RESPONSES TO ENVIRONMENTAL STRESS TRIGGER DIFFERENTIAL EXPRESSION AND CELLULAR DAMAGE IN *BORRELIA BURGDORFERI*

by

JENNIFER S. TREGLOWN

(Under the Direction of Frank C. Gherardini & Timothy R. Hoover)

ABSTRACT

Regulation of coordinated gene expression is required for survival of *Borrelia burgdorferi* in different hosts. The sigma factor RpoS is involved in the regulation of gene products needed for stationary phase response. Analysis of the upstream promoter region of *rpoS* revealed a putative σ^{54}-dependent promoter, which was confirmed to be required for expression of *rpoS* as the cultures entered stationary phase. Interestingly, QRT-PCR and immunoblots indicated the presence of a second promoter that was independent of σ^{54}. 5'RACE and RT-PCR linkage experiments suggested that the second promoter for *rpoS* was located in *flgI*, the gene immediately upstream of *rpoS*. Primer extension mapped this σ^{54}-independent promoter to 13 bp from the 3'-end of *flgI*.

σ^{54}-dependent transcription requires an activator protein to stimulate open complex formation. Analysis of the *B. burgdorferi* genome revealed a two-component system consisting of a putative σ^{54}-activator (SisR) and the putative cognate histidine kinase (SisK) located directly upstream of SisR. QRT-PCR showed that the histidine kinase is required for the maximum

expression of *rpoS* during stationary phase. In addition, phosphorylation assays demonstrated that SisK and SisR are part of the same two-component system, suggesting that they are necessary for σ^{54}-dependent transcription.

Reactive oxygen species (ROS) are encountered in the tick vector and the mammalian host therefore, *B. burgdorferi* possesses proteins necessary to detoxify these compounds. NapA was first identified as a Dps/Dpr homolog and initial studies showed that expression increased when cells are grown at high levels of oxygen (> 12%) or when anaerobically grown cells are exposed to ROS. NapA complemented an *E.coli ahpCF* mutant when exposed to *t*-butyl hydrogen peroxide, suggesting alkyl hydroperoxide reductase activity, however this may not be the primary function of the enzyme.

Immunoelectron microscopy revealed that anaerobic *B. burgdorferi* cultures exposed to ROS develop membrane blebs around the spirochete. Lipid analysis showed the levels of linoleic acid decreased when the cultures were treated with ROS, suggesting that the major target for damage caused by ROS in *B. burgdorferi* may be lipids, rather than proteins or DNA as occurs in most bacteria.

INDEX WORDS: Lyme disease, *Borrelia burgdorferi*, *rpoS*, σ^S, σ^{54}, differential expression, SisR, SisK, two-component system, oxidative stress, NapA, lipid peroxidation

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A Dissertation Submitted to the Graduate Faculty of the University of Georgia in Partial Fulfillment of the Requirements for the Degree

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Major Professor:	Timothy R. Hoover
Committee:	Duncan Krause
	Margie Lee
	John Maurer
	Rob Maier

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
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DEDICATION

To my parents, Susan and Glenn Treglown, for all of their love and support throughout the years.

I could not have done it without you. All my love and respect.

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Introduction and Literature Review

Introduction

Lyme disease is the most common arthropod-borne disease in the United States and Europe and has a wide spectrum of clinical manifestations ranging from localized infection to systemic disease (3). The first record of Lyme disease dates back to the 1880s, transmission by an infected *Ixodes* tick was proposed in 1909 by Dr. Afzelius, who described an expanding, ringlike rash known as erythema migrans (EM) (7, 8). In 1975, Steere *et al* investigated an usual cluster of non-rheumatoid pediatric arthritis in the small Connecticut town of Lyme and named it Lyme arthritis (276, 278). Subsequently in 1979, it was renamed Lyme disease when additional symptoms associated with neurological problems and severe fatigue were described (272, 275, 278). Currently, Lyme disease presents as a multi-system disorder which is contracted through the bite of an *Ixodes* tick (2, 243). Early symptoms include EMs, limb pain, flu-like symptoms, swollen lymph nodes, peripheral neuropathy and lymphocytic meningitis (7, 173, 192, 243, 300). If untreated, these early symptoms resolve and late Lyme disease begins after a latent period which is defined as the onset of chronic arthritis and infection of the central nervous system (39, 101, 173, 300).

In the early 1980s, Burgdorfer and Barbour discovered the causative agent of the disease when a previously unidentified spirochete was isolated from the midgut of an *Ixodes* tick (57). Serum from patients with Lyme disease also was found to contain antibodies to this agent (23).

Based on DNA relatedness, morphology, and physiology the new spirochete was placed in the genus *Borrelia* and given the species designation of *burgdorferi* after definitive identification by Dr. Willy Burgdorfer (137). Three genospecies of *Borrelia* have been shown to cause Lyme disease in the United States and Europe; ***B. burgdorferi*** sensu stricto, ***Borrelia afzelii*** and ***Borrelia garinii*** (20, 21, 137, 219).

Lyme Disease Manifestations

Lyme disease is a multisystem disease that can be broken into two distinct stages of clinical manifestations, early and late Lyme disease. Early Lyme disease occurs within days to a few weeks following the bite of an infected tick and can remain localized to the initial site of infection or disseminate to multiple sites (40, 192, 270). One of the earliest symptoms and distinguishable characteristics of Lyme disease is the development of an expanding rash known as erythema migrans (EM). EM occurs in approximately 60-80% of infected individuals and arises as the spirochete disseminates under the skin from the site of the infected tick bite (7, 39, 40, 243, 270, 276). The cutaneous lesion usually develops within one week of the tick bite and appears either as a solid or blotchy red expanding rash, or a clear central spot of skin surrounded by expanding circular red rash resembling a bull's eye (7, 39, 40, 243, 270, 276). It can be warm to the touch, but is not associated with itching or any pain (7, 39, 40, 243, 270, 276). This is one of the major reasons why the rash can go undetected if located on areas of the body that are out of sight. The rash, when present, can range in size from a few centimeters to as large as 40 centimeters in diameter and if untreated will persist for three to five weeks at which time it will spontaneously resolve (7, 39, 40, 243, 270, 276). However, with antibiotic treatment, the lesion will resolve within days (41, 274).

As the infection spreads, the second phase of early Lyme disease begins. Secondary annular lesions can develop as the bacteria disseminate and often appear irregular in shape (192). Other symptoms include joint pain, chills, fever, vomiting, fatigue, loss of appetite, and headaches (40, 180). These flu-like symptoms are often not attributed to Lyme disease if the patient does not develop the EM. The bacteria spread throughout the body via the bloodstream and lymphatic system to the brain, meninges, myocardium, retina, muscle, liver, spleen, bone, and joints (40, 88, 268, 270). In 15-20% of untreated individuals neurological complications will develop (78, 192, 269). The most common symptoms that occur during dissemination include meningitis, radiculoneuritis, facial palsy similar to Bell's palsy, tingling or numbness in extremities, swollen lymph glands, peripheral neuropathy, and lymphocytic meningitis (78, 192, 269).

Cardiac complications are rare and appear in only 4-8 % of Lyme disease patients. The most common of these are fluctuating degrees of atrioventricular blockage, ranging from mild first-degree heart blockage to complete heart blockage and myocarditis, which typically occur within 1 to 3 months after the initial infection (5, 40, 213). Without treatment, cardiac involvement is typically brief and will resolve within months (213).

Late Lyme disease occurs within weeks, months, or as late as a year from the initial infection and is a chronic disorder that cycles through periods of symptoms and remission (40, 84, 192). The most common of these symptoms is intermittent attacks of large joint arthritis (knees, tendons, bursae) (40, 84, 192). Lyme arthritis is described as severe swelling with pain and stiffness of one or both knees and can last for a few hours to several days (163, 272). Other symptoms include disabling neurologic disorders (disorientation, depression, mood swings, sleep disorder, dementia, confusion, dizziness and memory loss), encephalitis, cranial neuropathy,

unilateral or bilateral radiculopathy, lymphocytosis, and pleocytosis in the cerebral spinal fluid in addition to the demyelinated polyneuropathy (40, 78, 84, 192).

Chronic Lyme arthritis occurs in approximately 11% of untreated individuals with EMs and is defined as one or more years of continual joint inflammation (106, 279). Patients with chronic Lyme arthritis have synovial lesions containing deposits of fibrin, an increased number of mononuclear cells, elevated levels of collagenase, and the production of interleukin-1 and prostaglandin E_2 (109, 245, 263). Some patients may be genetically predisposed to chronic Lyme arthritis due to an increased frequency of the major histocompatibility complex (MHC) class II alleles human lymphocyte antigen DR (HLA-DR) HLA-DR4 & HLA-DR2 (106, 142, 271). In 1990, Steere *et al* published a study involving 80 patients with Lyme arthritis, where 89% of those with chronic Lyme arthritis had HLA-DR4, HLA-DR2, or both, compared to only 27% of patients with acute forms of arthritis (271). This study also showed that 22 of 38 patients with the HLA-DR4 allele suffered from persistent arthritis even after antibiotic treatment, even though no spirochetes were detected in their joints (271).

This higher incidence of HLA-DR4 associated with continual arthritis following antibiotic treatment was termed antibiotic-treatment resistant Lyme arthritis and has lead to further studies (271). Gross *et al* investigated an autoimmune etiology and found T cells from patients with treatment-resistant Lyme arthritis preferentially recognized outer surface protein A (OspA) of *B. burgdorferi* sensu stricto (106). The immunodominant epitope was identified to be $OspA_{165\text{-}173}$ which has significant homology to human leukocyte function-associated antigen-1 (hLFA-1), suggesting that cross-reactivity may be involved in chronic forms of the disease (106). It is hypothesized that OspA initiates an inflammatory response in the joints by recruiting OspA reactive T_H1 cells (106). These cells stimulate the release of INF-γ, which increases expression

of ICAM-1 on synoviocytes and nonprofessional antigen-presenting cells (APCs). This in turn leads to the recruitment of hLFA-1 expressing T_H1 cells, that will be processed and presented by synovial cells and macrophages with MHC class II to further stimulate the OspA reactive T cells (106). The stimulated T cells will target the synovial cells for destruction, beginning a brutal cycle of inflammation and stimulation, which will continue even after the elimination of the spirochete.

Late skin manifestations such as acrodermatitis chronica atrophicans, morphea, granulome annulare and Parry-Romber syndrome have been associated with Lyme disease (16, 17, 60, 192). Approximately 10% of patients in Europe (*B. afzelii*) develop acrodermatitis, which presents as a reddish blue plaque or nodule overlying a localized edema. The inflammation may last for many years, but gradually becomes atrophic. *B. afzelli* have been cultured from these lesions years after the onset of the disease (16, 17, 20, 60, 192).

Keratitis and uveitis are late ocular symptoms of Lyme disease that can occur years after the initial infection (180, 192). Keratitis is described as multiple, focal, nebular opacities at varying levels of the stroma and can progress to edema and scarring (180, 192). The eye lesion is comparable to lesions that occur during the late stages of syphilis. The involvement of multiple systems during late Lyme disease impedes treatment, therefore it is necessary to diagnose and treat the disease before the bacteria enter the latent phase.

Diagnosis

Diagnoses of the different stages of Lyme disease are difficult because most of the clinical manifestations are not solely associated with Lyme disease. Lyme disease has been referred to as the great pretender or imitator because the symptoms can appear like many other

diseases. It has been misdiagnosed as chronic fatigue syndrome, multiple sclerosis, menopause, depression, Alzheimer's disease and Lou Gehrig's disease (267, 268). As successful treatment requires early and accurate diagnosis, difficulty in identifying the disease presents a major problem in dealing with Lyme disease.

Despite significant efforts to improve diagnostic tools, the correct identification of EMs remains one of the simplest and most effective ways to identify the disease. However, EMs only develop in ~60 % of patients with Lyme disease and can be mistaken for another skin rash or disease (7, 39, 40, 270, 276). In addition, since the rash is painless, it can be easily overlooked, especially when the tick bite occurs in a hidden location on the body. Other early Lyme disease manifestations include flu-like symptoms that are often mistaken for influenza or a common cold. Late Lyme disease is difficult to diagnose based on symptoms, which can vary among individuals. Reiter's syndrome or septic arthritis can mistakenly be diagnosed rather than Lyme disease. Neurological symptoms associated with Lyme disease can be confused with multiple sclerosis, Gullian-Barré syndrome or neurosyphilis (267, 268). Therefore, in the absence of EM, diagnosis of Lyme disease relies predominately on laboratory tests and the physician obtaining a detailed medical history that includes potential exposure to areas considered endemic for the disease.

Laboratory tests identify spirochetes by direct detection, serology and polymerase chain reaction (PCR) (24, 192). Direct observation of *B. burgdorferi* from infected individuals is an invaluable diagnostic tool, however, the detection is not reliable due to the low numbers of spirochetes found in infected tissues and difficulty in culturing them (77, 192). *B. burgdorferi* can be observed with microscopy from the rash using a phase-contrast or dark-field microscope (77, 192). Standard bacterial stains, such as the Gram stain, do not visualize *B. burgdorferi*,

therefore specialized techniques like Giemsa, acridine orange or modified Dieterle must be used (77, 192). Direct observation is an important diagnostic tool and it can be used to confirm that the EM on the patient is caused by Lyme disease and not another infection. However, in patients that do not develop an EM there are generally not enough spirochetes in one particular area to observe.

Culture is considered the gold standard to document an infectious disease. Culturing *B. burgdorferi* from patient samples is a good diagnostic technique for Lyme disease, however, this is also hindered by the low number of spirochetes found in human body fluids and organs (192). One exception is the early skin lesion (EM). Skin punch biopsies from EM can produce viable cultures of spirochetes (77, 192). Unfortunately, such patients' cultures can take weeks to months to grow to detectable levels before spirochetes can be seen and most clinics are not equipped to grow *B. burgdorferi*. The technique used to acquire the samples and the types of sample obtained are variables that influence whether or not spirochetes can be cultured. Spirochetes have been cultured from joint synovial fluid, cerebrospinal fluid and large-volumes of blood with varying degrees of success (77, 130, 192, 307, 308). Due to the time needed to culture spirochetes and the small number present, other diagnostic techniques are typically used to diagnose Lyme disease.

Serology of *B. burgdorferi* is currently one of the most commonly used laboratory techniques. Serological tests include indirect immunofluorescence (IFA), enzyme-linked immunosorbent assays (ELISA) and immunobloting to detect the presence of anti-spirochete antibodies. ELISA assays are the most widely available and commonly performed tests, and have replaced IFA which are both labor intensive and subjective in interpretation (54, 77, 176, 192). However, both ELISA and IFA are prone to both false positives and false negatives. False

positives generally result from the cross reactivity of *B. burgdorferi* proteins with proteins of other infectious agents (spirochetal, bacterial, rickettsial, rocky mountain spotted fever, EBV) (54, 77, 176, 192). False negatives can occur if the individual does not mount a good immune response, when tests are performed too soon after infection and prior to seroconversion, or when antibiotics are used very early in the course of the infection before an antibody response occurs (54, 77, 176, 192). ELISA and IFA are useful techniques for diagnosing Lyme disease and steps have been made to alleviate some of the problems with these methods.

Elevated IgM antibody responses to spirochetes occur within 8 days after the onset of the disease (46, 65). After the first 4 to 6 weeks of infection, some individuals develop an elevated IgG antibody response to the spirochete. ELISA assays utilize sonicated whole cells or purified protein extracts to detect IgM or IgG antibodies to *B. burgdorferi* in the patient sera (54, 77, 176, 192). To help alleviate the problem of cross reactivity new ELISA tests have been developed using an immunodominant antigen, outer surface protein C (OspC) and OspA, which are expressed on the bacterial cell surface early during infections (54, 77, 176, 192). Immunoblotting is used to confirm the ELISA results.

In 1995, the Center for Disease Control (CDC) and the Association of State and Territorial Public Health Laboratory Directors (ASTPHLD) recommended specific guidelines for serological diagnosis of Lyme disease (4). The first step in this two-test approach is to perform initial screening with ELISA assays where borderline or positive tests are confirmed by immunoblots. If the initial infection is suspected to have occurred within the previous month, the immunoblots are used to detect both IgM and IgG antibodies against P21-24 (OspC), P39 (BmpA) and P41 (Fla) (4). These assays are considered positive if bands representing two of the three proteins are present. For infections greater than a month in duration, immunoblots are used

to detect IgG for P18, P21 (OspC), P28, P30, P39 (BmpA), P41 (Fla), P45, P58, P66 or P93 (4). IgG immunoblots are considered positive if bands representing five of the ten proteins are present.

