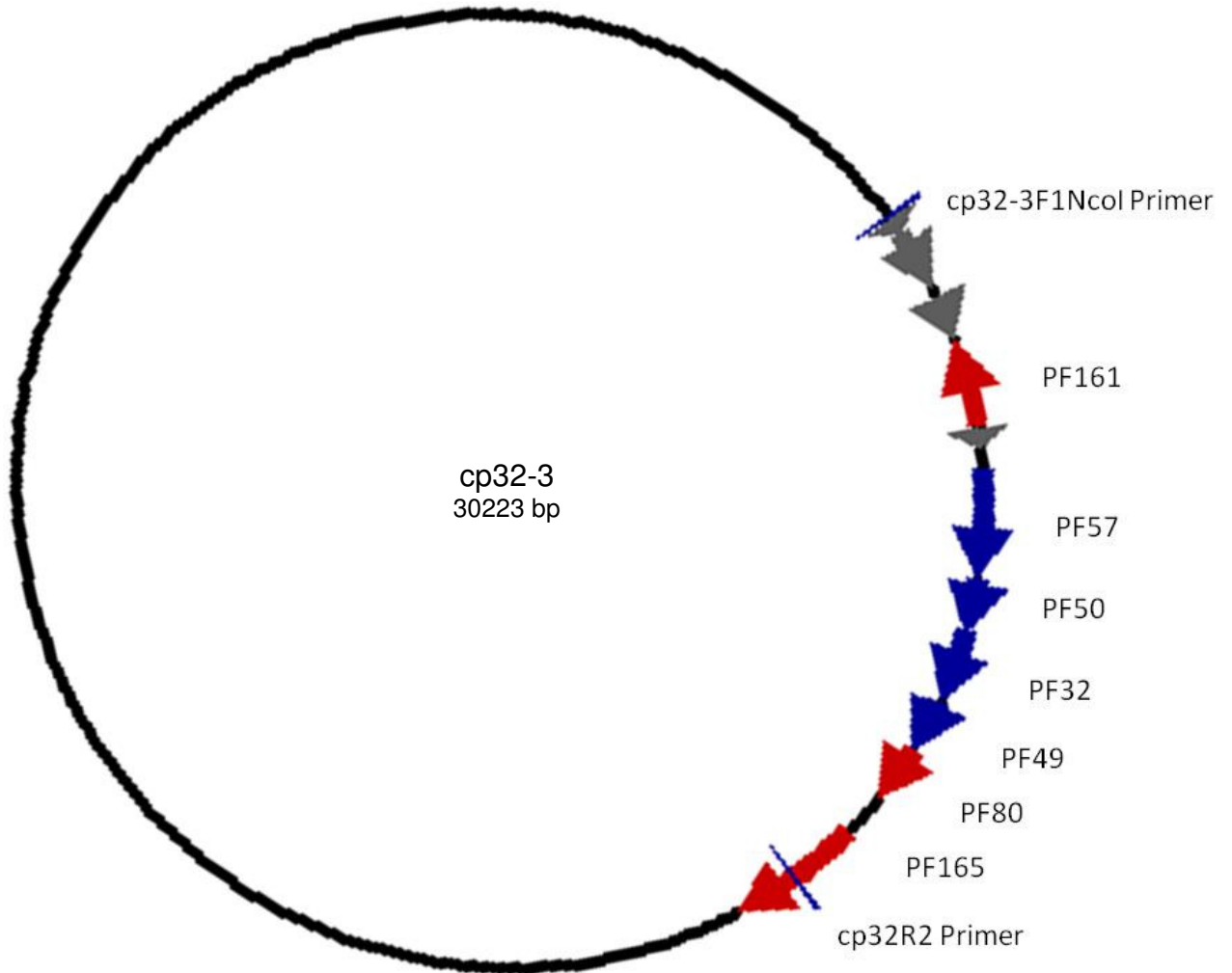


Figure 12: cp32-3 Putative Maintenance Region. The 8.4 kb region of DNA representing the putative maintenance region from the cp32-3 plasmid was amplified using cp32-3F1NcoI/cp32R2 primers. The resulting fragment was used in the construction of future plasmids.



### cp32-3 AMPLIFIED FRAGMENT

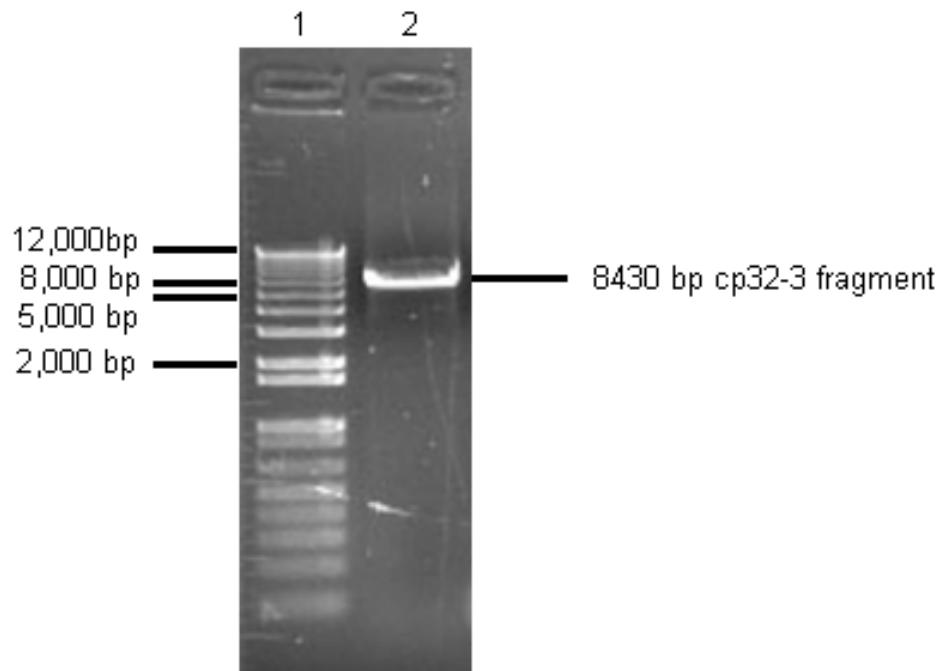


Figure 13: cp32-3 Amplified Fragment. The DNA fragment representing the putative maintenance region of ccp32 plasmids was amplified from cp32-3 and visualized by gel electrophoresis to confirm appropriate size. The resulting 8430 bp fragment was used in the construction of future plasmids.

Lane1:1kbp DNA Ladder  
Lane2: *B. burgdorferi* 5A4 cp32-3 fragment (cp32-3F1NcoI/cp3R1NcoI)

### pNCO1T VECTOR COMPOSITION

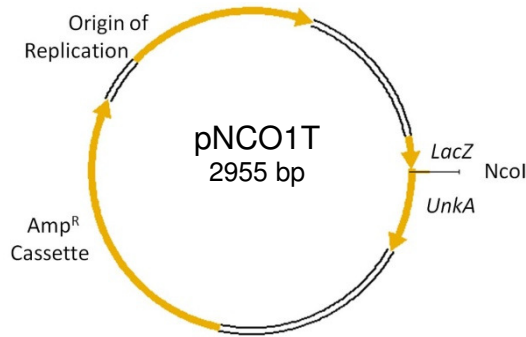


Figure 14: pNCO1T Vector Composition. The pNCO1T plasmid was cut at the *NcoI* site to prepare the plasmid for the insertion of the cp32-3 fragment of DNA that represents the punitive maintenance region of cp32-3.

### pNCO1Tcp32-3 VECTOR COMPOSITION

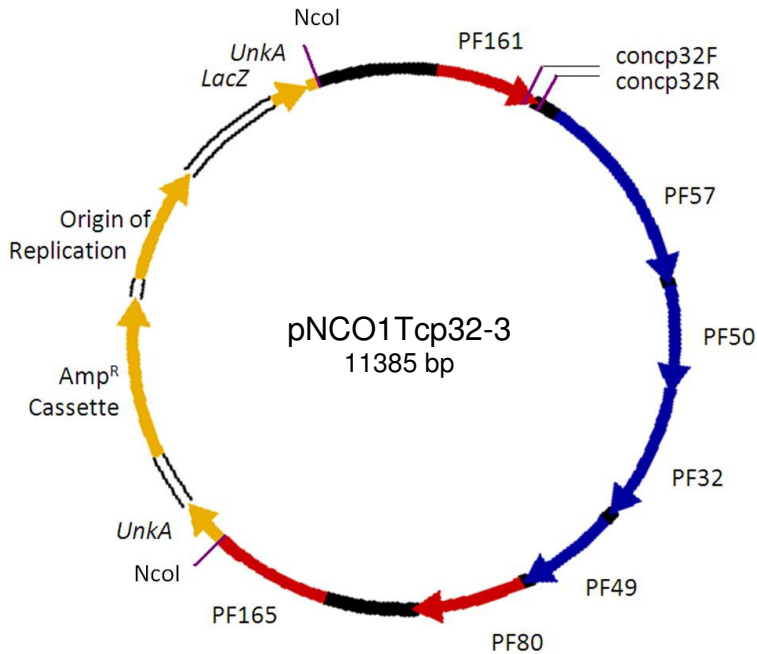


Figure 15: pNCO1Tcp32-3 Vector Composition. The pNCO1Tcp32-3 plasmid is the result of cp32-3 fragment insertion into the *NcoI* site of the pNCO1T plasmid. After the plasmid was introduced into competent *E. coli* cells for amplification, the extracted plasmid was tested via confirmation PCR with concp32F and concp32R primers to confirm the presence of the inserted cp32-3 fragment.

## BLUE VERSUS WHITE TRANSFORMANT SCREENING

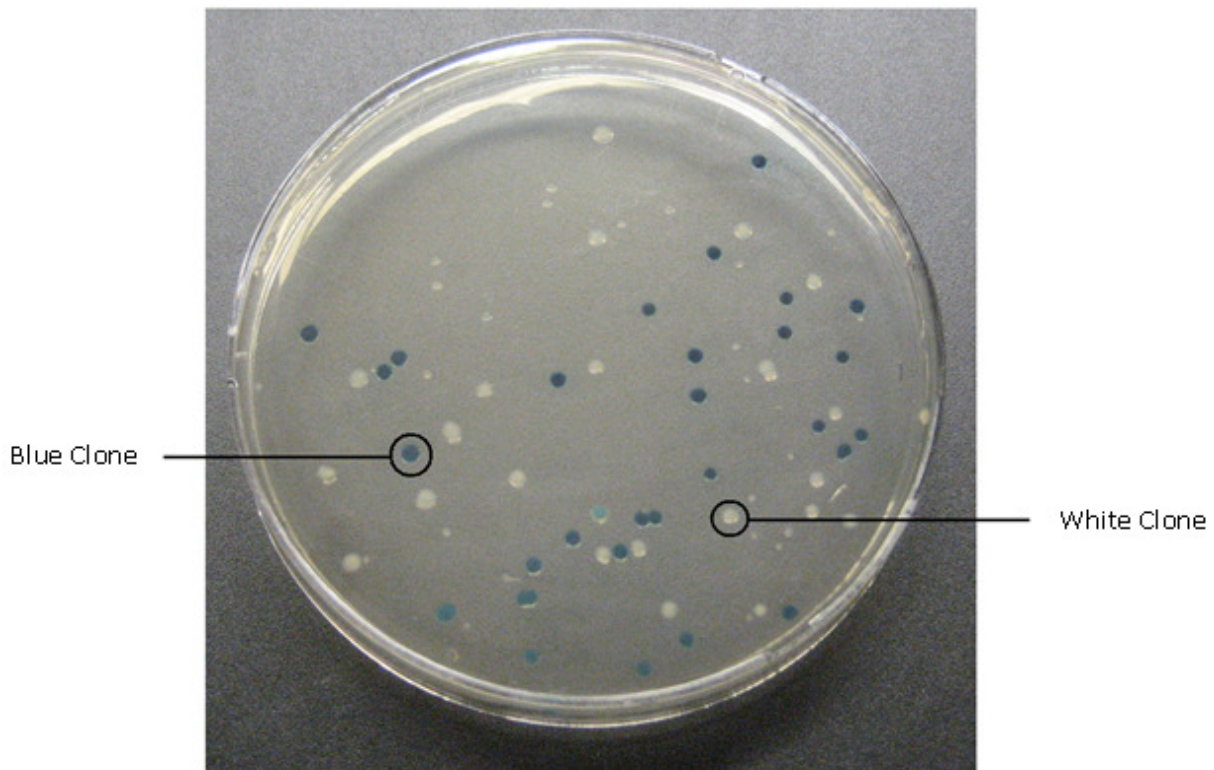


Figure 16: Blue Versus White Transformant Screening. *E. coli* colonies transformed with the pNCO1Tc32-3 were grown on a LB plate supplemented with ampicillin, IPTG, and X-gal. Blue clones are the product of an unsuccessful transformation as they indicate an intact *lacZ*. The white colonies were chosen for further testing to confirm presence of correctly constructed pNCO1Tc32-3 plasmid.

Although the colony color difference suggests successful transformants, confirmation PCR was performed to ensure the presence of the inserted cp32-3 fragment. The plasmid was extracted and fragment presence was confirmed using the confirmation primers concp32F and concp32R (Table 4). The procedure successfully amplified the approximately 500 bp region of DNA located toward the end of the PF161 gene extending toward PF57. The amplified fragment was run through an agarose gel and then compared in size to a DNA ladder. The size of the fragment was also compared to the position of a positive control confirmation PCR of original cp32-3 fragment DNA.

Next, a confirmation digestion reaction was performed by digesting the plasmid with *Nco*I. The two fragments were visualized by agarose gel electrophoresis to confirm successful plasmid construction. The two fragments, the original linear pNCO1T and the cp32-3 fragment, were compared to the DNA ladder and correctly located at approximately 3 kbp and 8.4 kbp respectively (Figure 17).

## **CREATION OF pG22**

The vector pNCO1T can replicate in *E. coli*, but not in *B. burgdorferi*; however, the insertion of the putative maintenance region of cp32-3 may make the vector reproducible in spirochetes. The use of the pNCO1T vector is further complicated because it does not have a selection marker that is available for use in the borrelial system. The vector pBSVGE22 contains a selectable gentamycin acetyltransferase gene that is constantly expressed under a borrelial *FlaB* promoter (Figure 18) (Stewart *et al.*, 2001). It also contains a copy of the *B. burgdorferi* gene *bbe22* that is necessary to restore infectivity to the highly transformable borrelial strain 13A, which has been