An antigen detection system for *B. burgdorferi* has also been developed, however it has encountered mixed reviews and has not yet been approved for clinical testing by the Food and Drug Administration. In experimental infections, *B. burgdorferi* has been observed in the bladder and *B. burgdorferi* antigens or nucleic acids are shed into the urine of animals (104, 112, 246). The proprietary Lyme urine antigen test (LUAT) and Lyme dot blott assay (LDA), developed by IGeneX Inc (Palo Alto, CA), are based on an antigen capture-inhibition ELISA that uses rabbit polyclonal antibodies to recognize the *B. burgdorferi* antigens: 23-25 kDa (OspC), 31 kDa (OspA), 34 kDa (OspB), 39 kDa and 93 kDa (112). The test results are interpreted according to the antigen level in the samples; negative <20 ng/ml, borderline 20-31 ng/ml, positive 32-45 ng/ml and strong positive >45 ng/ml (112). In 2001, Klempner *et al* reported that test results for 10 healthy control subjects were not reliable (146). The 10 samples were divided into 5 replicate samples and sent to the IGeneX Inc for testing. The results showed that 8 of the 10 samples yielded both negative and positive results and LDA is not a reliable diagnostic technique for Lyme disease (146). Instructions for IGeneX state that any positive sample should not be considered positive until confirmed by another method such as immunoblot or PCR.

PCR can be very effective for the direct detection of *B. burgdorferi* in various body fluids or tissues of infected individuals (54, 228). However, this is very expensive and most clinics do not have the necessary equipment. This technique has had varied success in detecting *B. burgdorferi* DNA from samples of blood (2-26%), cerebrospinal fluid (20-40%), urine (20-40%),

skin (70-90%) and synovial fluid (20-40%) from patients infected with Lyme disease (77, 143, 174, 189, 192, 228). *B. burgdorferi* gene targets for PCR include single-copy chromosomal targets (*flaB*, *recA,* ribosomal genes 16S, r23S and *rpoB*) along with genes present on multiple copy plasmids (outer surface proteins *ospA* and *ospB,* and *vlsE)* (77, 143, 174, 189, 192, 228). Because this technique is sensitive to the level of detection of one spirochete in a patient sample, there is the increased possibility for contamination and false-positives. Early diagnosis of Lyme disease is important for the detection of spirochetes in the host and for the successful treatment of the disease.

Treatment

Currently, the most effective treatment for Lyme disease is antibiotic therapy. However, a number of factors must be considered when deciding on the appropriate treatment. These include length of illness, immunocompetency, presence of coinfection, age, patient tolerance and blood levels achieved with the antibiotic (77, 192). Antibiotic resistance in *B. burgdorferi* has been investigated *in vivo*, *in vitro*, as well as in clinical reports. *B. burgdorferi* is resistant to aminoglycosides, ciprofloxacin, rifampin, most first generation cephalosporins and some clinical isolates are erythromycin resistant (40, 134, 136, 138, 154, 192, 199, 220, 293). In contrast, it is sensitive to penicillin and penicillin derivatives along with some macrolides and second generation cephalosporins (9, 40, 134, 136, 138, 154, 199, 220, 273, 277, 310). To effectively treat *B. burgdorferi* located in deep tissue, CNS, eye, and within tendons, higher doses are often required (9, 40, 199, 220, 273, 277, 310).

Oral penicillin was the classic treatment for Lyme disease, however, further clinical research has shown that other antibiotics demonstrate superior absorption and longer half-lifes in

the serum (27, 79, 80, 192, 199, 306). An oral treatment regimen of either 100 mg doxycycline two times per day, 500 mg amoxillin three times a day or 500 mg cefuroxime axetil twice a day for 21-30 days is effective for EM and early Lyme disease in adults (27, 79, 80, 192, 199, 306, 309). Because of the possibility of simultaneous erlichiosis infection, doxycycline is the preferred oral antibiotic due to its effective treatment of both infections (27, 79, 80, 192, 199, 306, 309). However, doxycycline should not be prescribed for the treatment of pregnant women or children under the age of 8 years (192, 306, 309). For treatment of early Lyme disease in children oral amoxilillin (50mg/kg/d) or oral cefuroxime axetil (30mg/kg/d) is prescribed for three to four weeks (192, 306, 309). For pregnant or lactating women 250 mg of amoxillin or cefuroxime axetil twice daily is recommended instead of doxycycline (192, 306, 309).

Treatment of disseminated and late Lyme disease is much more difficult. The duration of treatment is longer, the response is often slower and treatment failure is higher due to the presence of *B. burgdorferi* in the CNS, eye, within tendons and deep tissue (192, 306). For patients with neurological complications, intravenous treatment regimens can last many months and includes 2 grams daily of ceftriaxone or 20 million units per day of penicillin G in divided doses (192, 306, 309). Nonsteroidal anti-inflammatory agents, intra-articular corticosteroids or both can be used for treatment in patients with the HLA-DR4 allele and persistent Lyme arthritis following antibiotic treatment (192, 306, 309). Because effective treatment of Lyme disease involves the extended use of high concentrations of antibiotics, treatment complications such as the Jarisch-Herxheimer reaction can occur.

The Jarisch-Herxheimer reaction is uncommon but can occur after treatment of Lyme disease, relapsing fever or syphilis (24, 192, 240, 300, 306). The reaction typically occurs within hours to a few days after the first treatment with antibiotics and is caused by the release of toxins

after a large number of organisms are killed (24, 192, 240, 300, 306). The reactions include an increase in disease symptoms, rheumatic pains, fever, chills, headache, diarrhea, endocarditis and swelling of the joints (24, 192, 240, 300, 306). Within 5-10 days of continued antibiotic treatment, the body will dispose of the endotoxin and the Herxheimer reaction gradually lessens.

Prevention

Lyme disease infects humans though the bite on an infected tick and the risk of tick bites can be greatly reduced using simple measures. Immature *Ixodes* ticks usually stay close to the ground in the soil, leaf litter and vegetation and can be found in the woods, brushy areas or overgrown fields (1, 192, 306). If these areas cannot be avoided while hiking, camping, gardening, hunting or working, there are precautions that lessen the chances of contacting the vector. Clothing is a natural barrier to ticks and tucking long pants into socks, wearing long sleeves, light-colored gloves (while gardening) and enclosed shoes keeps exposed skin to a minimum (1, 192, 306). Ticks can climb clothing to reach exposed skin around the neck and head. Wearing light-colored clothes will aid in finding and removing the ticks before they bite (1, 192, 306). The single most effective method for preventing Lyme disease is the removal of the tick before the bacteria can be transmitted (1, 192, 306). *Ixodes* ticks must feed for 36-48 hours before transmitting *B. burgdorferi* (1, 192, 210, 306). Therefore, locating and removing ticks greatly reduces the chances of contracting Lyme disease. Even though antibiotic treatment is usually not needed, any area where a tick was removed should be observed for sign of EM development (1, 192, 306). Chemical repellents provide additional protection. Permethrin or repellents containing DEET (*N*,*N*-diethyl-meta-touamide) are particularly effective (1, 192, 306).

Vaccination offers another mean to prevent Lyme disease, and several *B. burgdorferi* proteins have been proposed as potential vaccine candidates. In 1998, the Food and Drug Administration approved Lymerix (produced by SmithKline Beecham). This vaccine is based upon a lipidated recombinant OspA (rOspA) (118, 175, 319). Lymerix is administered by three intramuscular injections over a two year period and clinical trials yielded 76% efficacy (118, 175, 319). It was determined that OspA antibodies were able to neutralize *B. burgdorferi* in infected ticks and prevent the transmission of the infection (118, 175, 319). Recently, after a number of vaccinated individuals reported symptoms worse than those associated with Lyme disease and due to the bad publicity from the potential side-effects (even though undocumented), SmithKline Beecham decided to no longer sell the vaccine (249, 306). These symptoms included incurable arthritis and neurological impairment (249, 306). It is believed that these individuals have the human leukocyte function-associated antigen HLA-DR4, and this is causing their symptoms to become more severe (113, 249, 306). Other outer surface proteins, OspB and OspC, along with other proteins, decorin binding protein (Dbp) from *B. burgdorferi* are being investigated as potential vaccine candidates (46, 96, 110, 111, 302, 304).

Causative agent

In the early 1980's, researchers from the National Institute of Allergy and Infectious Diseases in Hamilton, Montana were investigating tick-borne Rocky Mountain spotted fever and serendipitously found a new microbe. The bacterium was isolated from *Ixodes* ticks and subsequently from patients with Lyme disease (23, 57). This spirochete was placed in the genus *Borrelia* based upon DNA relatedness, physiology, and morphology and was given the species name *burgdorferi* after Dr. Willy Burgdorfer who first discovered and cultured it (24, 26, 55,

135, 244). *Borrelia*, a member of the family Spirochaetaceae, are helically shaped and have a outer membrane that covers the protoplasmic cylinder (24, 26, 135, 244). The cylinder consists of a peptidoglycan layer and cytoplasmic membrane (24, 26, 135, 244). There are also periplasmic flagella placed between the outer membrane and protoplasmic cylinder that give the spirochete its unique spiral shape (24, 26, 135, 244). The outer cell membrane is elastic and is easily separated from the protoplasmic cylinder with phosphate buffered-saline (26, 135). Various proteins that are encoded by linear and circular plasmids are located in the outer membrane. Some of these genes have been shown to rearrange, resulting in varied composition and antigenicity of the proteins (26, 99, 135). Between the outer membrane and the protoplasmic cylinder are the periplasmic flagella responsible for the unique motility of the spirochetes and allow the bacteria to move in viscous environments and disseminate during infection. The flagella are inserted subterminally, extend toward the opposite end of the cell and are comprised of four components: filament, hook, neck and basal disk (26, 99, 135).

B. burgdorferi was first isolated in modified Kelly's medium, a complex growth medium used to grow *B. hermsii* (23, 145). Kelly's medium contains a buffer system, salts, glucose, pyruvate, gelatin, sodium bicarbonate, and *N*-acetyl glucosamine (NAG) (145). In 1974, Stoenner modified this medium by adding yeastolate and a tissue culture medium (CMRL-1066) that contains amino acids, vitamins and other growth factors to help with the growth of *Borrelia* from blood cultures (285). The medium was later adapted further by removing gelatin and the glutamine from CRML-1066 and renamed BSKII (Barbour-Stoenner-Kelly) (23, 145, 285). BSKII is a rich, undefined growth medium that allows spirochetes to reach densities of approximately 2×10^8 cells per ml with a doubling time of 8-12 hours when incubated at 34 °C

under an atmosphere of 3-5% O_2-5% CO_2-90% N_2 (23). Investigators are currently developing minimal media to determine what components of BSKII are essential for *B. burgdorferi* growth.

B. burgdorferi sensu lato complex consists of ten genospecies. Three of these are associated with Lyme disease in humans: *B. burgdorferi sensu stricto, B. garinii,* and *B. afzelii* (26, 89, 135). The other seven *Borrelia* species are either nonvirulent to humans or the tick vector does not feed on humans (26). Characteristics of the genus *Borrelia* show that all species which are pathogenic to humans are transmitted by arthropod vectors, vary in diameter, length, tightness of coils and number of periplasmic flagella (26, 135). *B. burgdorferi* is the narrowest and longest of the *Borrelia* at 20-30 μm in length and 0.2-0.3 μm in diameter, whereas other members of the genus vary in diameter from 0.18-0.5 μm and 0.8-30 μm in length (26, 135). The number of flagella per cell end varies from 1-30 among the spirochetes where, *B. burgdorferi* contains 7-15 flagella (26, 99, 135). The three infectious *Borrelia* species responsible for human disease are associated with unique clinical presentations. *B. burgdorferi* is associated with arthritis, *B. afzelii* with cutaneous manifestations, and *B. garinii* with neurologic manifestations (20, 26). Not only are their clinical manifestations different, but also they are geographically distinct. *B. afzelii* and *B. garinii* are only found in Europe, while *B. burgdorferi sensu stricto* is only found in the United States (20, 26).

In 1997, sequencing of the *B. burgdorferi* chromosome was completed and analysis of the sequence revealed the absence of genes encoding enzymes necessary for the synthesis of enzyme cofactors, amino acids or nucleotides (99). Also missing from the genome were genes encoding enzymes for the tricarboxylic acid cycle, respiratory chain or oxidative phosphorylation (99). Therefore, *B. burgdorferi* has limited metabolic capabilities and produces energy by fermenting glucose by the Embden-Meyerhof pathway to release lactic acid (99, 133). Glycerol

and N-acetylglucosamine (NAG), may also be consumed by *B. burgdorferi* as a carbon source (99, 133). The cuticle of ticks and mammalian tissues contains chitin, which has NAG as a major constituent, and this may serve as a carbon source for *Borrelia* (26, 99). Along with being a potential carbon source, NAG is also the major constituent of peptidoglycan and necessary for cell wall construction (26, 99). *B. burgdorferi* metabolizes NAG to fructose-6-phosphate, which then enters glycolysis using *N*-acetylglucosamine-6-phosphate deacetylase and glucosamine-6-phosphate isomerase (99). The limited metabolic capabilities of *B. burgdorferi* demonstrate why a complex growth medium is needed to culture the cells *in vitro*. The growth medium must be supplemented with rabbit serum to supply the spirochete with long-chain fatty acids since *B. burgdorferi* is incapable of elongating the short-chain fatty acids (23, 135, 171). To this end, the membrane of *B. burgdorferi* reflects the components of the media given that the unaltered fatty acids are incorporated into the membrane (23, 135, 171). Lipids can be toxic to the cells, therefore, bovine serum albumin (BSA) is also included in the growth media as a lipid carrier (23, 99, 135, 171).

Transmission

B. burgdorferi is transmitted to mammalian hosts via the bite of an infected tick of the genus *Ixodes* (57, 58, 210). Most Lyme disease in the United States is localized to the northeastern, mid-Atlantic, upper north-central region and some California counties (22). *Ixodes scapularis* is responsible for the transmission of Lyme disease in northeastern and north-central United States (13). On the Pacific coast the bacteria are transmitted by the bite of an infected *Ixodes pacificus*, while in Europe and Asia the primary Lyme disease vectors are *Ixodes ricinus* and *Ixodes persucatus*, respectively (13, 58, 168, 275). *B. burgdorferi* is transmitted to *Ixodes*

ticks in the bloodmeal during feeding on an infected mammal (57, 58, 210). The chance that the tick will become infected depends on the animal that is infected and how long the infection is sustained in the animal. For example, a white-footed mouse (*Peromyscus leucopus*) will remain infected with *B. burgdorferi* for several months and will transmit the pathogen to ticks that feed upon it (13, 22, 173).

The two-year life cycle of *Ixodes* tick includes three stages: larva, nymph and adult. At each of these stages a bloodmeal is taken, and the ticks can become infected. As the tick matures it will feed on larger animals, usually starting with mice, then slightly larger mammals and finally a deer (19, 34, 37, 254, 264). Humans are an incidental host in this life cycle and a dead-end vector for the spirochete, due to the fact that there is no human-to-vector transmission (19, 34, 182, 254, 264). The life cycle of the tick begins in the spring, when eggs laid the previous fall hatch (19, 34, 254, 264). In the larval stage, the ticks prefer to feed on the white-footed mouse or other small rodents, which present the first possible source of infection since infected rodents are spirochetemic during the spring and fall (19, 34, 254, 264). *Ixodes* ticks are long feeding ticks that take a blood meal for 24-48 hours (19, 264). Tick saliva contains immuno-suppressants, anti-coagulants, anti-hemostatics, and analgesics that facilitate the long feeding and could aid the transmission of the spirochete (49, 232, 238, 254). During this extended feeding, *B. burgdorferi* is transmitted to the larvae in the blood meal and the spirochete can be found in the tick midgut at densities as high as 2×10^3 spirochetes/tick (167, 211). After the bloodmeal is digested, the larvae remain dormant through the autumn and winter months. During this time, <300 spirochetes have been found in the tick midgut (211). The following spring, the larvae molt into the nymph form (19, 34, 254, 264).

Nymphs are responsible for most of the transmission to humans due to their small size and at this stage ticks actively feed in the spring and summer when humans are most likely to be outdoors and wearing less protective clothing (19, 34, 254, 264). Nymphs prefer to feed on animals larger than mice, such as raccoons, chipmunks, and squirrels (19, 34, 264). However, the ticks can feed again on the reservoir animal, the white-footed mouse. This keeps the animal spirochetemic so the larva developing in the fall can become infected. Ingestion of the bloodmeal stimulates surviving spirochetes in the tick midgut to replicate (up to a density of 6 x 10^4 spirochetes), penetrate the midgut wall, and migrate to the salivary glands, where they can be transmitted to the new host with the tick's saliva (211, 238). When the feeding is complete, the nymph will drop off, molt, and enter the adult stage of the life cycle in the fall (19, 34, 254, 264).

The adult stage of the *Ixodes* tick is the final stage that completes the two-year life cycle. The adult ticks will mate while on larger animals, particularly the white-tailed deer (*Odocoileus virginianus*) (48, 177, 192, 212). The female adult ticks feed on the deer to have sufficient protein to produce eggs, and then drop to the leaf litter, lay the eggs, and dies, completing the life cycle of the female *Ixodes* tick (19, 34, 264). Transovarial transmission of spirochetes is rare and not believed to play a role in the transmission of *B. burgdorferi* to ticks (59, 161, 209). Therefore, ticks must become infected during their life cycle.