## pNCO1Tcp32-3 DIGESTION CONFIRMATION

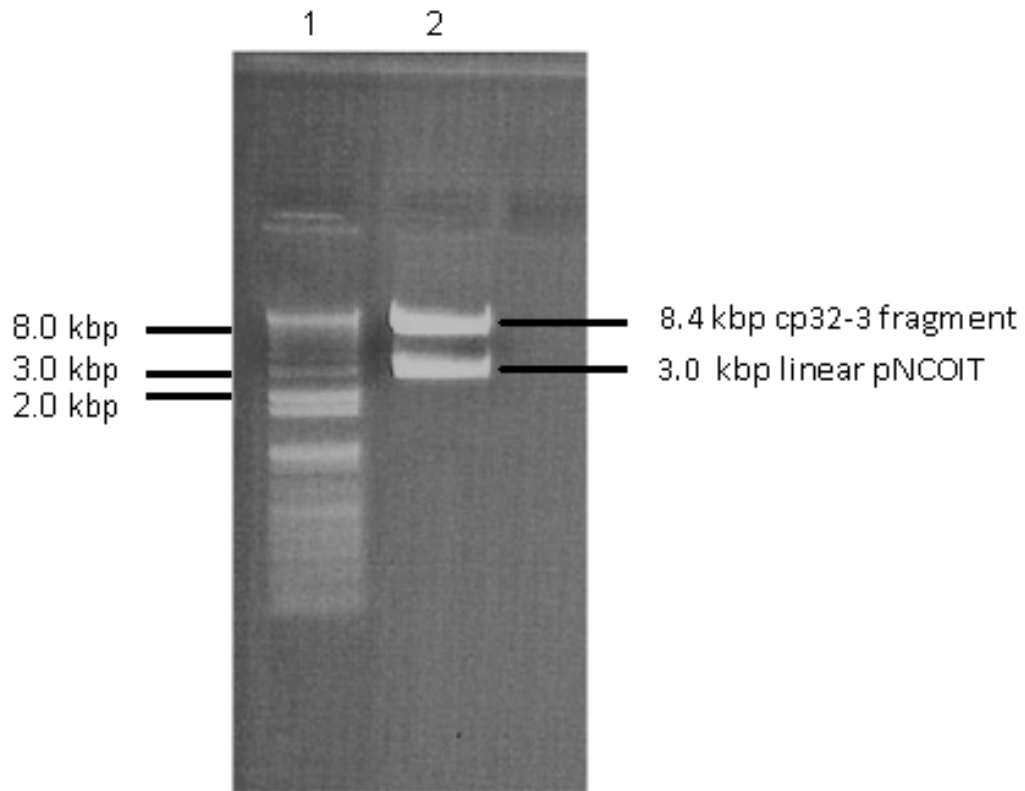


Figure 17: pNCO1Tcp32-3 Digestion Confirmation. The plasmid DNA extracted from *E. coli* after transformation was confirmed by digestion the plasmid with *NcoI*. The resulting fragments at 3 kbp and 8.4 kbp correspond to the original portion of pNcoIT and the cp32-3 fragment, respectively.

Lane 1: 1 kbp DNA Ladder  
Lane 2: pNcoITcp32-3 DNA (*NcoI*)

repeatedly used in our laboratory (Xu *et al.*, 2007a; 2007b). pBSVGE22 also contains the necessary elements to allow for successful replication and maintenance in *E. coli* cells.

As these necessary components only comprise half of the 6409 bp plasmid, the vector was reduced in size as to aid in transformation efficiency. The plasmid DNA was amplified via PCR with Gen32F and Gen32R primers (Table 4) to shorten the plasmid by 3625 bp. The shortened plasmid was run through an agarose gel and compared to a DNA ladder (Figure 20). The fragment was positioned appropriately for its 2784 bp size. This 2784 bp fragment, which retained the origin of replication site, copy of *bbe22*, and the gentamycin acetyltransferase cassette, was designated pG22 when circularized (Figure 19).

### **CREATION OF pG22cp32-3plus**

The pG22 plasmid, in linear form, was digested with *Nco*I to form cohesive ends compatible with the *Nco*I digested 8.4 kbp cp32-3 fragment amplified from pNCO1Tcp32-3. A ligation procedure was used to fuse the two fragments together to form a plasmid of 11.2 kbp in length designated pG22cp32-3plus (Figure 21). The following analyses were conducted to confirm pG22cp32-3plus was constructed as designed after replication and extraction from transformed *E. coli* cells.

First, the confirmation PCR was run using primers amplifying the gentamycin acetyltransferase gene, GenF and GenR (Table 4). The resulting DNA fragment was compared in size to the DNA ladder as well as the location of a positive control obtained using the same primers with pBSVGE22 DNA.

### pBSVGE22 VECTOR COMPOSITION

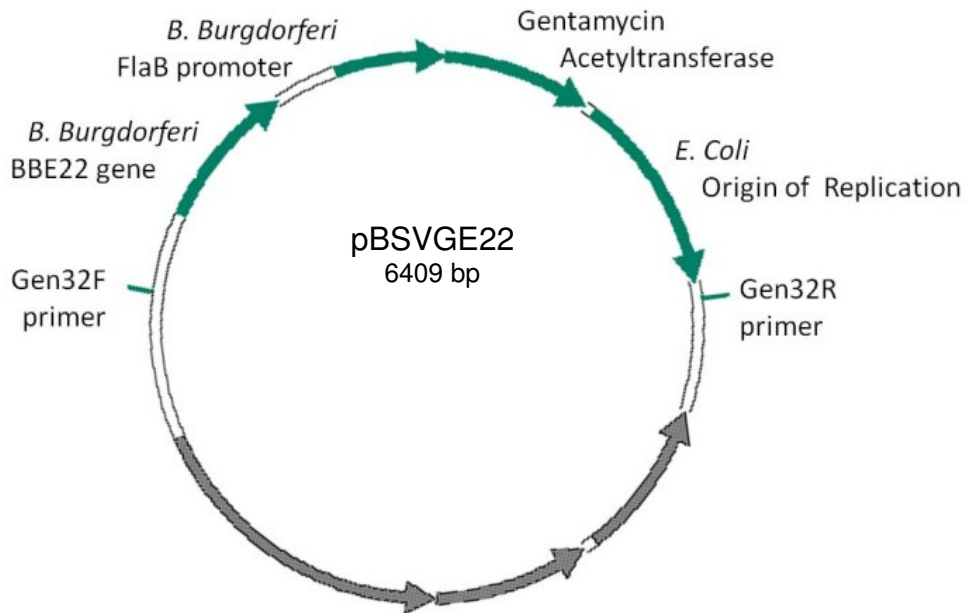


Figure 18: pBSVGE22 Vector Composition. The pBSVGE22 plasmid, at 6409 bp, was reduced in size to aid in transformation efficiency. The plasmid was reduced by amplifying a 2784 bp portion of the plasmid with the primers Gen32F and Gen32R.

### pG22 VECTOR COMPOSITION

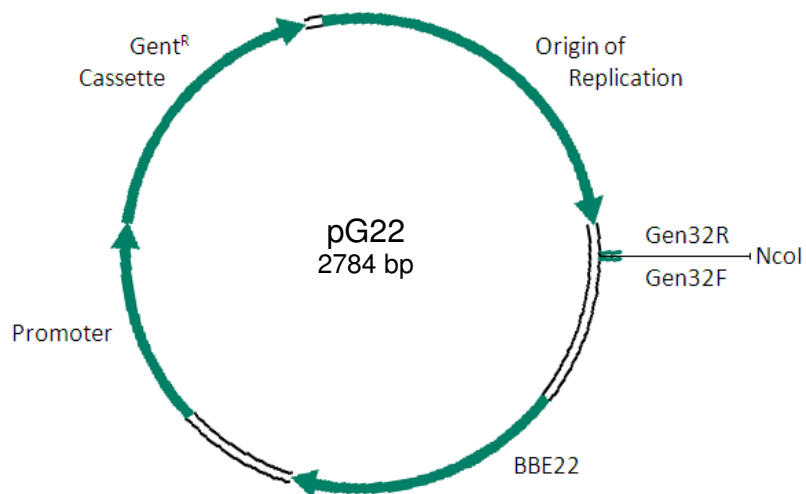


Figure 19: pG22 Vector Composition. pG22 is the 2784 bp product of pBSVGE22 amplification with Gen32F and Gen32R primers. As the amplification process introduced *NcoI* sites at the ends of the fragment, pG22 is ready for the insertion of the cp32-3plus DNA fragment.