Animal Models

The use of animal models allows the complex features of a multiple system disease, such as Lyme disease, to be studied in detail. The natural reservoir for Lyme disease, white-footed mouse, is easily infected, however the animal shows no outward signs of the infection. Therefore, other animals must be used to mimic various aspects and the infective cycle of human

Lyme disease. Investigators have developed animal studies in out-bred mice, rabbits, hamsters, rats, guinea pigs, dogs, and monkeys (15, 29, 33, 98, 122, 186, 205, 206, 251, 265). These animals have been important for the study of the infectivity of various *B. burgdorferi* strains, the immunology and pathogenesis of Lyme disease, and development of immunoprophylactic, diagnostic and chemotherapeutic protocols (15, 29, 33, 98, 122, 186, 205, 206, 251, 265).

The first animals used experimentally to study Lyme disease were rabbits, because they had been used to propagate other spirochetes and as animal models for infection (e.g. *Treponema pallidum*) (29, 56, 152). Male New Zealand white rabbits were infected by intradermal needle injection with as few as 1 x 10^3 bacteria (29, 56, 98, 152). Rabbits proved useful in characterizing various strains of *B. burgdorferi*, however the manifestations that developed are different in rabbits compared to humans. Symptoms in the rabbit model included persistent skin infection and visceral dissemination to the knee joint and CNS. However no other clinical manifestations developed and the spirochetes were uncultivable from the CNS (29, 33, 98, 205). One group reported that New Zealand rabbits developed EMs that were infiltrated with macrophages and neutrophils whereas, human EMs contain mononuclear leukocytes, however this was irreproducible by others (29, 33, 98, 205).

In 1986, Syrian (LSH) Hamsters were investigated as an animal model for Lyme disease. Using this system, the first dissemination of *B. burgdorferi* in an animal and the ability to infect multiple organs was observed (87). Hamsters are persistently infected but do not develop the disease as adults (29, 87, 205). The hamsters were spirochetemic twenty-fours hours after infection and spirochetes were detected in the spleen, kidney, or eye by culture and histology and in tissue samples from the liver and heart in young hamsters 1-9 months after inoculation (29, 87, 205). This animal model proved useful as bacteria disseminate throughout the animal, but is

limited by the fact that no other pathology or symptoms of the human disease are observed in the Syrian Hamsters (29, 87, 205).

Outbred Hartley guinea pigs had been shown to be useful in other human infections, such as leptospirosis (265). Skin lesions following tick bites have been documented in Hartly guinea pigs, thus susceptibility of the guinea pigs to *B. burgdorferi* infection was investigated (265). Guinea pigs less than three-month-old were found to be susceptible to *B. burgdorferi* infections, while those older than six months could not sustain an infection (265). *B. burgdorferi* disseminated to the heart, bladder, knee, spinal cord, and muscles, although no clinical arthritis was observed (29, 265). Guinea pigs were found to have the highest percentage of positive knee joint cultures (79%) compared to other animal models for *B. burgdorferi* (29, 265). Unfortunately, there is no long-term infection of the guinea pigs because the host immune response ultimately clears the infection.

An animal model that was able to sustain Lyme disease was needed to follow long-term manifestations. Lewis Rats were inoculated by intraperitoneal injection and dissemination of the bacteria was followed. Although no EM was observed, *B. burgdorferi* was detected after 30 days in the blood, 60 days in the brain, and 360 days in the spleen, liver, kidneys and heart tissues (185). Other symptoms included an exudative arthritis, tendonitis and bursitis in multiple joints after 30 days and acute arthritis within 180-360 days (185). Inconsistencies in peripheral joint manifestations, chronic and recurrent arthritis lesions, and myocardial involvement were also observed in this model (29, 185, 205). Similarities in cardiovascular and musculoskeletal lesions in infected rats and humans suggested that the rat model was reliable (29, 185, 205).

Lewis rats susceptibility to joint and heart disease at any age led to the investigation of the mouse model for *B. burgdorferi* infections. Mice can be infected by a very low dose by

either syringe or tick bite, will develop the disease at any age, and the infection will persist in mice less than 3 months old for over one year. The disease in mice, like humans, is intermittent which is associated with the presence of spirochetes in actively inflamed lesions (29, 30, 32, 162, 205). Various breeds of outbred mice have been used to look at clinical manifestations and symptoms of Lyme disease, and both disease susceptible and resistant haplotypes have been identified.

Studies in severe combined immunodeficiency (SCID) mice, which have impaired B and T cell functions, established that *B. burgdorferi* infection caused arthritis and carditis in the absence of functional T and B cells (205, 241, 262). Spirochetes were cultured from the blood and joints of these infected animals (205, 241, 262). When the joints and tissues were examined synovial hyperplasia, infiltration of mononuclear cells into the synovium, formation of an inflammatory exudate over the synovial lining, cartilage destruction and joint erosion was observed (205, 241, 262). However, neurological lesions were not recorded (205). The SCID mouse model demonstrated that the evolution of joint and heart disease is not immune dependent, however the regression of the disease is immune mediated (30-32).

Infection of CH3/HeJ mice has been thoroughly investigated as a model for *B. burgdorferi* infection. In these mice the bacteria develop disseminated infection with carditis, cystitis and arthritis and have become the small animal of choice for studying the pathology of arthritis and carditis and investigating experimental vaccines (29, 30, 32, 162, 205). These animals allow for studies into the mechanism of persistence of the spirochete during chronic infection, test correlating genetic markers, and test the potential of isolated mutants to produce disease (29, 30, 32, 162, 205).

The mechanism controlling the development of experimental Lyme arthritis remains a mystery. Simon *et al* tested genetically different inbred strains of mice and showed that all mice with the H-2^k haplotype developed moderate to severe arthritis, where those mice with the H-2^d haplotypes did not develop the severe arthritis or any clinical signs, but were readily infected (205, 262). However, either haplotype was able to generate protective antibodies to the spirochetes suggesting that genetically determined variations in the immune response might play a role in the progression of Lyme disease (205, 262). The arthritis observed in mice can also be attributed to a mitogen that *B. burgdorferi* produced\s which stimulates the proliferation of B cells and the production of immunoglobulin from naïve mice (248). C3H/HeJ mice show 10-fold increase in IgG levels, 2-fold increase in IgM levels and 15-fold increase in peripheral lymph node B-cell numbers when compared to BALB/c mice (248). This evidence for mitogenic activity could contribute to the inflammatory and immune response to the *Borrelia* infection.

These rodent models have shown that antibody alone, or with complement, mediates the killing and clearing of *B. burgdorferi in vivo* (178, 205, 239, 262). The mouse model has become an important tool and the small animal of choice in studying immune response and spirochete phenotype. However, all of the symptoms and manifestations of Lyme disease in humans are not observed in these small animals (29, 31, 205, 239, 262).

Non-human primates were investigated as a possible animal model to determine if they could mimic the complete human form of Lyme disease. In 1993, Philipp *et al* observed Rhesus monkeys for 13 weeks after inoculation with *B. burgdorferi* and reported that the monkeys mimic the early-localized and early-disseminated phases of Lyme disease (206). EM was observed in both needle and tick inoculated monkeys and *B. burgdorferi* were recovered from

skin biopsies, blood and conjunctiva (206). Other symptoms included lethargy, an enlarged spleen and CSF pleocytosis (205, 206). IgM and IgG antibody levels to *B. burgdorferi* appear at similar rates and levels in both the monkey model and humans (198, 206). In 1995, Roberts *et al* evaluated chronic Lyme disease in Rhesus monkeys and found chronic arthritis in 5 of the 6 monkeys (234). Knee and elbow joints were most continently affected along with articular cartilage necrosis and degenerative arthropathy lymphocyte infiltrate (234). Spirochetes observed in the joint suggested that chronic arthritis is caused by the persistence of *B. burgdroferi* in the joints (205, 206, 234).

Rhesus monkeys mimic several aspects of both early and chronic Lyme disease observed in humans, thus is a useful model for investigating the immunology, neurology, and pathogenesis of Lyme disease (198, 205, 206, 234). The development of immunoprophylaxis requires specifics of both an antigen and the antigen target that is the goal for this mechanism. Therefore, the Rhesus monkey model will be important in developing diagnostic and chemotherapeutic protocols for the treatment of Lyme disease (198, 205, 206, 234).

While there are multiple animal models for the study of *B. burgdorferi* pathogenesis, each has its own advantages and disadvantages, but thus far, none mimic all of the features of Lyme disease observed in humans and imitate the unremitting DR4 Lyme arthritis.

Genetic Composition

Initial studies into the genetic makeup of *B. burgdorferi* suggested that it possessed an unusual replicon in addition to linear plasmids with covalently closed ends (25). It was unique among prokaryotes when Ferdows *et al* demonstrated that *B. burgdorferi* might have a linear chromosome (95). The genome sequence for *B. burgdorferi* strain B31 was published in 1997

and confirmed that it harbored a linear chromosome, and numerous linear and circular plasmids (99). The unique linear chromosome of *B. burgdorferi* has a G+C content of 28.6%, is 910,725 base pairs in length, and contains 853 open reading frames (ORFs) (99). Analysis of the coding sequences on the chromosome showed that 59% could be assigned putative function based upon homology with previously identified bacterial genes, while 12% matched hypothetical proteins, and 29% had no matches in the database (99). The ORFs identified genes encoding proteins for DNA replication, recombination and repair, transcription, translation, solute transport, and energy metabolism (99). Interestingly, no genes encoding proteins involved in cellular biosynthetic reactions for the synthesis of amino acids, fatty acids, lipids, enzyme cofactors, or nucleotides were identified (99). In addition genes associated with the tricarboxylic acid cycle or respiratory chain were not identified (99). Analysis revealed genes required for nucleotide salvage and the Embden-Meyerhoff pathway, suggesting that *B. burgdorferi* utilizes glucose as a primary energy source and ferments it to lactate as an end product (99).

When the *B. burgdorferi* chromosome sequence was released, no origin of replication was identified. However, 66% of the genes on the chromosome are transcribed away from the center. Similarly this transcriptional bias has also been observed in *Mycoplasma genitalium* and *M. pneumoniae*, which have the origin of replication in the center of the chromosome (67, 99). Bacterial origins are usually located near *dnaA*, which was mapped to the center of the *B. burgdorferi* chromosome (99). CG skew analysis, (C – G)/(G + C), shows a clear break at the putative origin, and an octamer, TTGTTTTT, that resembles *E. coli* Chi sites, has been identified near the midpoint (99). In 1999, the origin of replication for the linear chromosome was mapped to the center of the linear chromosome and in the 240 bp sequence between *dnaA* and *dnaN* where the CG skew originates (208).

The chromosomal telomeres have similar 26 base pair inverted repeats and are unique from each other or other telomeres (99). The right end of the telomere has approximately 7,500 base pairs containing few ORFs compared to the rest of the chromosome and has high homology to the left ends of linear plasmids lp17 and lp28-3 (99). These observations suggest that the right end of the linear chromosome has exchanged DNA with the telomeres of the linear plasmids (99). Recent reports suggest that the replication of the *B. burgdorferi* linear chromosome and plasmids includes a telomere resolution step that involves a site-specific DNA breakage and reunion reaction that regenerates the hairpin ends (72, 148). Bidirectional replication from an internal origin gives a final linear product with hairpin telomeres predicted to be circular with the two DNA monomers covalently linked at the telomere (208). Telomere resolution is promoted in *B. burgdorferi* by ResT and occurs when the circular dimer is processed by a DNA breakage and a reunion reaction that regenerates the telomeres (149, 296).

When the genome sequence was initially released the number of plasmids varied, and it was observed that isolates could contain up to 20 different plasmids (99). Further investigation revealed as many as 23 total plasmids, and Casjens *et al* subsequently completed the remaining approximate 300,000 base pairs of the genome project that was previously unassembled (69). Casjens *et al* determined that *B. burgdorferi* strain B31 M1 contained 12 linear and 9 circular plasmids ranging in size from 5 to 56 kb (69). Plasmids were designated 'lp' for linear plasmids or 'cp' for circular plasmids and according to their approximate size in kb. The linear plasmids included lp5, lp17, lp21, lp25, lp28-1, lp28-2, lp28-3, lp28-4, lp36, lp38, lp54, lp56, and the circular plasmids included cp9, cp26, cp32-1, cp32-3, cp32-4, cp32-6, cp32-7, cp32-8, cp32-9 (69). Strain B31 M1 did not contain cp32-2 and cp32-5 which had been identified from other *B. burgdorferi* isolates (282). Even though the approximate size and number of plasmids varies in

B. burgdorferi from strain to strain, infectious strains appear to have the same basic profile of plasmids when examined by Southern blot (42, 69, 70, 202). *B. burgdorferi* strain B31 M1 adds 610,694 base pairs of plasmid DNA to bring the total genome size to 1,521,419 base pairs (69). The plasmids have an overall G+C contents ranging from 20.7% to 31.6%, and encode approximately 535 genes and 167 pseudogenes (69). Interestingly, only 8% of the plasmid encoded genes have similar homology outside the *Borrelia* genus (69). Traditionally plasmids are considered dispensable genetic information, however, some of the genes encoded on *Borrelia* plasmids are universally present in nature, thus, some plasmids likely carry metabolically important genes and virulence factors (69, 226).

The linear DNA molecules of *B. burgdorferi* are similar to those in *Streptomyces* (74, 75, 117). However, the telomeres of *B. burgdorferi* are not sensitive to protease treatment suggesting that there is no terminal protein bound like the telomeres of *Streptomyces* (68, 74, 117). In fact, both the terminus of the chromosome and the linear plasmids of *B. burgdorferi* are covalently closed single-stranded hairpin loops (66, 99, 116). The question as to why *Borrelia* has linear DNA remains unanswered. However, there is evidence that the free ends of the linear DNA molecules are recombinogenic (66). Many of the linear plasmids appear very similar, especially the lp28 family (lp28-1,-2,-3,-4). The linear plasmids have unique features, including an 11 kb block of a 63 bp direct repeat on lp21, perfect inverted repeats of the telomeres and the long repeating cassettes of *vls* (69, 124). Other plasmids also show evidence that recombination events have occurred over time. The most noticeable rearrangement involves lp56, which is a linear plasmid that contains a unique cp32 insertion (69). On lp21 there is all but one ORF from lp5 present and both cp9 and lp54 contain sections with homology to the cp32 plasmids (69).

Nine circular plasmids make up the cp32 family and each is highly similar. The circular plasmids contain a gene encoding RepC that has homology to Gram-type positive bacteria, and it was first suggested that replication may occur by a rolling circle method (69, 86, 99). However, Picardeau *et al* suggest that due to the plasmids base composition asymmetry pattern, the plasmids may replicate bidirectionally from the origin near the skew point (207). There is also evidence that the cp32 plasmids are bacteriophage (69, 90, 91). Isolated bacteriophage from *B. burgdorferi* has been shown to package a 32-kb molecule that hybridizes to the cp32 family plasmids of the genome (91). Recently, Eggers *et al* demonstrated the ability of ϕBB-1 to package and transduce a kanamycin resistance cassette to three different strains of *B. burgdorferi* (90). This exhibits the first direct evidence for lateral gene transfer in *B. burgdorferi* and could play an important role in investigating plasmid genetics and developing new genetic systems for studying *B. burgdorferi* (90).

Infectivity and Virulence

When the genome sequence for *B. burgdorferi* was released, no known virulence factors were identified by homology, and it was observed that continued growth and passage *in vitro* lead to the loss of infectivity when the spirochete was introduced into a new host (26, 99). Thus, scientists have investigated the possible correlation between infectivity and virulence with plasmid content and the identification of unrecognized virulence factors.

In 2000, Norris *et al* followed the presence/absence of 21 plasmids by PCR in 19 clonal isolates of *B. burgdorferi* and evaluated the infectivity of these strains through isolation of bacteria from needle inoculated mice (226). A number of plasmids (cp26, 32-1, 32-2, 32-4, 32-6, 32-7, 32-8, 32-9, lp28-3, 36, 38, 54) were present in all strains, so it could not be established if

any of these plasmids were required for infectivity (226). Also no correlation in infectivity of the 19 strains was observed when various combinations of the cp32's were present/absent (226). A high infectivity (all tissue samples collected were culture positive) phenotype was retained in the absence of cp9, cp32-3, lp21, lp28-1, lp28-4, and lp56 (226). Interestingly, two plasmids were identified as required for the colonization and short-term maintenance in mammalian tissues by *B. burgdorferi* (159, 160, 226). Lp25 was only present in clones with high or intermediate infectivity (some of the tissue cultures were positive) and absent in all with a low infectivity (all of the tissue cultures were negative) phenotype (226). Strains containing lp25 but lacking lp28-1 have an intermediate infectivity phenotype requiring a 50% higher infective dose, and spirochetes are found primarily in the joints of the infected mouse (159, 160, 226). However, in 2001, Labanderia-Rey *et al* reported that the loss of lp28-4 can also lead to a moderately attenuated strain of *B. burgdorferi* (160). Possible virulence factors on lp25 and lp28-1 have been investigated to explain their requirement for virulence in *B. burgdorferi*.

Encoded on lp25 is *pncA*, which has 46% similarity to a well-characterized nicotinamidase of *Salmonella typhimurium* that catalyzes the deamination of nicotinamide to nicotinic acid (225, 320). *B. burgdorferi* uses PncA to convert nicotinamide to nicotinic acid, which is a precursor for NAD, and when Purser *et al* restored PncA to a strain lacking lp25, infectivity in a mouse returned to levels comparable with wild type *B. burgdorferi* (225).