## pBSVGE22 PLASMID REDUCTION

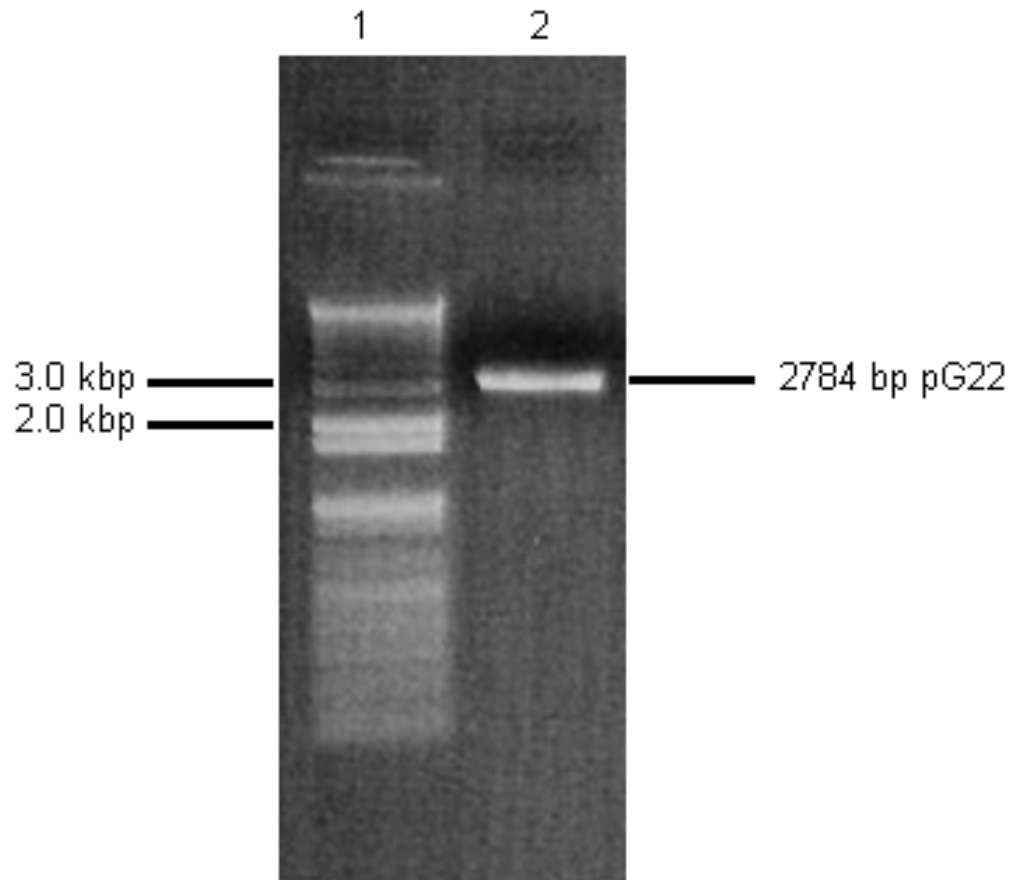


Figure 20: pBSVGE22 Plasmid Reduction. The pBSVGE22 plasmid was amplified with Gen32F and Gen32R primers to shorten the plasmid while retaining the plasmid's necessary function. The resulting plasmid, pG22, is 2784 bp in length and appears as a single band on an agarose gel.

Lane 1: 1kbp DNA Ladder  
Lane 2: pBSVGE22 (Gen32F/Gen32R)



### pG22cp32-3plus PLASMID COMPOSITION

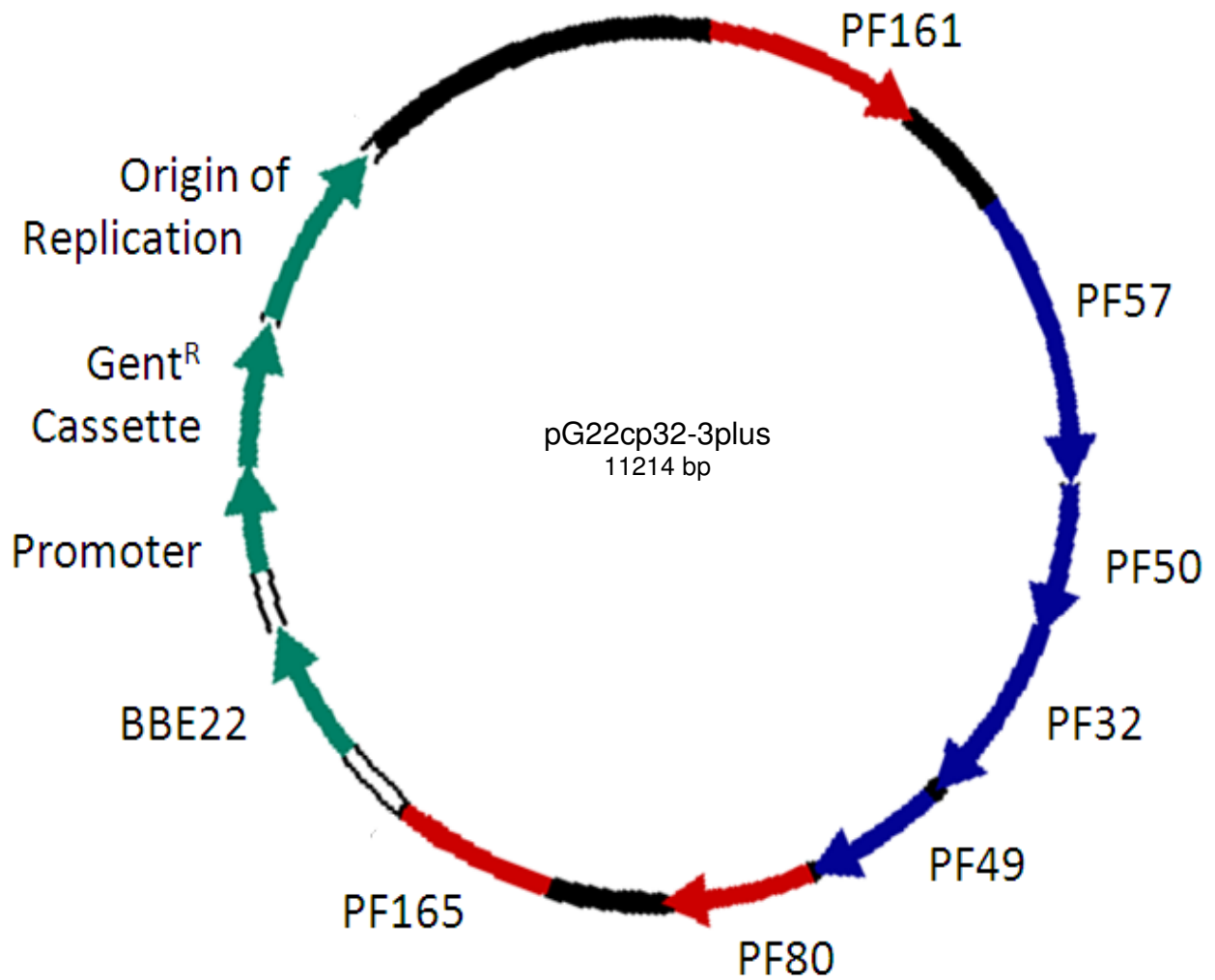


Figure 21: pG22cp32-3plus Plasmid Composition. The pG22cp32-3plus plasmid is the result of ligating the pG22 plasmid with the cp32-3plus fragment obtained from pNCO1Tcp32-3. The 11,214 bp pG22cp32-3plus plasmid will be introduced into *Borrelia* in order to push out the native cp32-3 plasmid.

As a second confirmation step, a second confirmation PCR was performed with confirmation primers to cp32 as described before. Similarly, the resulting DNA fragment was viewed after agarose gel electrophoresis. The fragment was compared to the DNA ladder as well as the positive control PCR fragment obtained from original cp32-3 fragment DNA.

Based on these analyses, the plasmid pG22cp32-3plus contained all necessary components to replicate in both *E. coli* and *B. burgdorferi*. In addition, the plasmid also contained a selectable marker that can be used in both genera of bacteria as well as the cp32-3 DNA fragment of interest. With all these components present, the plasmid is ready for transformation into *Borrelia*.

### **CREATION OF BORRELIA BURGDORFERI TRANSFORMANTS**

The pG22cp32-3plus was introduced into *B. burgdorferi* strain 13A by electroporation. This particular borrelial clone is highly transformable due to the lack of lp25 and lp56, the two plasmids that may carry restriction enzymes (Lawrenz *et al.*, 2002). The plasmid lp25, not lp56, is required for mammalian infection as it carries *bbe22*, a gene that codes for a nicotinamidase essential for survival of *B. burgdorferi* in the mammalian environment (Purser *et al.*, 2003). The copy of the *bbe22* gene present on the pG22cp32-3plus plasmid will restore the bacteria to its naturally infectious state.

Once pG22cp32-3plus was introduced, transformed bacteria were grown under gentamycin selection pressure at 50 mg/μL. The antibiotic added to the media eliminated spirochetes that had not received pG22cp32-3plus. The transformed bacteria were screened for growth using the color change in BSK-H media. Bacterial growth

lowers the media pH changing the color from red-orange to yellow-orange (Figure 22). The tubes containing yellow-orange media were selected for confirmation.

The growing colonies were tested to confirm the presence of the gentamycin acetyltransferase cassette. The confirmation PCR procedure using GenF and GenR primers (Table 4) to rule out false positive transformants due to spontaneous mutation to a gentamycin resistant phenotype (Figure 23). All transformants that tested positive for the cassette are true transformants that had acquired pG22cp32-3plus.

### **cp32-3 MAINTAINED IN PRESENCE OF pG22cp32-3plus**

All transformants that acquired the pG22cp32-3plus plasmid were tested by PCR to determine if the native cp32-3 plasmid was present. The confirmation PCR procedure utilized the BSS41F and BSS41R primers (Table 4). In all cases, cp32-3 was present (Figure 24).

To afford the bacteria enough time for the vector pG22cp32-3plus to displace the cp32-3 plasmid, the cultures were maintained in fresh media supplemented with 50 mg/ $\mu$ L of gentamycin for an additional two weeks. After this period of growth, the colonies were serially diluted into 96-well plates to produce single clones.

The 14 clones were again visually screened for growth by observing media color change. The growing spirochetes were again tested for the presence of the gentamycin acetyltransferase cassette using confirmation PCR using the GenF and GenR primers (Table 4). Confirmation PCR was also employed to screen the transformants for the presence of the cp32-3 plasmid as described above. While all of the 14 clones still possessed the pG22cp32-3plus plasmid, the bacteria also maintained the cp32-3 plasmid.

## MEDIA COLOR CHANGE INDICATES GROWTH

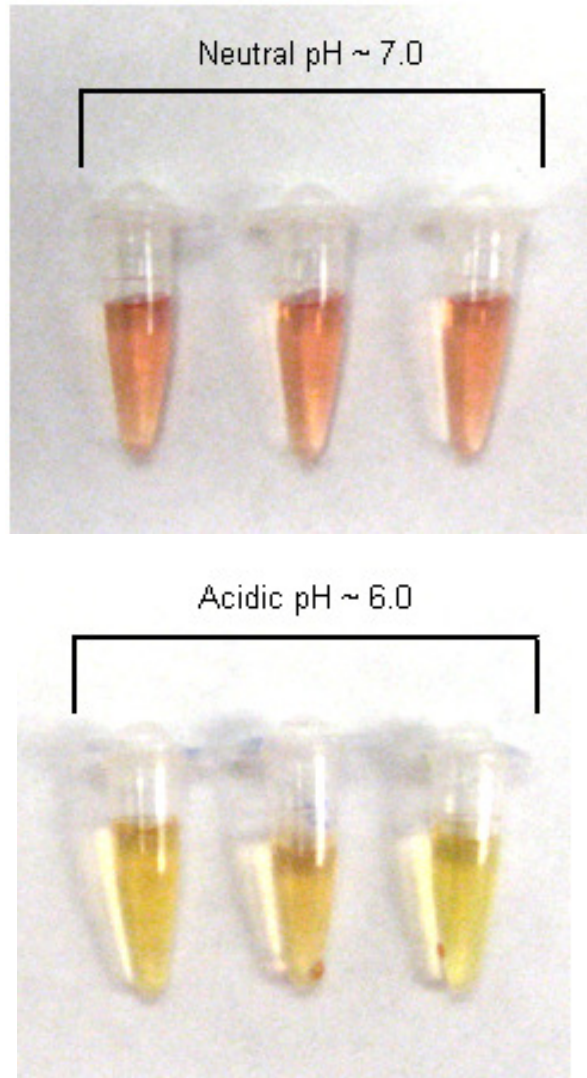


Figure 22: Media Color Change Indicates Growth. Spirochetal growth is indicated by a color change in the BSK-H media when the pH is lowered. The phenol red indicator dye changes from red-orange color (shown at right) indicating a pH of approximately 7.0 to yellow-orange color (shown at left) indicating a pH of approximately 6.0.

## PCR SCREENING FOR pG22cp32-3plus TRANSFORMANTS

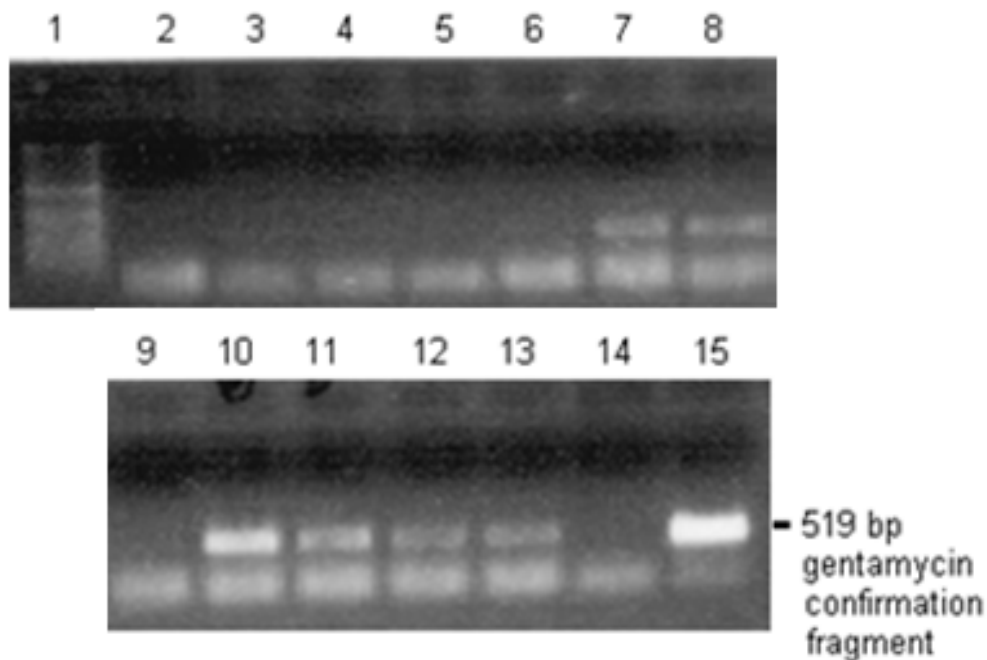


Figure 23: PCR Screening For pG22cp32-3plus Transformants. Borrelial cells transformed with pG22cp32-3plus must be tested to confirm the presence of the gentamycin acetyltransferase cassette to ensure true transformants. The cassette presence was confirmed via PCR with GenF and GenR primers. False positive transformants due to spontaneous mutation were ruled out and excluded when the band of DNA representing the 519 bp GenF/GenR fragment did not appear on the agarose gel (lanes 2-6 and 9).