Lp28-1 contains a variable membrane proteins (*vmp*)-like sequence (*vls*) locus similar to the *vmp* locus described in *B. hermsii* (214, 315). This *vls* expression site (VlsE) is thought to be required for survival within the tissues and skin of hosts (159, 160). Identified by Zhang *et al*, the *vlsE* expression locus is located at the telomere of lp28-1 and is comprised of 15 silent *vls* cassettes upstream in the opposite direction of the gene (315). Each cassette is flanked by a 17

bp direct repeat and they are 95% identical at the nucleic acid level and approximately 85% similar at the amino acid level (315). VlsE, a 35 kDa surface exposed lipoprotein, which undergoes antigenic variation by rearranging segments of the 15 silent cassettes within the central cassette region of the expressed *vlsE* gene (315-317). Antibodies from infected mice and humans demonstrate that immunodominant epitopes are located within the conserved domains of VlsE (169, 183). However, McDowell *et al* provide direct evidence that the variable regions of the central domain influence the overall antigenicity of VlsE and are targeted by the host immune system during infection (183). This suggests that the ability of *B. burgdorferi* to evade the host immune response system is due in part to antigenic variation within VlsE.

VlsE expression *in vitro* remains at a low level, however when *B. burgdorferi* cells are incubated with human tissue cells the expression increases (124). Increased expression was also observed when *B. burgdorferi* cells were incubated with endothelial membranes (124). Recently, Seshu *et al* have shown that VlsE expression increases as the dissolved oxygen concentration decreases (258). In these experiments, oxygen was depleted from the media by adding Oxyrase, an oxygen consuming membrane preparation to the growth media. Taken together these data suggest that VlsE is regulated in response to oxygen concentration.

While VlsE expression is affected by oxygen concentration *in vitro*, recombination between the expression locus and silent cassettes is not. The sequence of *vlsE* was observed in *in vitro* cultures for 84 days and no sequence changes occurred, suggesting that *vlsE* recombination is induced by an unknown factor absent in the growth media (317). To determine when the recombination is induced, genetic variation in the *vlsE* locus was followed though the mouse-tick-mouse cycle. In infected mice, *vlsE* undergoes promiscuous recombination between the expression locus and the silent cassettes, leaving the flanking segments of the expression loci and

cassettes unchanged (315-317). These changes in the *vlsE* sequence were observed by PCR as early as 4 days postinfection in both C3H/HeN and SCID mice (317). The short amount of time required for antigenic variations to arise suggests that induction of sequence variation of *vlsE* is caused by a specific factor or factors rather than immune selection (317). In the tick vector, *vlsE* was expressed at very low levels, and the variations generated in the mouse were maintained through the various tick stages (127, 196). Even though the recombinations in *vlsE* were unchanged in the tick vector, there were no dominant alleles observed between the various stages of tick development and individual ticks (127, 196). The exact function of VlsE remains unknown, however, these results suggest that *vlsE* recombination is induced by a signal that is present in the mammalian host, not in the tick vector and recombination appears to be independent of immune selection yet required to avoid clearing by the host immune system. Investigators believe there are more virulence factors that play a critical roles in the pathogenesis of Lyme disease that remain unidentified.

The majority of the putative virulence determinants identified in the *B. burgdorferi* genome are encoded on the plasmids that were always present in the various clones screened in the infectivity study. Much of the interest focuses on plasmid encoded membrane antigens that are able to stimulate an immune response in the infected mammal (94, 110, 291). These membrane lipoproteins have the opportunity to interact with the host and elicit an immune response, which maybe responsible for the pathology of Lyme disease. The best-characterized *B. burgdorferi* lipoproteins include VlsE, OspA (Outer surface protein A), OspB, OspC, OspD, Erps (OspEF related proteins), and decorin binding protein (DpbA & DbpB) (107, 108, 122, 136, 176, 253, 277, 302).

Encoded in an operon on lp54, OspA and OspB were the first lipoproteins investigated as possible virulence factors. Both OspA and OspB are expressed at high levels in cells grown *in vitro* and have been shown to stimulate host immune cells to release interleukin-1, IL-2, and IL-6, tumor necorsis factor alpha, gamma interferon, and prostaglandin E (94, 113, 299). OspA, the most abundant outer surface protein, is a polymorphic lipoprotein that has a molecular weight from 30-32 kDa (131, 139, 301). The apparent size of OspA varies among *B. burgdorferi* strains and *ospA* genes were found to be about 75-86% identical (131, 139). OspB is the second protein encoded on the *ospAB* operon and has a molecular weight that varies between 33–36 kDa, similar to variations in the size of OspA (139, 305). Sequence analysis of various *B. burgdorferi* strains revealed that OspA and OspB share approximately 60% amino acid similarity, and their C-terminal ends share a 280 bp sequence (43, 131). This observation supports the idea that these genes resulted from an evolutionary duplication of an ancestral *osp* gene (43, 131).

One important factor contributing to the antigenicity of OspA is the lipid modification of the protein. OspA contains a post-translationally added tripalmitoyl-*S*-glceryl-cysteine (Pam_3Cys), which is essential for induction of an immune response in mice (52). It was determined that a lipidated OspA, non-lipidated OspA, and an OspA fusion protein with part of the nonstructural protein 1 of influenza virus were able to induce interleukin-1β, IL-6 and tumor necrosis factor-α (94, 113, 299). Lipidated OspA was also able to induce high levels of IL-10 within 24 hrs (113). The purified recombinant lipidated OspA induced a strong protective response even in the absence of an adjuvant, thus, OspA was an excellent vaccine candidate (94, 113, 299).

Experimental animals respond with a vigorous antibody response to OspA following inoculation, however, human hosts express IgG antibodies to OspA late in the infection (10, 82).

In 1995, Schwan *et al* investigated the expression level of OspA in infected ticks. In unfed ticks, *B. burgdorferi* produces high levels of OspA but this expression is down regulated when the bloodmeal enters the tick midgut and is not expressed when *B. burgdorferi* migrates to the salivary glands (256). During this down regulation of OspA, another outer surface protein, OspC, is being expressed at higher levels in the tick midgut (256). The factors that induce the change in expression from OspA to OspC in the midgut of the tick include the temperature change due to the bloodmeal and some other yet unidentified factor in the bloodmeal (256, 257, 281). OspC expression is also regulated by the RpoN-RpoS regulatory pathway in *B. burgdorferi* (123). Interestingly, antibodies against OspA and OspB, especially the C-terminal epitope of OspA, appear in the human host late in the infection, and the reappearance of OspA and OspB has been observed in cases with severe and prolonged arthritis (10). This suggests a role for OspA and OspB in enhancing the severity and duration of joint inflammation during Lyme arthritis.

Recently Pal *et al* reported that OspA is required for adhesion to the tick intestinal cells, and OspA expression allows *B. burgdorferi* to stay attached to the surface of the tick midgut and colonize the vector (200). This study also suggests that the down regulation of OspA during the bloodmeal enables the spirochetes to detach from the tick midgut and disseminate to the salivary glands (200). This explains the requirement for OspA expression in the tick midgut and the repression of OspA expression after the bloodmeal and during invasion of a human host.

As OspA is down regulated in the tick, OspC expression increases. Antibody responses to OspC are elevated in infected animals early during the infection suggesting that it is required by *B. burgdorferi* early in the transmission (303). OspC is an immunodominant lipoprotein of *B. burgdorferi* encoded on lp26 with a molecular weight between 20-25 kDa. OspC has been

investigated as a possible virulence factor as it is expressed at high levels in the tick as the bacteria are being transmitted to the new host and it elicits an immune response in humans with Lyme disease and infected animals (103, 221, 224, 256).

OspC from *B. burgdorferi* shares 43% identity to Vsp33 (variable short membrane protein 33) from *B. hermsii* (179). Interestingly, expression of both OspC and Vsp33 is induced *in vitro* by a shift in growth temperature (255, 256, 281). However, OspC expression increases at 37°C while Vsp33 is induced at 23°C (255, 256, 281). In the tick vector, *B. hermsii* expresses Vsp33 before the ingestion of the bloodmeal while *B. burgdorferi* OspC expression is induced after the bloodmeal (253, 255, 256). Additionally, *B. hermsii* resides in the salivary glands rather than the tick midgut, where it is ready for transmission to a new host. This difference in localization has been attributed to the different feeding behaviors of the two tick vectors. *B. burgdorferi* is transmitted by *I. scapularis* which feeds for 3-4 days compared to *B. hermsii* that is transmitted by *Ornthodoros hermsi* which feeds in a few minutes (253, 255). Due to the relatively short feeding time for *O. hermsi*, *B. hermsii* must be ready for rapid transmission to the new host while *B. burgdorferi* has time to upregulate the expression of proteins necessary for transmission.

Originally, it was thought that both OspC and Vsp33 allow the spirochetes to migrate from the salivary glands into the new host. Pal *et al* demonstrated that OspC strongly binds to the tick salivary glands and *ospC* knockouts in *B. burgdorferi* strain 297 were unable to invade the salivary glands (201). However, Grimm *et al* showed that an *ospC* mutant in *B. burgdorferi* B31 strain A3 was able to colonize the tick vector and migrate from the midgut to the salivary glands during tick feeding (105). Both *ospC* mutants were unable to infect mice by needle inoculation or natural tick challenge (105, 201). These data suggest that OspC is required for the

initial stage of infection in the new host, possibly allowing the spirochete to evade the innate immune response during dissemination (105).

Whether or not OspC immunized animals show protection from challenge with various *Borrelia* strains is controversial (103, 132, 221, 223, 224). Active immunization with OspC was shown to protect gerbils when challenged with *B. burgdorferi* (102, 221). However, others have shown that mice immunized with OspC have not been protected when subsequently challenged (223). The gene encoding OspC from 22 various *Borrelia* strains were aligned and revealed 48 conserved amino acids (294). Conserved regions include helices near the N or C termini, and the highest degrees of diversity were observed in the central portion of the protein (variable domains V1 and V2) (132, 170, 294). Interestingly, Jobe *et al* reported that the C terminal end of OspC is the region that IgM and IgG antibodies from Lyme disease patients recognize (132). While there are some questions about the therapeutic value of OspC, further research to investigate the function of OspC and its true usefulness as a vaccine is necessary.

Two other lipoproteins, OspE and OspF, are differentially expressed *in vitro* when the temperature is shifted from 23°C to 32°C (256, 281). Further investigation found that OspEF are encoded by a single bicistronic operon, and unlike the other Osps, they are members of a very large family of proteins referred to as the OspEF-related proteins (Erps) (99, 282). To date, 17 *erp* genes have been identified on the cp32 family of plasmid/prophage (92, 99, 114, 282). The *erp* genes have several distinct features including nearly identical promoter sequences, highly conserved leader polypeptide sequences, simultaneous expression, and their products all localize to the outer membrane (92, 283). Investigators were first interested in the Erp proteins because an antibody response to the various proteins was observed within a few weeks of initial infection (11, 291).

In humans and many other animals, the host must protect itself from its own alternate complement pathway. One method of protection is to bind complement inhibitor factor H. This protein prevents opsonization of the host cell by not allowing the deposition of factor C3b on the cell surface and promotes the deactivation of C3 convertase and the breakdown of C3b (155). *B. burgdorferi* has developed a way to use the host's defense mechanism to prevent opsonization and killing by complement, allowing the bacteria evade the alternate pathway (12, 184, 280). Investigators determined that the various Erp proteins were able to bind factor H from a number of hosts with varying specificities (12, 184, 280). This suggests that the multiple Erp proteins are necessary for the survival of *B. burgdorferi* in various animal hosts (12, 184, 280).

In addition to surface proteins that bind factor H, other proteins may be necessary for *B. burgdorferi* to colonize the host. *B. burgdorferi* binds heparin sulfate, dermatan sulfate and decorin, a proteoglycan that covers collagen fibers in the host dermis (97, 108). Two surface proteins that bind decorin have been identified (107). The genes encoding decorin binding protein A (DpbA, 19 kDa) and decorin binding protein B (DbpB, 20 kDa) are in an operon on lp54, and were tested for their ability to bind various classes of glycosaminoglycans (GAG), proteoglycans expressed on mammalian cell molecules (97, 107). Results demonstrated that DbpA and DbpB each promote binding to dermatan sulfate and mammalian cells but with different GAG-binding specificities (97). This differential binding of DbpA and DbpB could be a mechanism for the bacteria to discriminate between various mammalian cell types promoting colonization of different tissue sites (97).

With 6% of the genome involved in motility and chemotaxis and approximately 130 surface lipoproteins identified in *B. burgdorferi* there are many proteins that maybe involved in virulence (99). Identification of *B. burgdorferi* virulence factors has been slow due to the lack of

necessary genetic tools to manipulate the Lyme disease spirochete. Within the last 4 years, mutagenesis techniques and the development of shuttle vectors has opened up the genetic studies in *B. burgdorferi* (47, 235-237, 284). These new tools have helped to define the roles of these proteins and will aid in the identification of other virulence factors associated with in Lyme disease.

Regulation of Gene Expression

To colonize different hosts successfully *B. burgdorferi* must regulate specific combinations of genes at the appropriate times and in the correct host. Regulation of gene expression in *B. burgdorferi* has been shown to be influenced by temperature, pH, cell density, oxygen, and host factors (65, 128, 129, 197, 227, 252, 256, 258, 281). Schwan and Stevenson showed that expression of OspA and OspC is effected by a temperature shift from 37°C to 23°C, with OspC predominating at 37°C and OspA at 23°C (252, 256, 281). In 2003, whole genome arrays were used to investigate other genes whose expression is influenced by temperature. Two hundred and fifteen ORFs were found to be differentially expressed, 133 ORFs expressed higher levels at 37°C and 82 ORFs expressed higher levels at 23°C (197). Sixty-two percent of those ORFs are of unknown function and, interestingly, 63% of the ORFs are plasmid encoded, suggesting that the genes encoded on the plasmids play an important role in how *B. burgdorferi* adapts to different host environments (197).

Another environmental cue that influences differential protein expression is pH. By two-dimensional nonequilibrium pH gradient gel electrophesis (2D-NEPGE), 37 proteins were observed to have altered expression when grown in BSK-II at pH 6.0, pH 7.0, or pH 8.0 (65). Differential expression of *B. burgdorferi* proteins has also been observed during different growth

phases. For example, as cells enter post-logarithmic phase, at least 20 antigens are upregulated, including p35, p7.5, BmpD, GroEL, and OspC (128, 129, 227). Most of the proteins identified are plasmid encoded, including OspC, BBA03, BBA24 and RevA (63-65). Interestingly, the expression of OspC is affected by temperature, pH, oxygen, and is regulated by RpoS (64, 65, 123, 258).

Defining the regulatory mechanisms involved in controlling the expression of these genes in response to various environmental cues is essential for understanding how *B. burgdorferi* adapts to the different environments and survives in the tick vector and mammalian hosts. Few known regulatory proteins were identified from the analysis of the *B. burgdorferi* genome sequence. Those that were identified included an *Borrelia* oxidative stress response regulator (BosR), two XylR family members that can activate and repress gene expression, and a few other regulatory proteins putatively involved in carbon and phosphate metabolism (51, 99). Despite the fact that temperature has been shown to play a role in protein expression, no heat shock sigma factors such as σ^{32} or σ^{28}, were identified even though a full complement of typical heat shock proteins have been described (76, 99). Three sigma factors were identified in the *B. burgdorferi* genome, the "housekeeping" σ^{70}, the stationary phase and stress response sigma factor, σ^{S}, and the alternative sigma factor, σ^{54} (99). Two complete histidine-kinase/response regulators, two-component systems, were also identified, and one of these systems is suggested to be required for σ^{54}-dependent gene expression (314).

In *B. burgdorferi* the gene encoding σ^{S}, *rpoS*, is controlled by σ^{54}, which suggests a unique regulatory cascade in which an environmental signal or cellular cue is sensed through a two-component system that mediates σ^{54}-dependent expression of σ^{S} during stationary phase (123). *B. burgdorferi rpoS* mutant strains are not adversely affected during stationary phase, but

are more sensitive to osmotic shock as well as reactive oxygen species, such as *t*-butyl hydrogen peroxide (93). Thus, the regulatory cascade that controls expression of σ^S may protect *B. burgdorferi* from a variety of environmental assaults the bacterium encounters during its transmission cycle.

What seems clear is that *B. burgdorferi* regulates gene expression in response to the various conditions encountered during transmission. It is also likely that there are other important regulatory proteins yet to be identified and that the functions of identified regulatory proteins and sigma factors differ from those of their homologs in other bacteria. For example, *B. burgdorferi* BosR was initially identified as a homolog to a *Bacillus subtilis* protein, PerR, a peroxide sensitive, global repressor which contains a structural zinc and a second peroxide responsive metal ion (iron) (115). Recently, Boylan *et al* showed that despite the sequence similarity between the two proteins, BosR functions primarily as a zinc-dependent transcriptional activator in *B. burgdorferi* rather than a repressor of the oxidative stress response (51).