Lane 1: 1 kbp DNA Ladder  
Lanes 2-13: *B. burgdorferi* A13 pG22cp32-3plus (GenF/GenR)  
Lane 14: sterile autoclaved ddH<sub>2</sub>O (GenF/GenR)  
Lane 15: pG22cp32-3plus (GenF/GenR)

## PCR SCREENING FOR cp32-3 PLASMIDS

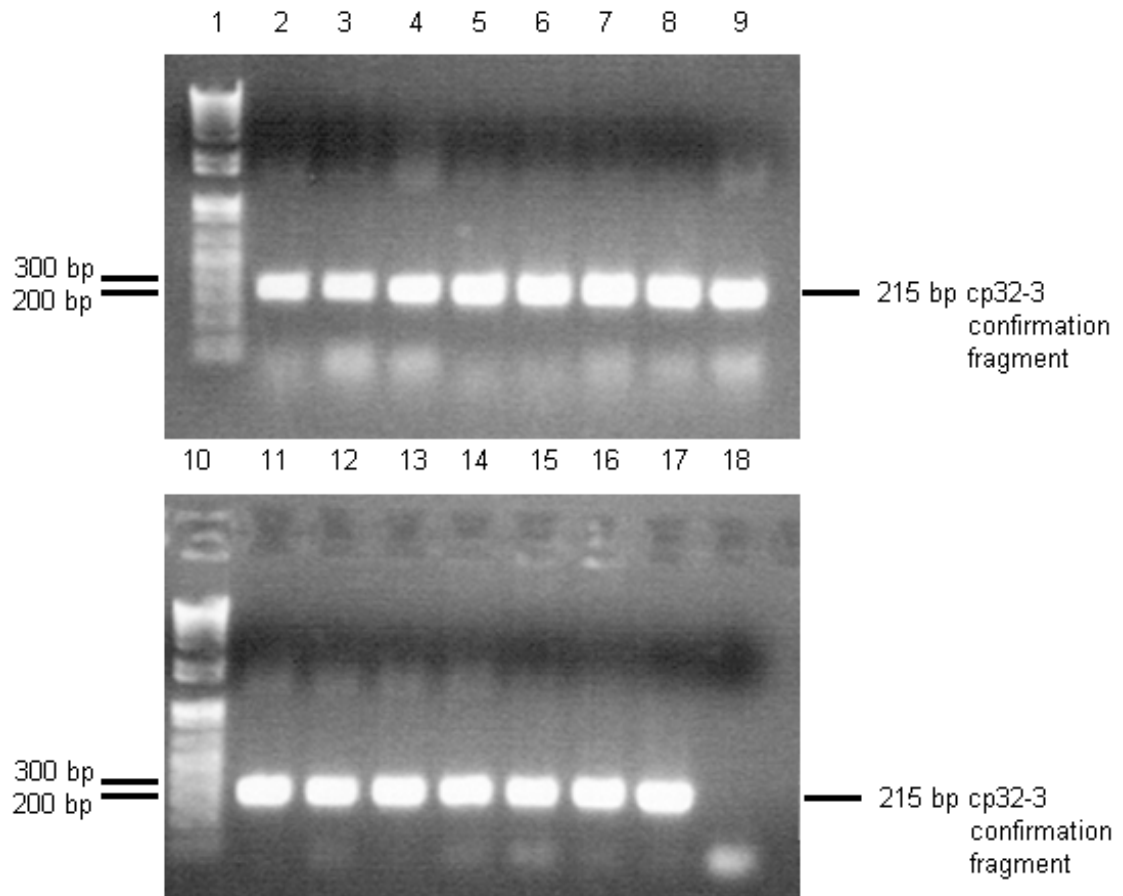


Figure 24: PCR Screening for cp32-3 Plasmids. Single clones of *Borrelia* transformants with pG22cp32-3plus grown in 50 µg/mL of gentamycin. The cultures were tested for the presence of the native cp32-3 plasmid. In all cases the bacteria had eliminated the plasmid.

Lane 1 & 10: 1kbp DNA Ladder  
Lanes 2-9 & 11-16: *B. burgdorferi* A13 pG22cp32-3plus (BBS41F/BBS41R)  
Lane 17: *B. burgdorferi* 5A4 (BBS41F/BBS41R)  
Lane 18: sterile autoclaved ddH<sub>2</sub>O (BBS41F/BBS41R)

## **INCREASED SELECTION PRESSURE FORCES cp32-3 EXCLUSION**

In an attempt to force the plasmid to selectively exclude the cp32-3 plasmid, the transformants were subjected to increased selection pressure in the form of varying and increasing gentamycin concentrations of 300 mg/ $\mu$ L to 1400 mg/ $\mu$ L. With this additional pressure, cultures were monitored for growth. Cultures grown at 500 mg/mL were allowed to incubate for four days in media containing 550 mg/mL of gentamycin.

At the conclusion of this growth period the culture was divided and the gentamycin concentration was increased to 750 mg/mL and 950 mg/mL. The culture was allowed to grow to 100 spirochetes per field of view in media supplemented with 50 mg/mL of gentamycin. The cultures were serially diluted into 96 well plates to select single clones. Single clones were selected visually using the color change in the media produced by growing bacteria.

In the same manor as before, the growing transformants were tested to confirm the presence of the introduced plasmid (Figure 25). After the pG22cp32-3plus plasmid presence was confirmed, the transformants were tested for the presence of the cp32-3 plasmid. In all cases the confirmation PCR performed with BBS41F and BBS41R primers (Table 4) showed the cp32-3 had indeed been lost in all of 17 clones examined (Figure 26). As an additional precautionary measure, the transformants were subjected to yet another round confirmation PCRs. The PCRs were also performed with primers specific to cp32-4, cp32-6, cp32-7, cp32-9 plasmids. Results confirmed the presence of all cp32 plasmids, except for cp32-3 (Figure 27).

## PCR CONFIRMATION OF pG22cp32-3plus PLASMID PRESENCE

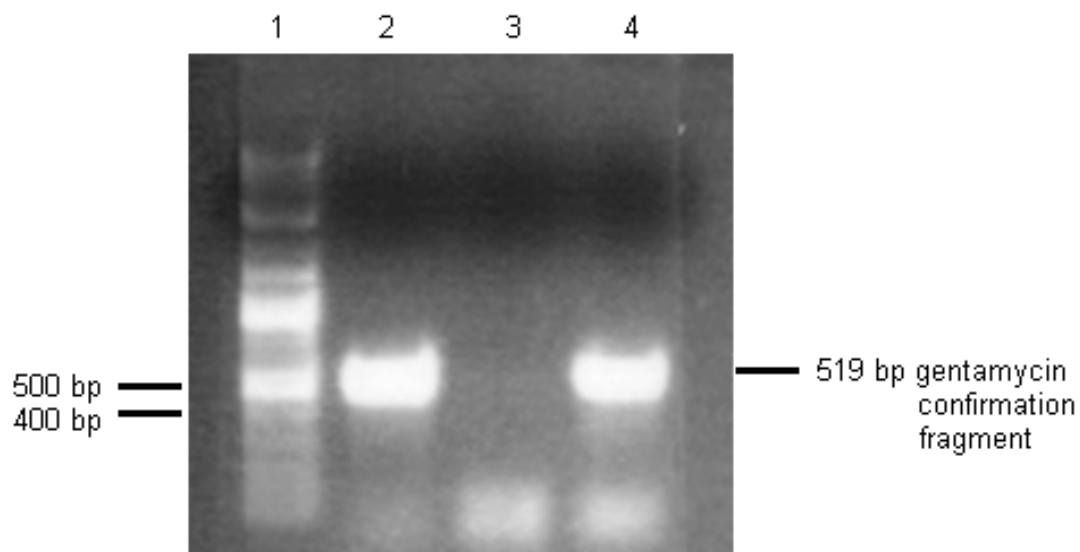


Figure 25: PCR Confirmation of pG22cp32-3plus Presence. Single clones of borrelial pG22cp32-3plus transformants grown in high concentrations of gentamycin were tested via PCR for the presence of the gentamycin acetyltransferase cassette using GenF and GenR primers.