Oxidative Stress Response in *Borrelia burgdorferi*

Interestingly, BosR does not appear to be the only regulatory system committed to the oxidative stress response in *B. burgdorferi*. Preliminary analyses of σ^{54} and σ^S mutants demonstrate an increased sensitivity of these strains to reactive oxygen species (ROS) compared to wild-type strain B31 (93). This suggests that this regulatory cascade may also play a role in protecting *B. burgdorferi* from ROS encountered in different hosts and host environments. For example, during the initial stages of the infection in the skin, *B. burgdorferi* is challenged by ROS released by neutrophils in response to the bacterial infection (147, 292). Additionally, during transmission, *B. burgdorferi* must respond to and survive ROS generated in the tick

salivary glands during the feeding cycle as they are transmitted to a new host (J. Garcia-Lara, unpublished data). Clearly, successful survival and colonization of a new host requires that *B. burgdorferi* express ROS scavenging enzymes regulated by BosR, σ^{54} or σ^{S}.

Possible targets for ROS damage in bacteria include DNA, RNA, proteins, and lipids. The most extensive damage is thought to be due to hydroxyl radicals (OH^-) generated when H_2O_2 reacts with iron via the Fenton reaction (126, 286, 295). OH^- then reacts with DNA introducing single and double stranded breaks, which leads to increased mutation rates and cell death (62, 172, 295). Likewise, metalloproteins, particularly those containing Fe-sulfur centers, and proteins containing reduced cysteine residues are also targets of ROS (61, 126, 222, 286, 295). Damage to active sites and bound ligands can dramatically affect the function of key enzymes in metabolic/biosynthetic pathways (44, 61, 126, 141). Much more unlikely in most bacteria is ROS mediated damage to phospholipids, lipoproteins or glycolipids (125). This is due to the fatty acid composition of lipids in bacteria such as *E. coli.* Since lipid damage requires the oxidation of unsaturated fatty acids (e.g., linoleic acid etc) not found in the lipids of most bacteria, lipid peroxidation is not thought to be a major contributor to oxidative damage in these bacteria (125, 126).

Preliminary analyses of the internal chemistry and chemical composition of *B. burgdorferi* strongly suggest that the targets of ROS are very different in this bacterium. First, *B. burgdorferi* does not contain any intracellular iron minimizing the possibility of DNA being a major target for damage via the Fenton reaction (218). This seems to be the case since high concentrations of ROS (50 mM H_2O_2 or *tert*-butyl hydrogen peroxide) have little to no effect on the mutation rate in strain B31 (J. Garcia-Lara, unpublished data). Second, while damage to cysteine residues in proteins most likely occurs, ROS attack on iron-sulfur proteins seems

unlikely since none have been identified in the genome of *B. burgdorferi* (218). Finally, in contrast to most bacteria, *B. burgdorferi* phospholipids, lipoproteins and glycolipids contain unsaturated fatty acids, such as linoleic, linolenic, and arachidonic acids, which are derived from the host (35, 38, 121, 171). Lipid peroxidation initiated by the attack of free radicals on these polyunsaturated fatty acids could decrease the membrane fluidity and**,** if these reactions propagate, lipid peroxides and their degradation products (e.g., aldehydes) in turn could damage proteins (125). This would dramatically affect the function of transmembrane proteins and membrane bound lipoproteins involved in the maintenance of membrane potential and solute transport, decreasing cell survivability. Thus, it seems most likely that lipids and proteins rather than DNA are the primary targets of ROS in *B. burgdorferi*.

Bacteria have adopted several strategies to deal with ROS. Whatever the source of ROS, the biochemical approach cells employed to neutralize these incomplete reduction products is accomplished in two steps. First, singlet oxygen radicals (O_2^-) are reduced by superoxide dismutases (SOD) or superoxide reductases (SOR) to H_2O_2 and the reduction is completed when hydroperoxidases reduced peroxide to H_2O (45, 125, 126, 286). Three major classes of SODs have been described (Fe, Mn, and Cu/Zn types) and while these enzymes are widely distributed among aerobic and microaerophilic bacteria, it should be noted that some bacteria, particularly anaerobes, do not necessarily contain SODs (45, 125, 126, 191, 286). These bacteria can contain SORs to begin the reduction of O_2^- (191). Further reduction of the end-product of SODs and SORs (H_2O_2) requires other enzymes such as catalases, peroxidases, alkyl hydroperoxide reductases (AhpR), and glutathione peroxidases to complete the detoxification of ROS (125, 126, 286). The exact role/function of other putative ROS protective enzymes, such as the DNA

binding protein induced in stationary phase (dps) homologs or bacterial ferritins, remains to be determined.

The *B. burgdorferi* genome contains the genes encoding few ROS protective enzymes. To date, only the genes encoding a putative manganese dependent SOD and a Dps-homolog (NapA) have been identified (99). However, how *B. burgdorferi* deals with ROS is only beginning to be investigated and additional protective enzymes may be identified. Identifying and characterizing some of the products of genes regulated by BosR and by the σ^{54}/σ^{S} regulatory cascade will lead to a better understanding of the oxidative stress response in *B. burgdorferi.* For example, BosR has been implicated in regulating the expression of the neutrophil activating protein (NapA), glutamate transporter (GltP), an NADH oxidase (Nox), thioredoxin reductase (TrxR), SOD, the decorin binding proteins (DbpA and DbpB) and itself (51, 259). Initial studies of NapA show that its expression increases when cells are grown at high levels of O_2 (>8%) or when anaerobically grown cells are exposed to ROS such as *t*-butyl hydrogen peroxide (258). This protein has homology with NapA from *Helicobacter pylori* and another Dps homolog from *B. subtilis*, MrgA. Typical characteristics of Dps/Dpr proteins are that they bind Fe, bind DNA (Dps but not Dpr) and are induced when cells are exposed to ROS or other cellular stress conditions (14). The initial characterization and potential role of *B. burgdorferi* NapA is described further in Chapter 3.

Transcriptional Activation with σ^{54}-RNA Polymerase Holoenzyme

The ability of bacteria to regulate transcription initiation allows for the coordinated expression of genes at appropriate times. The DNA sequence upstream of a bacterial gene or operon encodes the promoter region, which determines specific recognition by RNA polymerase.

Before transcription can occur, core RNA polymerase ($\alpha_2\beta\beta'$) must combine with the dissociable σ subunit to form RNA polymerase holoenzyme ($\alpha_2\beta\beta'\sigma$) (53, 230, 287). The addition of the σ subunit to the RNA polymerase confers specificity for the recognition of promoter sequences (53, 230, 287). To date, two classes of sigma factors have been identified; the σ^{70} class, which contains most sigma factors, and the alternative sigma factor group σ^{54}, which has only one member (53, 230, 287). Both groups of sigma factors bind the same RNA core polymerase despite different amino acid sequences and mechanisms of transcription (53, 151, 287).

One unique aspect of σ^{54} is the specificity for a distinct, well conserved promoter that has GG and GC doublets at –24 and –12 positions, respectively, relative to the transcriptional start site, instead of the typical -35/-10 boxes observed in σ^{70} class promoters (28, 53, 151, 287). One hundred and eighty six σ^{54}-dependent promoter sequences were compiled in 1999, from which the consensus DNA sequence YTGGCACGrNNNTTGCW was generated (28). The spacing between the conserved GG and GC doublets is absolutely critical since the addition or deletion of a single base between these elements results in inactivation of the promoter. However, functional divergent promoters have been described (28). For example, *Myxococcus xanthus* gene 4521 has an A at the –25 position where the G at -24 is 99 % conserved (28, 311). The G at the –13 position and the C at the –12 position are 96 % conserved among σ^{54}-dependent promoters with a mapped transcriptional start site (28). However, functional divergences in the GC doublet have been observed in *Pseudomonas aeruginosa oprE*, *Alcaligenes eutrophus koxK*, and *Rhizobium leguminosarum* biovar viciae *nifH* (28, 313).

Another unique characteristic of σ^{54} is the mechanism and specific requirements for transcription initiation with σ^{54}-RNA polymerase holoenzyme (σ^{54}-holoenzyme). Transcriptional initiation involves discreet steps, the first of which is binding of RNA

polymerase to the promoter to form a closed promoter complex. Closed complexes formed with σ^{70}-RNA polymerase holoenzyme (σ^{70}-holoenzyme) are generally transient and can only be observed by trapping them at low temperatures (<4°C) (119, 242). This closed complex rapidly undergoes isomerization to form an open complex in which DNA is locally denatured at the transcriptional start site (119, 151, 242). The open complexes are competent to initiate transcription. Activators that function with σ^{70}-holoenzyme generally activate transcription by binding to sites near the promoter and recruiting RNA polymerase to the promoter (231). In contrast, σ^{54}-holoenzyme binds the promoter to form a stable closed complex, but the closed complex is unable to undergo isomerization to an open complex in the absence of an activator and ATP hydrolysis (28, 53, 230, 260, 287).

With the increasing number of completed genome sequences, σ^{54} and potential activators have been identified in a widespread variety of bacterial genera including, *E. coli*, *B. subtilis*, *Chlamydia pneumoniae*, spirochetes (*B. burgdorferi* and *T. pallidum*), extreme theromphiles (*Aquifex aeolicus*), green sulfur bacterium *Chlorobium tepidum* and many others (287, 288). σ^{54} was first identified in *Salmonella typhimurium* as the σ factor responsible for the transcription of genes involved in nitrogen metabolism and is often referred to as σ^{N} (100). Subsequent analysis of the roles of σ^{54} in other bacteria has shown that this sigma factor is involved in a wide range of functions. Depending on the bacterium, σ^{54} has been shown to control hydrogen uptake, degradation of xylene and toluene, transport of dicarboxylic acids, pili and flagella synthesis, arginine catabolism, alginate and rhamnolipid production, mannose uptake, glutamate biosynthesis, and heme biosynthesis (28, 230, 287-289). Interestingly, σ^{54} has also been implicated in controlling the expression of another sigma factor, σ^{S}, in *B. burgdorferi*, *Pseudomonas syringae* pv. *tomato*, and *Enterobacter cloacae* (123, 288). Even though the

regulation of other bacterial σ factor genes have been reported, σ^{54} of *E. coli* and *K. pneumoniae* are constitutively expressed (71, 81). One exception has been observed in *Bradyrizobium japonicum* were there are two functional homologs of σ^{54} and the expression of $rpoN_1$ is regulated by oxygen and $rpoN_2$ is negatively autoregulated (156).

As indicated previously, initiation of transcription by the σ^{54}-holoenzyme is absolutely dependent on an activator protein. Productive interactions between the activator and σ^{54}-holoenzyme lead to isomerization of the closed complex to an open complex, which is competent to initiate transcription. Dicarboxylic acid transport protein D (DctD) was crosslinked to Cys-307 of σ^{54} and to the β subunit of *E. coli* core RNA polymerase (164). In 2001, Region I of σ^{54} was demonstrated to bind the central ATP-hydrolyzing domains of the transcriptional activators PspF and NifA in the presence of ADP-aluminum fluoride (73). Binding of Region I to this domain was shown to require the GAFTGA motif, a conserved signature sequence within the ATPase domains of σ^{54}-dependent activators (73).

σ^{54}-dependent activators generally bind 100–200 bp upstream of the promoter and contact the closed complex through DNA looping (157, 215). DNA loops may result from random and transient conformational changes in the DNA or can be stabilized by auxiliary proteins, such as integration host factor (120, 157). An exception to this was observed with the σ^{54}-dependent activator in *B. subtilis*, RocR, which can activate transcription while bound downstream of the promoter (36). σ^{54}-dependent activators are similar to eukaryotic enhancer-binding proteins in the respect that both bind at large distances from the transcriptional start. Hence, these σ^{54}-dependent activators are also referred to as bacterial enhancer-binding proteins (157, 215). The nitrogen regulatory protein C (NtrC) binding sites at *glnA* are able to function

when moved from the normal upstream location while, DctD binding sites fail to function when moved and are therefore referred to as upstream activation sites (229).

Activators of σ^{54}-holoenzyme typically contain two or more binding sites for enhancers, to which the protein can bind cooperatively (217). The *S. typhimurium glnA* enhancer, which is the best characterized bacterial enhancer, was examined by scanning force microscopy to observe the binding of σ^{54}-dependent activator NtrC and it was estimated that 6-8 subunits of NtrC bind to this enhancer (233). Two of the dimers bind directly to the DNA, where the other two dimers are held in place by protein-protein interactions with the enhancer bound proteins. AAA^{+} proteins typically have six subunits arranged in a ring but they can range from 5-8 subunits (190, 195). However, the number of subunits necessary to bind is subject to debate. The ATPase domain of NtrC1 from *A. aeolicus* was crystallized and forms a ring-shaped heptamer, while the ring-like structure of PspF from *E. coli* has six subunits (166, 318). A single dimer is unable to activate transcription at the *glnA* promoter, indicating that the ring formation is necessary for activity (217). While the enhancer appears to facilitate ring formation and interactions between the activator and σ^{54}-holoenzyme, it is not absolutely required for transcriptional activation. Activator mutants that fail to recognize the enhancer can still activate transcription if they are present at higher than normal concentrations (193, 312). In these cases, the activators appear to oligomerize and contact σ^{54}-holoenzyme from solution to activate transcription.

The number of σ^{54}-dependent activators present within a bacterial genome varies. Eleven activators have been identified in *E. coli*, while *A. aeolicus* has five and each activator has a specific function for the bacteria. *Chlamydia pneumoniae* and *B. burgdorferi* each have one possible activator of σ^{54}-holoenzyme, which could reflect the small genome size and restricted

metabolic capabilities of these bacteria (290). The putative activator of σ^{54} in *B. burgdorferi* was identified by homology to activators in the NtrC1 family and was named Sigma^{S} regulatory protein (SisR).

Regulators of σ^{54}-Dependent Activators

σ^{54} activators fall into different subgroups based on their mode of regulation (188, 260). About half of the over 600 σ^{54}-dependent activators in the Pfam database are response regulators of two-component systems and include, NtrC, DctD, *Caulobacter crescentus* flagellar transcription activator D (FlbD), and SisR (157, 158, 260). In response to an environmental signal or cellular cue, such as nitrogen concentration, the histidine kinase modulates the phosphorylation state of the response regulator at a conserved aspartyl residue in the receiver domain which is typically positioned at about residue 54 (157, 158, 260). The histidine kinase has autophosphorylation activity and, in some cases, regulated phosphatase activity that catalyzes the removal of the phosphate from the phosphorylated activator. Phosphorylation stimulates the activity of the activator, presumably by converting it from an off-state, which is the dimeric form of the protein, to the on-state, which is capable of forming the higher-order oligomeric complex needed for the ATP hydrolysis and transcriptional activation (166, 203).

Not all σ^{54} activators are regulated by phosphorylation or are part of a two-component system. Regulatory protein of the levanase operon, LevR, from *B. subtilis* is both positively and negatively regulated by phosphorylation, but is not part of a two-component system (83, 181). A third group of activators interacts directly with an environmental signal through their amino terminal domain. This group includes xylene catabolism regulator, XylR and phenol catabolic pathway positive regulator, DmpR, from *Pseudomonas putida* and FlhA from *E. coli*. These

regulators are generally stimulated by the binding of an inducer, typically an aromatic compound for which the activators control the transcription of genes required for the degradation of that compound (204, 261). In *B. japonicum*, *Rhizobium trifoli*, and *Sinorhizobium meliloti*, nitrogen fixation protein A, NifA, is thought to be controlled by redox (153, 187, 247, 250). In comparison, NifA in *Azotobacter vinelandii* and *K. pneumonia* is negatively regulated by interactions with NifL in response to fixed nitrogen and molecular oxygen (18, 260).

Structure and Functions of the Domains of σ^{54}-Activators

Activators of σ^{54}-holoenzyme consists of three distinct functional domains, an amino-terminal regulatory domain (A domain), a central transcriptional activation domain (C domain) and carboxyl-terminal DNA-binding (D domain) (188). The A domain is the least conserved domain and varies greatly in length from 12 amino acids for the hypersensitivity reaction and pathogenesis protein S (HrpS) of *Pseudomonas syringae*, to close to 400 residues for formate hydrogen-lyase transcriptional activator, FlhA, of *E. coli* (188, 260). An exception is the phage shock protein, PspF, from *E. coli*, which contains no A domain and is a constitutively active protein that is inhibited by interactions with the product of one of the genes it regulates, *pspA* (6, 140). Removal of the amino terminal domain of XylR, DctD and LevR results in a constitutively active protein (165, 181, 204). In contrast, removal of the amino terminal end of NtrC does not result in constitutive activity (85). The A domain is usually tethered to the C domain of the σ^{54} activator by a flexible glutamine rich linker, known as the Q-linker or B domain (188, 260).

The central activation domain, C domain, is comprised of approximately 240 residues and is the most highly conserved portion of σ^{54}-dependent activators. This domain contains an ATP-binding motif, is directly responsible for transcriptional activation and can be subdivided

into seven regions of high homology, C1-C7 (53, 157, 188, 260). The functions of some of the subdomains have been determined by comparison with other ATPases associated with diverse cellular activities (AAA^+) and investigation of the effects of point mutations on various σ^{54}-dependent activators. Region C1 contains a glycine-rich (G--G-GK-) structural motif known as a phosphate-binding loop at the nucleotide-binding site or Walker-type A sequence, similar to other ATP-binding proteins (188, 260). The C4 region has 3-4 hydrophobic residues followed by a conserved aspartate that is thought to coordinate the Mg^{2+} associated with ATP and is similar to the Walker-type B sequences found in other ATPases (188, 260). The C5 region (QaKLLRVLqe) is similar to segment three from adenylate kinase and although no mutants have been isolated in this region, it is suggested that region C5 is involved in binding and the hydrolysis of ATP (188).