Lane 1: 1kbp DNA Ladder  
Lanes 2: *B. burgdorferi* A13 pG22cp32-3plus (GenF/GenR)  
Lane 3: sterile autoclaved ddH<sub>2</sub>O (GenF/GenR)  
Lane 4: pG22cp32-3plus (GenF/GenR)



**PCR SCREENING FOR cp32-3 PLASMIDS  
AFTER INCREASED ANTIBIOTIC SELECTION PRESSURE**

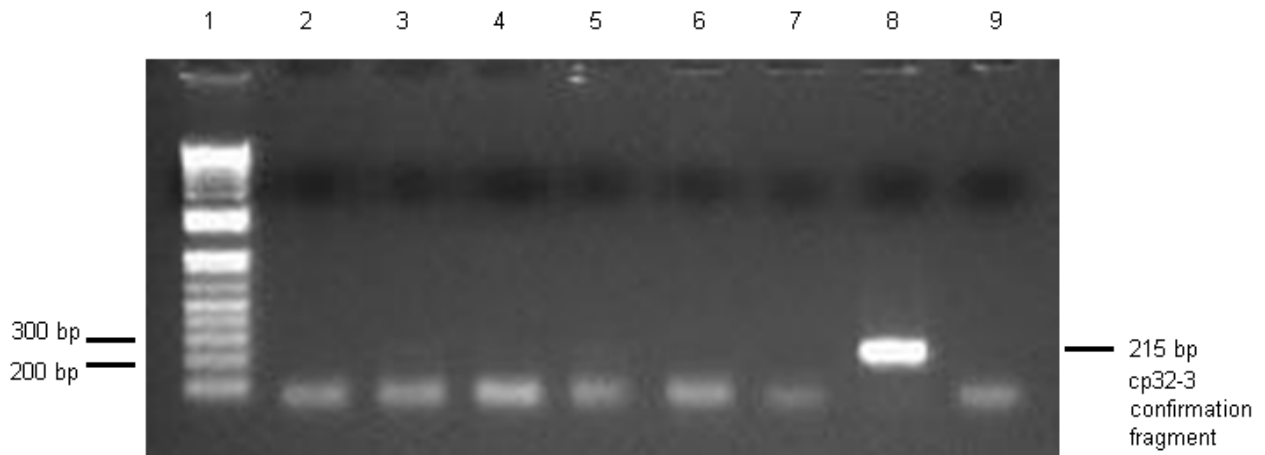


Figure 26: PCR Screening for cp32-3 Plasmids After Increased Antibiotic Selection Pressure. Single clones of borrelial pG22cp32-3plus transformants grown in high concentrations of gentamycin were tested for the presence of the cp32-3 plasmid. In all cases the bacteria had eliminated the plasmid.

Lane 1: 1 kbp DNA Ladder  
Lanes 2-6: *B. burgdorferi* A13 pGE22cp32-3plus (BBS41F/BBS41R)  
Lane 7: *B. burgdorferi* 5A4 (BBS41F/BBS41R)  
Lane 8: sterile autoclaved ddH<sub>2</sub>O (BBS41F/BBS41R)

**PCR SCREENING FOR cp32 PLASMIDS  
AFTER INCREASED ANTIBIOTIC SELECTION PRESSURE**

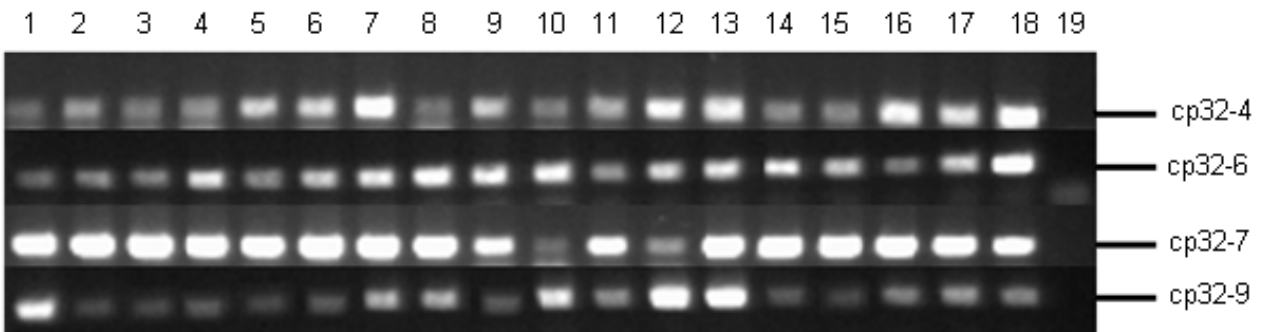


Figure 27: PCR Screening for cp32 Plasmids After Increased Antibiotic Selection Pressure. Single clones of borreliac pG22cp32-3plus transformants grown in high concentrations of gentamycin were tested for the presence of the cp32-3 plasmid. In all cases the bacteria had retained cp32-4, cp32-6, cp32-7, and cp32-9 plasmids.

Lanes 1-17: *B. burgdorferi* A13 pG22cp32-3plus

Lane 18: *B. burgdorferi* 5A4

Lane 19: sterile autoclaved ddH<sub>2</sub>O

## DISCUSSION

Lyme disease has been the most commonly reported arthropod disease in the United States for the last 23 years (Centers for Disease Control and Prevention, 1985). Since reporting began in 1980, the number of disease cases has dramatically increased. In 2005 there were 23,305 cases reported in 45 different states making the incidence of Lyme disease in the United States 7.9 cases per 100,000 (Centers for Disease Control and Prevention, 2007).

The disease produces a wide range of symptoms that can debilitate infected persons overtime, especially if the infection is allowed to progress. The onset of the infection produces flu-like symptoms usually accompanied by an erythema migrans rash (Steere *et al.*, 1983; Centers for Disease Control and Prevention, 1997). When left untreated, the disease produces more serious symptoms including: joint pain, fatigue, aching neck, numbness of extremities, facial palsy, headaches, and arthritis. The bacterial infection can also produce detrimental effects to the heart and central nervous system (Steere, 2001; Agüero-Rosenfeld *et al.*, 2005). The increased number of cases, as well as the severity of disease symptoms, validates the need to study Lyme disease's causative agent, *Borrelia burgdorferi*.

Casjens *et al* (2000) completed the genomic work by Fraser *et al.* (1997) to produce the full compilation of the *Borrelia* genome. The bacteria have an unusual segmented genome comprised of a single linear chromosome complemented with up to 21 extrachromosomal plasmids. As the chromosome is 910 kbp in length, the extrachromosomal DNA contains 610,694 bp and makes up roughly 40% of the entire borrelial genome (Fraser *et al.*, 1997; Casjens *et al.*, 2000) .

The extrachromosomal DNA or plasmids are particularly interesting as they may encode for genes required by the bacteria; as a result the plasmids are faithfully maintained (Purser and Norris, 2000). Four of the bacteria's plasmids, lp28-1, lp25, lp56, and cp26, are recognized as required and necessary. Although the bacteria can lose lp28-1 spontaneously, the plasmid is required to be present to produce a persistent infection because of the *vlsE* region that gives the bacteria antigenic variation to a surface exposed protein (Purser and Norris, 2000; Labandeira-Rey and Skare, 2001). The lp25 plasmid is required for borrelial survival in the mouse due to the *pncA* gene that provides a critical step in synthesizing NAD for cellular energy (Purser and Norris, 2000; Purser *et al.*, 2003,).

Plasmid lp56 is required for cellular survival for several reasons. The plasmid contains the genes to encode for OspA, OspB, DbpA, DbpB, and CRASP-1. The proteins OspA and OspB aid the bacteria in tick colonization while DbpA and DbpB facilitate the bacteria with colonization and dissemination and mediate attachment to host tissue (Guo *et al.*, 1995; Fischer *et al.*, 2003; Ojaimi *et al.*, 2003). The CRASP-1 protein assists *Borrelia* in avoiding complement-mediated killing (Kraiczky *et al.*, 2004). The circular plasmid cp26 has been shown to be essential to *B. burgdorferi* bacterial viability (Byram *et al.*, 2004). The cp26 plasmids encodes for *ospC* and *resT* genes which produce proteins that help the spirochete adhere to tissue and resolve circular dimmers, respectively (Kobryn and Chaconas, 2002; Caimano *et al.*, 2004).