Region C3 has become a diagnostic of σ^{54}-dependent activator proteins. For example, *Rhodobacter capsulatus* NtrC is very similar to other enhancer-binding proteins, however it lacks the C3 portion and has been shown to interact with σ^{70}-holoenzyme instead of σ^{54}-holoenzyme (50). As indicated previously, the GAFTGA motif within the C3 region is required for the stable binding of the activator to σ^{54} in the presence of ADP-aluminum fluoride (73). Cross-linking studies with point mutations of DctD have also revealed the importance of the C3 region for interactions between the activator and σ^{54}-holoenzyme (298). In the crystal structure of NtrC1, the GAFTGA motif forms a stable loop that extends into the pore of the ring (166). Thus, it is tempting to speculate that the GAFTGA loop helps pull Region I of σ^{54} into the pore of the ring structure during the ATP hydrolysis cycle which results in the reorganization of the closed complex to a transcriptionally productive open complex.

Limited research on the remaining regions of the central domain has been done. Amino acid substitutions in regions C6 and C7 of DctD and C7 of NtrC showed disrupted ATPase activity suggesting a role in ATP hydrolysis, although whether it is a direct or indirect role still needs to be determined (194, 297).

The carboxy-terminal, D domain, of σ^{54}-dependent activators is the shortest domain, between 65 and 130 residues. One exception is LevR that has a D domain of 600 amino acids (188). σ^{54}–dependent activators typically bind ~100- 200 bp upstream of the σ^{54} -dependent promoter and the helix-turn-helix DNA binding motif has been shown to reside in the D domain (188, 260). DNA binding appears to have two important roles; it facilitates oligomerization of the activator and provides target specificity in bacteria that have multiple σ^{54}-dependent activators (217, 260). Interestingly, σ^{54}-dependent activators, *H. pylori* FlgR and *Chlamydia trachomatis* CtcC, are functional but lack the DNA-binding domain (150, 266). It is hypothesized that CtcC is capable of bypassing DNA binding to activate transcription by forming oligomers in solution close to σ^{54}-holoenzyme.

In *B. burgdorferi* the σ^{54}-dependent activator, SisR, is part of a two component regulatory system. The sensor protein, sigma^{S} histidine kinase (SisK), is encoded by ORF BBO764, which is directly upstream of the response regulator *sisR* (ORF BBO763). Recently, Yang *et al* generated a single point mutation within the central C4 domain of SisR to remove the σ^{54}-dependent activation ability of the protein (314). This single amino acid change eliminated σ^{S}-dependent lipoprotein expression by disrupting the cascade involved in the σ^{54}-dependent expression of σ^{S} (314). SisR knockout mutants could not be isolated, suggesting that disruption of SisR is lethal due to the loss of other another unknown function(s) of the protein, such as repression of unknown genes. To understand how SisR is activated and how the complete

regulatory cascade functions, it is necessary to investigate and characterize its cognitive histidine kinase, SisK, which senses the environmental signal or cellular cue to activate the regulatory cascade. The initial characterization and potential role(s) of *B. burgdorferi* SisK/SisR is described further in Chapter 2.

It should be pointed out that in some well studied two-component systems other factors have been shown to be required for signal transduction. One such example is the *S. typhimurium* and *E. coli* NtrB/C two-component system that controls the expression of genes involved in nitrogen assimilation in these bacteria. In bacteria, the nitrogen regulated response is the response to poor nitrogen conditions where ammonia is not the primary available nitrogen source. The nitrogen regulated response involves the coordinated regulation of genes involved in the transport and degradation of various nitrogen compounds. In *S. typhimurium* the Ntr response includes proteins involved in the transport of glutamine, arginine, aspartate, lysine, onithine, glutamate, histidine, and the glutamate synthetase (GS) enzyme encoded by *glnA* (230). The *glnAntrBC* operon has two promoters, *glnA*p1 which is a minor σ^{70} –dependent promoter and *glnA*p2 that is the primary σ^{54}–dependent promoter. Under excess nitrogen conditions, σ^{70}–holoenzyme activates transcription of *glnA* by *glnA*p1 and cotranscribes *ntrBC* (230). In these conditions, NtrC acts as a repressor by binding to sites overlapping the *ntrB* promoter to interfere with initiation of transcription and to maintain low intracellular levels of both NtrB and NtrC (216, 230). In limited nitrogen conditions, NtrB phosphorylates NtrC at Asp-54 and NtrC-P activates σ^{54}–dependent transcription from *glnA*p2 while blocking the *glnA*p1 by binding to a overlapping region of the σ^{70}–dependent promoter (216, 230). The nitrogen status of the cell is sensed by uridyltransferase, which transmits the signal via the P_{II} protein to the sensory kinase, NtrB (144, 230, 260). In nitrogen limiting conditions NtrB autophosphorylates, and transfers the

phosphate to NtrC to generate the active form of NtrC (216, 260). In nitrogen excess conditions, the phosphatase activity of NtrB prevents the activation of NtrC (144, 216, 260). NtrC is still able to bind the promoter in the inactive form. However, only NtrC-P is capable of activating transcription as the phosphate group is required for ATPase activity, oligomerization, and the formation of the open complex (216, 217, 260).

Summary

B. burgdorferi must regulate specific combinations of genes in the appropriate host and at the correct time to survive and colonize the mammalian and arthropod host. Numerous environmental signals and cues, including temperature, pH, cell density, oxygen, and host factors, influence gene expression (65, 128, 129, 197, 227, 252, 256, 258, 281). However, few regulatory proteins in *B. burgdorferi* have been identified, and the characterization of these regulatory systems has just started. Two of the known global regulators include σ^S and BosR. This research project involved investigating the regulation of stress response in *B. burgdorferi*, in particular σ^S, and the role of NapA, a Dps/Dpr homolog, in that stress response.

Previously it was suggested that σ^S expression is dependent on another sigma factor, σ^{54} (123), creating a complex regulatory cascade involving a two-component system in conjunction with σ^{54}-holoenzyme to activate transcription of *rpoS*, and allowing expression of σ^S-dependent genes. Chapter 2 confirms that expression of *rpoS* as *B. burgdorferi* cultures enter stationary phase is σ^{54}-dependent. However, *B. burgdorferi* has an additional regulation of *rpoS* that is independent of σ^{54}, which I found to express *rpoS* as cultures were diluted and recovering in fresh medium.

To analyze the σ^{54}-dependent expression of *rpoS* further, I began investigating the two-component system that is required for activation of σ^{54}. A two-component system consists of a cognate histidine kinase that senses an environmental cue, autophosphorylates, and activates the response regulator by transferring the phosphate group. The genome of *B. burgdorferi* revealed a putative histidine kinase (SisK) and a response regulator (SisR) that could be involved in the activation of σ^{54}. The characterization of the cognate histidine kinase and response regulator is discussed in Chapter 3.

The survival of *B. burgdorferi*, as cultures enter stationary phase is not the only stress that the bacteria must overcome for survival. During the ticks feeding cycle ROS are encountered in the tick salivary glands (J. Garcia-Lara, unpublished data) and in the skin *B. burgdorferi* is also challenged by ROS released by neutrophils (147, 292). Recently, BosR was described as a global regulator of the oxidative stress response in *B. burgdorferi* and NapA was one of the proteins identified to be regulated by BosR (51). Initial studies of NapA show that expression increases when cells are grown at high levels of O_2 (>8%) or when anaerobically grown cells are exposed to ROS, such as *t*-butyl hydrogen peroxide (51, 258). Therefore, I investigated the role of NapA in *B. burgdorferi* response to oxidative stress (Chapter 4).

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Chapter 2

Differential Expression of σ^S in *Borrelia burgdorferi* from σ^{54}-Dependent and σ^{54}-Independent Promoters[1]

[1] Treglown, J. S., J. G. Frye, A. Elias, P. Rosa, F. C. Gherardini, T. R. Hoover. 2004. To be submitted to *Journal of Bacteriology*.

Abstract

As in other bacteria, the sigma factor RpoS (σ^S) functions as a global gene regulator in *Borrelia burgdorferi* (11, 43). Expression of *B. burgdorferi* σ^S has previously been shown to be dependent on the alternative sigma factor, σ^{54}, which is encoded by *rpoN* (21). In this study, we show that the *rpoS* gene has two promoters, one that maps to the σ^{54}-dependent promoter and a second that is independent of σ^{54}. Quantitative RT-PCR assays revealed that levels of *rpoS* transcript in stationary phase were significantly reduced in a strain lacking σ^{54}. In addition, western blot analysis showed that levels of σ^S were dramatically reduced in a *B. burgdorferi rpoN* mutant strain in cells harvested during stationary phase. Cultures of *B. burgdorferi rpoN* mutant strain that were diluted in fresh medium, however, displayed relatively high levels of both *rpoS* transcript and σ^S antigen, the levels of which were indistinguishable from those found in the wild-type parental strain. These data confirm the differential expression of *rpoS* from σ^{54}-dependent and σ^{54}-independent promoters, with the σ^{54}-dependent promoter being primarily responsible for expression of σ^S during stationary phase. In addition, these findings suggest roles for σ^S in *B. burgdorferi* in response to an unknown stress upon dilution into fresh medium as well as a role in stationary phase.

Introduction

Lyme disease, which is caused by the spirochete *Borrelia burgdorferi,* is the most prevalent reported arthropod-borne disease in the United States (1). *B. burgdorferi* is maintained in a rodent reservoir and is transmitted between individual hosts by a tick vector of the genus *Ixodes* (5, 39). While the larvae remain dormant for up to nine months, spirochetes remain present at low levels in the tick midgut (9, 29, 36, 38). In this natural environment,

B. burgdorferi encounters nutrient limitations resulting in a decline in the numbers of spirochetes in the tick midgut to a low, relatively stable number of bacteria (9, 29). When the tick feeds, *B. burgdorferi* undergoes rapid growth prior to transmission to the new host (3, 9, 15, 29, 32, 36). The spirochete alters gene expression during the transition from stationary phase to rapid growth which allows it to survive the various environments (6, 8, 9, 12, 13, 22, 29). Little is known, however, about the molecular mechanisms that coordinate these changes in gene expression.

Changes in gene expression due to environmental cues, such as temperature, pH, or growth phase, can be regulated by various sigma factors. In enteric bacteria, sigma factor RpoS (σ^S) regulates the expression of specific genes as cells enter stationary phase or in response to osmotic stress (17, 25). *E. coli* σ^S responds to nutrient starvation by transitioning to stationary phase, which allows them to survive this environmental stress (18, 19). In *B. burgdorferi*, levels of σ^S have been shown to increase as cultures enter stationary phase or in response to decreased pH (43).

Elias and coworkers demonstrated that *B. burgdorferi* σ^S participates in the stationary phase related adaptive response by altering protein expression (11). Inactivation of *B. burgdorferi rpoS* results in a slight increase in sensitivity to osmotic shock and altered expression of several proteins, such as outer surface protein C (OspC) and decorin-binding protein C (DbpC) (11). Hübner and colleagues suggested that sigma factor RpoN (σ^{54}) controls the expression of RpoS in *B. burgdorferi*, which in turn regulates the expression of OspC and DbpC (21). However, only indirect evidence was demonstrated to support this RpoN-RpoS regulation of OspC and DbpC.

To examine the role of σ^S in *B. burgdorferi*, we constructed an RpoN knockout strain (B31-ARpoN) and monitored the expression of *rpoS* versus *B. burgdorferi* wild-type (B31-A) as

cultures entered stationary phase. Levels of both *rpoS* mRNA and σ^S in *B. burgdorferi* wild-type cultures increased as cultures entered stationary phase. The transcriptional start site for *rpoS* as cells entered stationary phase was mapped to the σ^{54}-dependent promoter. Thus, providing the first direct evidence that expression of *rpoS* in *B. burgdorferi* during stationary phase is dependent on σ^{54}-RNA polymerase holoenzyme (σ^{54}-holoenzyme). Interestingly, in *B. burgdorferi* wild-type and *rpoN* mutant cells *rpoS* expression increased independent of σ^{54}, when cells were diluted in fresh medium. This suggests that transcription of *B. burgdorferi rpoS* originates from at least two promoters, one of which is independent of σ^{54}-holoenzyme. Transcription of *rpoS* in stationary phase cultures of *B. burgdorferi* appeared to occur predominately from the σ^{54}-dependent promoter; while in late log phase *rpoS* expression appears to be σ^{54}-independent.

Materials and Methods

Bacterial strains and growth conditions. *B. burgdorferi* strains were grown in a modified Barbour-Stoenner-Kelly (BSKII) (2) medium at 34°C under an atmosphere of 5% O_2/5% CO_2/90% N_2. The BSKII medium was supplemented with 2 μg/ml coumeromycin A1 (Sigma, St. Louis, MO) or 200 μg/ml kanamycin (Sigma) as indicated. Cultures were inoculated at a density of $5x10^6$ cells/ml and enumerated using dark-field microscopy until the desired cell densities were obtained. *Escherichia coli* strain Rosetta [BL21 (DE3) pLysS] (Invitrogen, Carlsbad, CA) was grown in LB medium at 37°C and supplemented with 100 μg/ml ampicillin (Sigma) or 100 μM isopropyl β-D-thiogalactopyraniside (IPTG, Sigma) as indicated.

Primer extension analysis of *rpoS* promoter. Total RNA was isolated from a *B. burgdorferi* high-passage strain B31-A (P. Rosa, Hamilton, MT) culture in stationary phase ($2x10^8$ cells/ml)

by a phenol extraction method using TRI-Reagent (Sigma) as described by the manufacturer, and the RNA was quantified by measuring absorbance at 260 nm. One μg of RNA was used as template for primer extension reactions with the Primer Extension System AMV (Promega, Madison, WI). A sequence ladder was generated using the *fmol* Cycle Sequencing System (Promega) using 1 μg chromosomal DNA isolated from *B. burgdorferi* B31-A. For primer extension and sequencing reactions, primers rpoS-4 and up-PE were labeled at the 5'-end with ^{32}P[γ]-ATP (3,000Ci/mmole) (ICN, Costa Mesa, CA) using T4 polynucleotide kinase (Promega). Reaction products were separated by electrophoresis on a 6% polyacrylamide, 7M urea gel. Reaction products were visualized by autoradiography.

Reverse transcriptase-PCR. Reverse transcriptase PCR (RT-PCR) was done with the Access RT-PCR system (Promega) according to manufacturer recommendations. For *rpoS* expression levels, oligonucleotide primers rpoS-1 and rpoS-2 were used. *flaB* transcripts, which have been reported to remain relatively constant throughout various growth phases for *B. burgdorferi*, were used as a control, and primers flaB-1, and flaB-2 (Table 1) were used (31). To identify the σ^{54}-independent *rpoS* promoter, RT-PCR linkage experiments were done using the oligonucleotide primers rpoS-4, flgI-1 and flgI-ds1 (Table 1). RNA was isolated as described above from cultures with cell densities of $1x10^7$, $5x10^7$, $1x10^8$, and $2x10^8$ cells/ml, as well as, from a culture that had been in stationary phase for 24h (cell density $2x10^8$). Products were separated on a 1% agarose gel by electrophoresis and visualized by ethidium bromide staining.

Quantitative RT-PCR was performed using the one-step reaction, TaqMan Gold RT-PCR Kit (PE Applied BioSystems, Foster City, CA) as previously described (42). Using reaction components and instrument parameters recommended by the manufacturer, primers and probes specific for *rpoS* and *flaB* were designed using the Primer Express 1.0 program (PE Applied

BioSystems) and synthesized by PE Applied BioSystems (Table 1). All components were supplied from PE Applied BioSystems and the final concentration of the reaction components were: 1 X TaqMan buffer A, 300 nM each deoxynucleotide triphosphate, 5.5 mM $MgCl_2$, 0.4 units/μl RNase inhibitor, 0.25 units/μl Multi scribe reverse transcriptase, 0.025 units/μl Amplitaq Gold DNA polymerase, 900 nM each PCR primer, and 250 nM probe. All reactions were carried out on the ABI PRISM 7700 Sequence Detection System (PE Applied BioSystems). Assays were done in duplicate, and each quantitation was performed in triplicate. A standard curve based on dilutions of known amounts of RNA was used to quantify the amount of each transcript generated. Each transcript was normalized by comparison to the constant, internal control *flaB*.

Generating mutations in *rpoN* in *B. burgdorferi*. To inactivate *rpoN* in *B. burgdorferi* strain B31-A, *rpoN* was amplified by PCR with primers rpoN-1 and rpoN-2 (Table 1). The resulting PCR product was digested with *Hind*III to produce an 827 bp internal fragment of *rpoN* which was introduced into the *Hind*III restriction site of pJLB12a, that bears a kanamycin resistance gene under control of the *B. burgdorferi flaB* promoter to generate the plasmid pRpoN (4). The resulting plasmid was transformed into *B. burgdorferi* high-passage strain B31A as described by Samuels *et al* (34). After electroporation cells were resuspended in 5 ml BSKII medium, incubated at 34°C for 24 h and plated on BSKII medium containing agarose and 200 μg/ml kanamycin (34). Chromosomal DNA from kanamycin-resistant mutants was analyzed by PCR using rpoN-1 and rpoN-2 primers (Table 1) to confirm insertion of the plasmid into *rpoN*.

Purification of hexahistidine-tagged *B. burgdorferi* σ^S. *B. burgdorferi rpoS* was amplified by PCR with primers rpoS-1 and rpoS-2 (Table 1). The resulting PCR product was digested with *Nde*I and *BamH*I and cloned into the T7 expression vector pET28a (Novagen, San Diego, CA)

digested with *Nde*I and *BamH*I to generate pRpoS. The resulting pRpoS plasmid was introduced into *E. coli* strain Rosetta [BL21 (DE3) pLysS] and selection for by kanamycin resistance. For overexpression *E. coli* strain Rosetta [BL21 (DE3) pLysS] cells containing pRpoS were grown in 1 L of LB at 37°C with shaking. When cells reached A_{600} of 0.5, expression of the hexahistidine-tag σ^S was induced with 1 mM IPTG for 3 h. The cells were harvested by centrifugation at 12,000 x g for 20 min at 4°C, and His-RpoS was purified by using Ni-nitriolotriacetic acid resin (Qiagen, Valencia, CA) following the manufacturer's protocol for the denaturing purification of insoluble proteins. One ml fractions were collected and analyzed by SDS-PAGE. Those fractions containing His-RpoS were pooled and stored at -20°C until needed. Purified His-RpoS was used to raise polyclonal antiserum in a New Zealand white rabbit (Cocalico Co., Reamstown, PA). Antiserum was cross-adsorbed with cell lysate from *B. burgdorferi* strain B31-A74 (*rpoS* mutant) (P. Rosa, Hamilton, MT) as described previously (7, 11).

Immunoblotting methods. *B. burgdorferi* B31-A, B31-A74 (11) and B31-ARpoN cells were grown as described above, washed twice with 20 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 50 mM NaCl, 1 mM phenylmethylsulfonyl fluoride buffer pH 7.6 (Haley's buffer) and resuspended in ice cold Haley's buffer. Cells were lysed with 10 3-sec pulses of a sonic probe using a 50 Sonic Dismembrator (Fisher Scientific, Suwanee, GA) set at 50% power. Total protein was determined by a modified Lowry assay (26). Proteins (20 μg) were separated on a SDS-12% polyacrylamide gel, electrophoretically transferred to 0.2 μm nitrocellulose membrane (Bio-Rad, Hercules, CA), and blocked with 5% non-fat milk in PBS for 1 h at room temperature. The primary antibody, cross-adsorbed α-σ^S, was applied to the blot and incubated for 1 h. The blot was washed 3 times for 10 min with TTBS (100 mM TrisCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) and secondary antibody (goat-α-rabbit peroxidases

conjugate, Sigma) was applied to the blot for 1 h (7). The blot was washed three additional times for 10 min with TTBS and was visualized by chemiluminescence using ECL reagents (Amersham Bioscience, Piscataway, NJ).

5' RACE system. *B. burgdorferi* low-passage stain B31-A3 (P. Rosa, Hamilton, MT) cells were grown in BSKII medium to late-log phase ($5x10^7$ cells/ml) and diluted into fresh BSKII medium. Cells were allowed to grow for an additional 12 or 16 hours to recover. Total RNA was extracted as described. The 5' RACE system for rapid amplification of cDNA ends (Invitrogen, Carlsbad, CA) was used following manufacturer's recommendations. cDNA was generated from 500 ng of RNA isolated from either 12 or 16 hours after dilution in fresh medium using oligonucleotide primer up-GSP1. After purification of the cDNA and TdT tailing of the cDNA, oligonucleotide primers up-GPS2 and up-nested were used to amplify the product. PCR products were then separated by agarose gel electrophoresis and visualized by ethidium bromide staining.

Results

***Borrelia burgdorferi rpoS* is expressed from a σ^{54}-dependent promoter.** The *B. burgdorferi* B31 genome sequence revealed a potential σ^{54}-dependent promoter located approximately 65 bp upstream of the putative *rpoS* start codon (Figure 1, panel A) (21, 40). To confirm that *rpoS* was transcribed from the σ^{54}-dependent promoter, primer extension reactions were performed using RNA isolated from *B. burgdorferi* strain B31-A culture in stationary phase ($2x10^8$ cells/ml), when *rpoS* expression should be at the highest. Using the rpoS-4 primer within the *rpoS* coding region, we detected an extension product that terminated 13 bp downstream of the GC doublet of the σ^{54}-dependent promoter sequence (Figure 1, panel B). This transcripts originates 12 to 14 bp

downstream of the GC doublet, which is consistent with the sequence of a σ^{54}-dependent promoter (27).

To confirm that the identified sequence was a *bona fide* σ^{54}-dependent promoter, a *B. burgdorferi* mutant that lacked σ^{54} was constructed by interrupting *rpoN* through plasmid integration. An 827 bp internal *Hind*III fragment of *rpoN* was cloned into pJLB12a, which bears a kanamycin resistance gene under control of the *B. burgdorferi flaB* promoter but does not contain a *Borrelia* origin of replication (4). The resulting plasmid (pRpoN) was introduced into *B. burgdorferi* high-passage strain B31A by electroporation. Transformants in which the plasmid had integrated into the chromosome were selected on BSKII medium containing kanamycin.

Chromosomal DNA from 60 kanamycin-resistant transformants was analyzed by PCR for insertion of the plasmid into *rpoN*. Two of these mutants yielded a 4.1 kb PCR product, which was expected for insertion of the plasmid into *rpoN* (data not shown). The insertion into *rpoN* was confirmed by sequence analysis, which established that the plasmid had integrated into the second *Hind*III site of *rpoN* resulting in a truncated gene (data not shown). RNA was isolated from a stationary phase culture ($2x10^8$ cells/ml) of the *B. burgdorferi* B31-ARpoN strain, and primer extension reactions were performed. No extension product from the σ^{54}-dependent promoter was detected (Figure 1, panel B), indicating that *B. burgdorferi rpoS* is transcribed from this σ^{54}-dependent promoter in stationary phase.

RT-PCR analysis of the *B. burgdorferi* B31-ARpoN strain. RT-PCR was used to confirm that disruption of *rpoN* altered expression of *rpoS* in *B. burgdorferi*. The presence of *flaB* mRNA was monitored as a positive control. *flaB* mRNA was comparable for the B31-A and the B31-ARpoN strains from mid-log phase ($5x10^7$ cells/ml) (Figure 2, panel A). In contrast, *rpoS*

mRNA was significantly reduced, but still present, in RNA samples prepared from the *rpoN* mutant strain compared to the wild-type strain. These data suggest the possibility of a second promoter in addition to the σ^{54}-dependent promoter. RT-PCR again was used to determine if *rpoS* was cotranscribed with *flgI*. RT-PCR linkage experiments detected RNA spanning the junction between *rpoS* and *flgI*, which is 389 bp upstream of the σ^{54}-dependent *rpoS* promoter (Figure 2, panel B). These data suggests that the *rpoS* transcripts observed in B31-ArpoN strain originated from inside the upstream flagellar gene, *flgI*.

Quantitative RT-PCR analysis was used to assess levels of *rpoS* transcript in the *B. burgdorferi* B31-A and B31-ArpoN strains at different growth phases. Levels of *rpoS* mRNA were quantitated and normalized to levels of *flaB* transcripts (31). Cells from cultures in log growth phase were inoculated into fresh medium at a density of ~$5x10^6$ cells/ml. Both strains grew with a generation time of approximately 8 h until the cultures achieved cell densities in stationary phase of ~$2x10^8$ cells/ml. RNA was isolated from cultures in which cell densities were $1x10^7$, $5x10^7$, $1x10^8$, or $2x10^8$ cells/ml. In addition, RNA was isolated from a culture that had been in stationary phase for 24 h ($2x10^8$ + 24 h). As cultures of the wild-type strain entered stationary phase ($2x10^8$ cells/ml), the levels of *rpoS* transcript increased 4-fold relative to *rpoS* mRNA in cells from late-log phase ($5x10^7$ cells/ml) (Figure 3, panel A). In contrast, levels of *rpoS* transcript in the *rpoN* mutant only increased ~1.5-fold following the transition from log to stationary phase, indicating that σ^{54} is required for maximal transcription of *rpoS* as cultures enter stationary phase. Interestingly, both the *rpoN* mutant and wild-type *B. burgdorferi* strains displayed relatively high levels of *rpoS* mRNA at the earliest time point taken ($1x10^7$ cells/ml). This pattern of *rpoS* expression was observed in three independent experiments and showed that the high levels of *rpoS* mRNA at the earliest time point ($1x10^7$ cells/ml) is σ^{54}-independent.

σ^S levels in *B. burgdorferi* cultures in log and stationary growth phases. Immunoblots were used to examine σ^S levels in *B. burgdorferi* at the various cell densities to determine if the high levels of *rpoS* mRNA at the earliest time point ($1x10^7$ cells/ml) could also be observed at the protein level. As observed with *rpoS* transcripts, σ^S levels in *B. burgdorferi* strain B31-A were high at the earliest time point examined ($1x10^7$ cells/ml), dropped during late log phase ($5x10^7$ cells/ml), then increased again as the culture entered stationary phase ($2x10^8$ cells/ml) (Figure 3, panel B). The pattern of σ^S accumulation in the *B. burgdorferi* strain B31-ARpoN varied from that observed in the wild-type strain. In the *B. burgdorferi* strain B31-ARpoN, like *B. burgdorferi* strain B31-A, the amount of σ^S was highest at the earliest time point ($1x10^7$ cells/ml) examined and decreased during late-log phase ($2x10^8$ cells/ml). However, the level of σ^S did not recover as the *B. burgdorferi* B31-ARpoN culture entered stationary phase as it did in the wild-type parental strain (Figure 3, panel B).

In other experiments, we inoculated cultures to a final concentration of $1x10^6$ cells/ml and harvested the cells 24 h later when they had reached a density of $2x10^6$ cells/ml. These cells expressed high levels of σ^S, (data not shown) suggesting that the transient increase in *rpoS* expression in mid-log phase is the result of dilution in fresh BSKII medium rather than cell density. This dilution effect may be caused by oxygen or a component of the medium that induces a stress response in *B. burgdorferi*.

Location of second promoter for *rpoS* expression. The 5' RACE system was used to identify the region from which the σ^{54}-independent transcripts of *rpoS* originated. cDNA was generated from RNA isolated from *B. burgdorferi* strain B31-A3 diluted in fresh BSKII and allowed to grow for 12 or 16 h. The cDNA was amplified following manufacturer's protocols using up-GSP2 and up-nested primers designed upstream of the σ^{54}-dependent promoter. Controls in

which no reverse transcriptase were included in the assay mixture were also performed to ensure that there was not any contaminating DNA in the RNA samples. Amplification with the 5' RACE system resulted in a product of approximately 270 bp that mapped about 40 bp inside the 3'-end of *flgI* (data not shown).

Primer extension was used to confirm the σ^{54}-independent transcription of *rpoS* and map the start of the putative transcript. *B. burgdorferi* strain B31-A3 culture diluted in fresh BSKII medium was allowed to grow for 12 or 16 h, and RNA was isolated. Using a primer upstream of the σ^{54}-dependent promoter, we detected an extension product that terminated ~13 bp from the 3'-end of *flgI* and about 402 bp upstream of the GC doublet located within the σ^{54}-dependent *rpoS* promoter (Figure 4).

Discussion

Global gene regulation in bacteria is often accomplished through changes in the levels or activities of alternative sigma factors. In *E. coli*, regulation of gene expression by σ^S is responsible for the transcription of a variety of genes as the cells enter stationary phase or during certain types of starvations and stress (18-20, 24). In enteric bacteria, increased expression of σ^S-dependent genes is the result of increases in the cellular levels of σ^S (18). These increased levels of σ^S result from higher rates of transcription and translation of *rpoS*, as well as increased stability of σ^S (24).

In *E. coli*, σ^S levels are controlled in a variety of ways, including transcription initiation and translational elongation (24, 41). Some of the major factors that influence σ^S levels are post-translational events that lead to increased stability of the protein. During exponential growth at 37°C, σ^S has a half-life of less than 2 minutes. As the cells enter stationary phase or encounter

certain stress conditions, the half-life of σ^S increases to greater than 30 minutes. The ClpXP protease is a cytpolasmic, ATP-dependent protease that is responsible for the rapid degradation of σ^S during exponential growth (37). This rapid degradation of σ^S by ClpXP requires RssB (also called SprE), a protein that shares homology with the family of response regulators and appears to specifically modulate the activity of σ^S as well as its degradation (24, 37). The heat shock protein DnaK has also been shown to have a positive role in the post-translational control of σ^S. DnaK appears to be involved in the transduction of at least two signals, heat shock and carbon starvation, that result in reduced σ^S turnover (33).

B. burgdorferi lacks most of the factors involved in the regulation of proteolysis of σ^S in enterics, specifically, the protease ClpXP and RssB (14). Further investigation, however, is needed to confirm that regulated proteolysis or other post-transcriptional control mechanisms are not important for modulating σ^S concentrations in *B. burgdorferi*. However, the amount of *rpoS* transcript, although not perfectly, correlated with σ^S levels (Figure 3), suggesting that proteolysis is not a major mechanism for regulating levels of σ^S in *B. burgdorferi* as it is in enteric bacteria (24).

In *E. coli*, the products of genes that are regulated by σ^S have a variety of functions, including virulence factors, septum formation, glycine betaine and proline transport, trehalose synthesis, protection against H_2O_2, synthesis and degradation of glycogen, actetate synthesis, and DNA repair (16, 18, 25). These genes often have more than one promoter, with only one of these promoters being dependent on σ^S. Alignment of σ^S-dependent promoters has suggested a –10 consensus sequence of TATACT, which is very similar to that of σ^{70}-dependent promoters. There is, however, no clear homology in the –35 region for known σ^S-dependent promoters.

This may reflect the need for additional *cis*-acting regulatory factors that can compensate for the low affinity of RNA polymerase for the –35 region (18).

The expression of σ^S in *B. burgdorferi*, has been shown to be responsible for changes in gene expression in response to environmental insults or during stationary phase (11, 43). Moreover, σ^S controls the expression of virulence related genes necessary for pathogenesis and survival of some bacteria and has been shown to regulate expression of important lipoproteins in *B. burgdorferi* (10, 11).

Transcription of *E. coli rpoS* involves multiple promoters, but none of these promoters are dependent on σ^{54} (23). Potential σ^{54}-dependent promoter sequences occur upstream of *rpoS* in *Enterobacter cloacae* and *Pseudomonas syringe*, but these sequences have not yet been shown to be *bona fide* σ^{54}-dependent promoters (40). Hübner and colleagues have suggested that σ^{54} controls the expression of σ^S in *B. burgdorferi*, which in turn regulates the expression of OspC and DbpC (21), but no direct evidence was reported to support this hypothesis. The mapping of the transcriptional start site of *rpoS* to the σ^{54}-dependent promoter that we report here provides evidence for σ^{54}-holoenzyme being directly responsible for the expression of *rpoS* in *B. burgdorferi*.

Transcription initiation with σ^{54}-RNA polymerase holoenzyme requires an enhancer-binding protein (28, 30, 35). The *B. burgdorferi* genome sequence contains only one open reading frame (locus BB0763) that encodes an enhancer-binding protein, and has been shown to be required for expression of *rpoS* (44). The *B. burgdorferi* enhancer-binding protein shares homology with response regulators of the two-component systems, and a potential histidine kinase is encoded by an adjacent open reading frame (BB0764) (Figure 1, panel A). This two-component system has been suggested to be part of a RpoN-RpoS regulatory cascade (44). We

postulate that this regulatory cascade involving σ^{54} and a two-component system activates transcription of *rpoS* as *B. burgdorferi* cultures enter into stationary phase, possibly in response to a signal or environmental cue that accumulates as cultures approach stationary phase.

An unexpected observation was a high level of *rpoS* expression in mid-log phase cultures of *B. burgdorferi* that was independent of σ^{54} (Figure 3). Yang and coworkers reported high levels of σ^{S} in *B. burgdorferi* strain 297 during stationary phase, but no significant amounts of σ^{S} in cells during mid-log phase ($2x10^{7}$ cells/ml) unless the cells were cultured at pH 6.8 instead of pH 7.5 (43). The cultures that were used in those experiments were a different *B. burgdorferi* strain and had been grown for approximately 16 generations before they were harvested, whereas in our experiments, cells had been cultured for only one generation prior to the first time point. Hübner and co-workers reported no expression of *rpoS* in their *B. burgdorferi* 297 *rpoN* mutant by RT-PCR or by examining protein expression profiles (21). However, proteins were from a different *B. burgdorferi* strain and only isolated at a late-logarithmic phase of growth in this previous study, a timepoint in which expression of σ^{S} is dependent on σ^{54}.