As part of the bacteria's extrachromosomal plasmids, a single bacterium can maintain up to seven different cp32 plasmids (Casjens *et al.*, 1997, Casjens *et al.*, 2000). These plasmids represent almost 20% of bacterial DNA. The nine plasmids that

make up the cp32 family are each roughly 30 kbp in length and have 80% similarity between them (Casjens *et al.*, 2000). By characterizing natural isolates of the bacteria, it was determined that the plasmids are well maintained during *in vitro* cultivation (Purser and Norris, 2000). No characterized isolate exists with all cp32 plasmids missing from its repertoire (Purser and Norris, 2000). This information suggests that at least some of the plasmids provide genetic information necessary for spirochetal survival in nature (Casjens, et al. 1997; Purser and Norris, 2000).

At present, relatively little is known about the true function of the majority of the cp32 plasmids' genes (Casjens *et al.*, 2000). Through research, three distinct hypervariable regions have been identified on each cp32 plasmid. The first hypervariable region, the putative plasmid maintenance region, contains the paralogous gene families, PF57, PF50, PF32, and PF49 that responsible for the cp32 family's replication and segregation (Casjens *et al.*, 2000; Stevenson and Akins, 2000; Eggers *et al.*, 2002). The second hypervariable region contains the *erp* locus that contain genes for the OspE and OspF surface exposed lipoproteins used to help the bacteria evade complement mediated killing (Stevenson *et al.*, 1995; Kraiczy *et al.*, 2001; Stevenson *et al.*, 2002; Kraiczy *et al.*, 2004).

The final hypervariable region, the 2.9 loci contains genes that encode the antigenic Mlp proteins that may assist in interacting with host tissues (Porcella, et al. 1996, Theisen 1996, Yang, et al. 1999). Most of the 2.9 loci also contain a *bdr* gene for proteins that potentially play a role in cellular regulation (Meyer and Barbour, 1999; Zückert *et al.*, 1999; Roberts *et al.*, 2000; Stevenson and Akins, 2000). Instead of a *bdr*

gene, cp32-1 and cp32-6 encode a *rev* gene to provide the bacteria survival advantages in diverse environments (Gilmore and Mbow, 1998; Stevenson and Akins, 2000).

Outside of the hypervariable regions the cp32 plasmids similarity increases dramatically to between 80-99% similar (Stevenson and Akins, 2000; Casjens *et al.*, 2000). Throughout the almost identical stretches of DNA, only one region of DNA has been identified and characterized (Stevenson and Akins, 2000). This region, similar amongst all cp32 plasmids, contains genes for the BlyA and BlyB pore-forming toxins that are involved in the cellular release of bacteriophage particles (Guina and Oliver 1997; Eggers and Samuels, 1999; Stevenson and Akins, 2000; Eggers *et al.*, 2001). Other than these four regions of DNA, little is known about the genes encoded on the cp32 plasmids, including whether the plasmids play a role in mammalian infection.

Studying the function of the cp32 plasmids by analyzing existing natural isolates is not possible because no isolate has been discovered lacking all members of the cp32 family. Since every isolate does carry some combination of the plasmids and the plasmids are difficult to knockout, another approach to study the plasmid family must be developed. Consequently, we have designed a process that artificially displaces the plasmids by utilizing the hypervariable region responsible for plasmid replication and segregation to construct a plasmid that is incompatible with its cp32 counterpart. Once introduced, the constructed plasmid will force the bacteria to lose its corresponding plasmid. The transformed bacteria can be used to gain insight into *Borrelia's* potential requirement for the plasmids as well as the subsequent role they play in infectivity or pathogenicity.

In addition to the four genes from the replication and maintenance region, PF57, PF50, PF32, and PF49, the constructed plasmid contains several other important components. The borrelial BBE22 gene is present to restore the transformable 5A13 strain of *B. burgdorferi* back to a naturally infectious state. A gentamycin acetyltransferase gene under the constitutively expressed borrelial *flaB* promoter is also located on the plasmid to serve as a selection marker for transformed bacteria.

After confirming proper construction, the pG22cp32-3plus plasmid was introduced into *Borrelia* 5A13. Ultimately, selection pressure in the form of high concentrations of gentamycin forced the bacteria to eliminate cp32-3 plasmid. The transformed bacteria were tested to confirm plasmid depletion.

Further studies should exploit the same plasmid construction process employed by this experiment to create constructs corresponding to every cp32 family member. Both individually and in combinations, these plasmids transformed into *Borrelia* will produce a multitude of isolates for studies to ascertain if the plasmids confer any advantage to the bacteria during the infectious process.

The borrelial transformants should be introduced into an established mammalian model to study the potential difference in infectivity and pathogenicity. The mouse model currently in use by the Liang lab is such a model that will allow the determination of the difference between the transformants with different cp32 plasmid contents (Xu *et al.*, 2005; Xu *et al.*, 2006). The model requires intradermal injections of the transformants into both wild-type and SCID mice.

To study infectivity, inoculated mice are sacrificed after 2 to 3 weeks. At the time of sacrifice, tissues from the mice are collected and cultured. The cultures are then

visually examined using dark-field microscopy for the presence of borrelial cells. If spirochetal cells are present the transformant is in fact infectious.

For information regarding the transformants pathogenicity, mice, two weeks postinoculation, are visually examined every other day for the development of arthritis. The mice are sacrificed at one month postinoculation and tissues are collected. The tissues are then prepared for analysis with quantitative polymerase chain reactions for the presence of *B. burgdorferi* DNA. The severity of pathogenicity is assessed by the degree of arthritis present compared to the quantity of borrelial DNA.

Ultimately, identifying plasmids that are associated with infectivity and pathogenicity is the first step to determining the location of *Borrelia's* essential genes. The information gathered from the experiments outlined above can only provide evidence for *B. burgdorferi's* requirement for the cp32 plasmid. Since these plasmids are extremely similar throughout their length, it is hypothesized that at least some of the cp32 plasmids will be required by the bacteria. If in fact a cp32 plasmid is required for infectivity, pathogenicity, or viability of the organism, the cp32 plasmids that are most closely related, such as cp32-3 and cp32-7, might serve the same function in the organism. In this case, the plasmids could be similar enough such that either plasmid could produce a strain of *Borrelia* with the same phenotype.

The previously mentioned experiments can determine the bacteria's requirement for the cp32 plasmid family. To further explore what gene or genes present on the cp32 plasmids are required by *B. burgdorferi*, more specific experiments must be performed. It will be through those experiments that the true reason for the plasmids presence will be discovered and the link to infectivity, pathogenicity, or viability be revealed.



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## VITA

Amanda Paige DeRouen Polito was born to Gordon Ray and Karen Labbe DeRouen in Lafayette, Louisiana, on November 19, 1982. Her family moved to Baton Rouge when she was a young girl. Several years later, she became a big sister to Lauren DeRouen. Although she attended several schools along the way, she graduated from Saint Joseph's Academy in May of 2001. That summer she began her college career at Louisiana State University to pursue a Bachelor of Science. Four years later, in May of 2005, she earned her degree, majoring in biochemistry with a minor in chemistry. The following fall Amanda began graduate school in the Department of Pathobiological Sciences at the Louisiana State University School of Veterinary Medicine. On August 19, 2006 Amanda married Thomas Anthony Polito, also of Baton Rouge, Louisiana. Almost one year later, Amanda gave birth to their first child, Riley Anthony Polito, on August 14, 2007. She plans to graduate on December 21, 2007, with a Master of Science degree in veterinary medical science.