Expression of *rpoS* in mid-log phase cannot be accounted for by residual expression from the stationary phase cells that we used to inoculate the cultures, as expression of *rpoS* in mid-log phase was independent of σ^{54}, whereas it was σ^{54}-dependent in stationary phase. This dilution effect may be caused by oxygen or a component of the medium that induces a stress response in *B. burgdorferi*. Further investigation is needed to identify the environmental signals that *B. burgdorferi* responds to following dilution into fresh medium as well as the signals that are sensed by the two-component system controlling σ^{54}-holoenzyme activity as cultures enter stationary phase.

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TABLE 1. Oligonucleotide primers used in this study

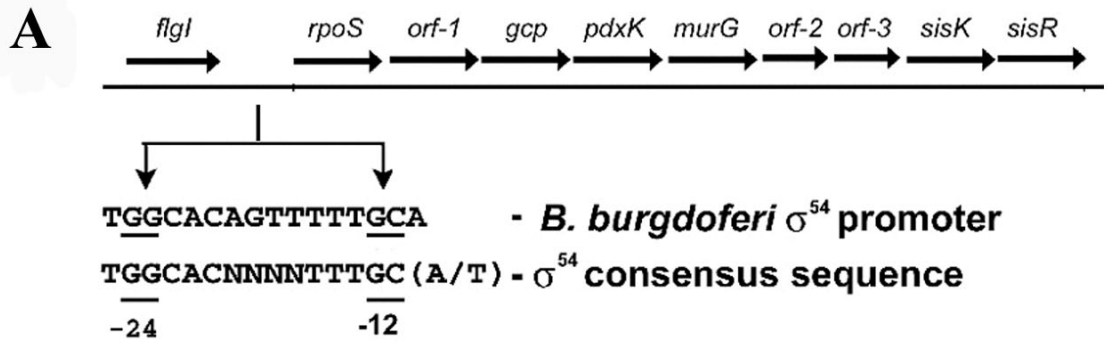
Primer	Primer Sequence (5'-3')
rpoS-1	GAAAATATAATACATATGAACATATTTAG
rpoS-2	TCATGAATGTCAAGCTTAATTTATTTC
rpoS-4	TATGTTTAAATCCTCATTACT
flaB-1	CCCATCGATAACGCTGCTAATCTTAGTAAA
flaB-2	CGCATCGATGAATTAACTCCGCCTTGAGAA
rpoN-1	GTGGATTTTTTTATACATTAATAT
rpoN-2	TCATTCAAGCTGTAATTAACC
flgI-1	CTTGGAGGAAATTGATGGAAACC
flgI-ds1	TGAAGATATGCTTTGCGAACA
up-GSP1	TAATTTCCATGCAAAAACTGTG
up-GSP2	GATATAATTTTGAGTTTAGGGGGAGT
up-nested	TTTGAGTTGTGCCATTTGT
up-PE	TAGATTTTGTGAATTAATTTTGGTTTCC
rpoS-215F	AAGAAGGCAACTTGGGATTAATAAGA
rpoS-308R	TGCTTAATCCAAAATGATGCATAAG
rpoS-242T	**FAM**[1]-CTGCTGAAAAATATGACCCGAATAAAAATACCAAATT-**TAM**[2]
flaB-586F	AATCTTTTCTCTGGTGAGGGAGCT
flaB-657R	TCCTTCCTGTTGAACACCCTCT
flaB-611T	**VIC**[3]-AAACTGCTCAGGCTGCACCGGTTC-**TAM**[2]

[1]-FAM = 6-carboxyflourescein

[2]-TAM = 6-carboxy-N, N, N', N'-tetramethylrhodamine

[3]-VIC = 6-carboxy-7, 2', 7'-tetrachlorofourescein

Figure 1. Primer extension analysis of *rpoS* transcripts. (A) Map of the *rpoS* region indicating the location of the σ^{54} promoter. The gene names are as identified in the genome sequence (TIGR). (B) Primer extension reactions were performed with RNA extracted from *B. burgdorferi* strains B31-A (1) and B31-ARpoN (2) at 2 x 10^8 cells/ml. The sequencing ladder was generated with the rpoS-4 primer. -24 and -12 doublets are indicated. Arrowhead (<) denotes the 5' end of the transcript.


A
flgI
rpoS
orf-1
gcp
pdxK
murG
orf-2
orf-3
sisK
sisR
TGGCACAGTTTTTGCA - B. burgdoferi σ54 promoter
TGGCACNNNNTTTGC(A/T) - σ54 consensus sequence
-24
-12

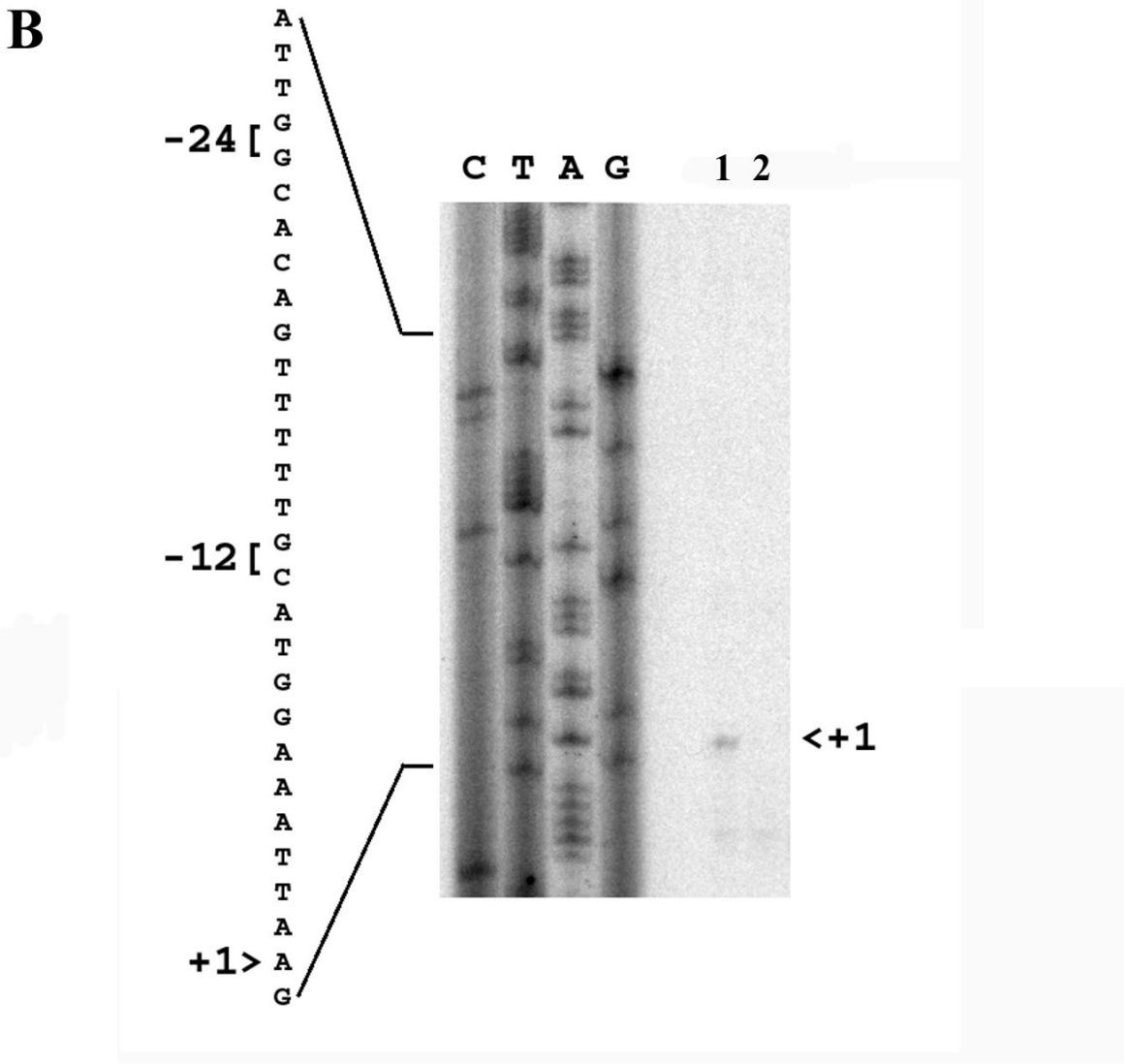

B
-24 [
-12 [
+1>
C T A G
1 2
<+1

Figure 2. RT-PCR analysis of *B. burgdorferi*. (A) *B. burgdorferi* strain B31-A and B31-ARpoN were grown to mid-log phase (5 x 10^7 cells/ml), RNA was extracted and RT-PCR was performed with primers for *rpoS* (lanes 2 and 6) or *flaB* (lanes 4 and 8). No reverse-transcriptase controls were also performed with primers for *rpoS* (lanes 1 and 5) or *flaB* (lanes 3 and 7). Size standards in kb are listed to the left. (B) RNA extracted from *B. burgdorferi* strain B31A after being diluted in fresh BSKII medium and grown for 12 or 16 h was used in RT-PCR linkage reactions with primers for flgI-1 and rpoS-4 (lanes 1 and 2), and flgI-ds1 and rpoS-4 (lanes 3 and 4). No reverse-transcriptase controls were also performed with the appropriate primers for lanes 1 and 3. Size standards in kb are listed to the left.

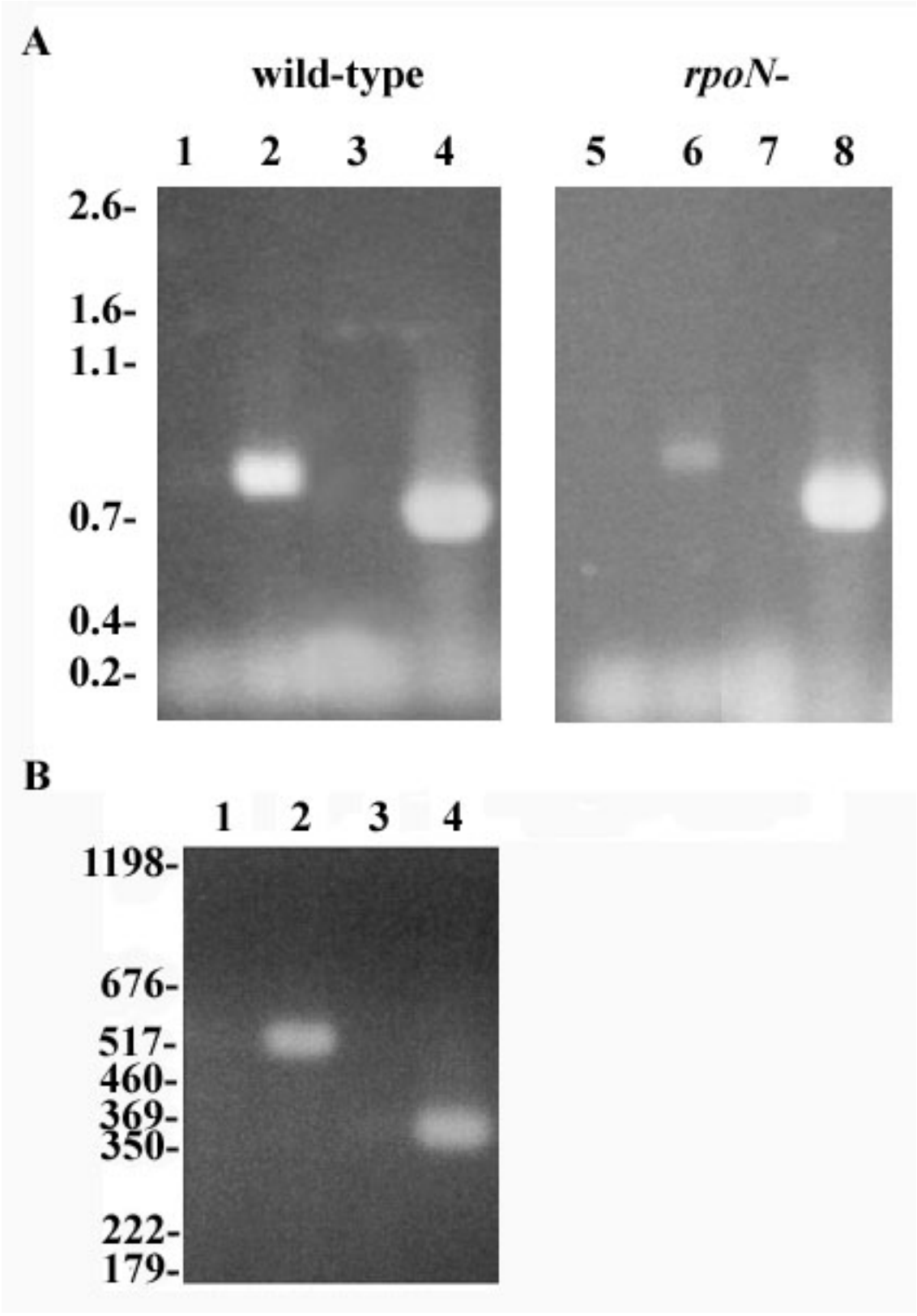
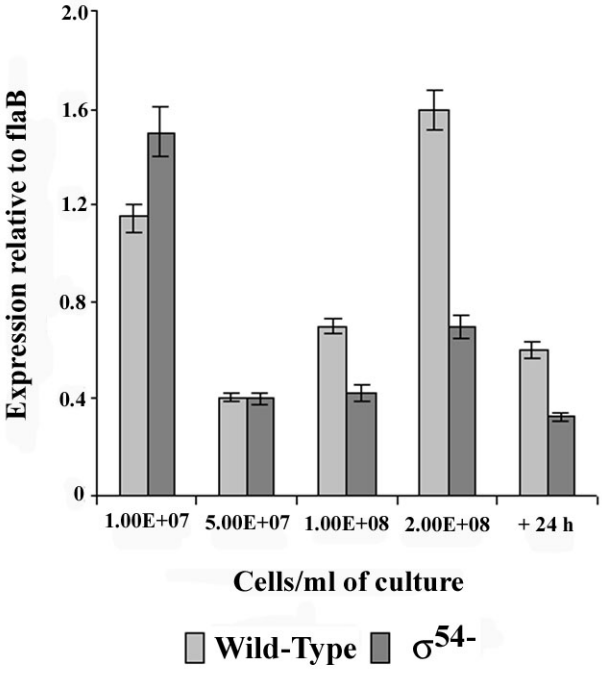

A
wild-type
rpoN-
1 2 3 4
5 6 7 8
2.6-
1.6-
1.1-
0.7-
0.4-
0.2-
B
1 2 3 4
1198-
676-
517-
460-
369-
350-
222-
179-

Figure 3. Quantitative RT-PCR analysis of *rpoS* transcripts and immunoblot analysis of σ^S. (A) RNA extracted from *B. burgdorferi* strains B31-A and B31-ARpoN were quantitated using specific primers and the Taqman system. Values have been normalized to the internal control, *flaB,* and are in arbitrary units. Numbers indicate cell densities per ml of culture. (B) Immunoblot analysis of σ^S levels in *B. burgdorferi* strains B31-A, B31-A74, and B31-ArpoN. Twenty μg of protein from *B. burgdorferi* B31 A (lane 1), B31A74 (lane 2), B31A (lanes 3-7), and B31-ARpoN (lanes 9-12) were separated by SDS-PAGE. Cell densities per ml of culture were: $1x10^7$ (lanes 3 and 8), $5x10^7$ (lanes 4 and 9), $1x10^8$ (lanes 5 and 10), $2x10^8$ (lanes 6 and 11), and $2x10^8$ + 24 hr (lanes 7 and 12). Blot was probed with polyclonal rabbit sera directed against purified recombinant *B. burgdorferi* σ^S. Size standards, in kDa, are indicated to the left.

A


2.0
1.6
1.2
0.8
0.4
0
Expression relative to flaB
1.00E+07
5.00E+07
1.00E+08
2.00E+08
+ 24 h
Cells/ml of culture
Wild-Type
σ54-

B

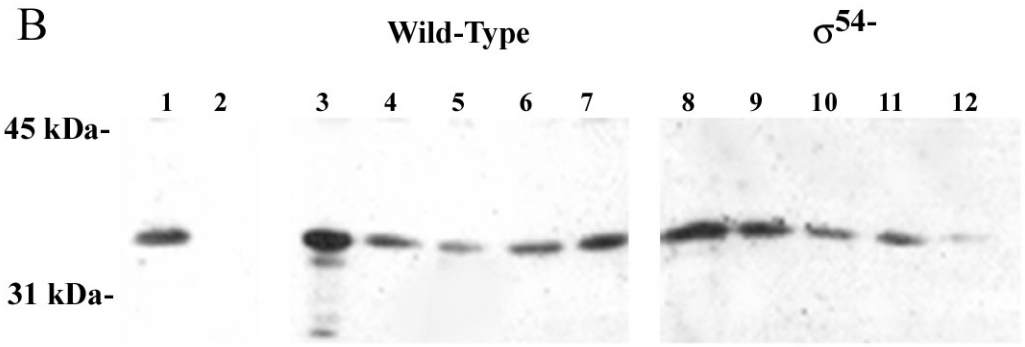
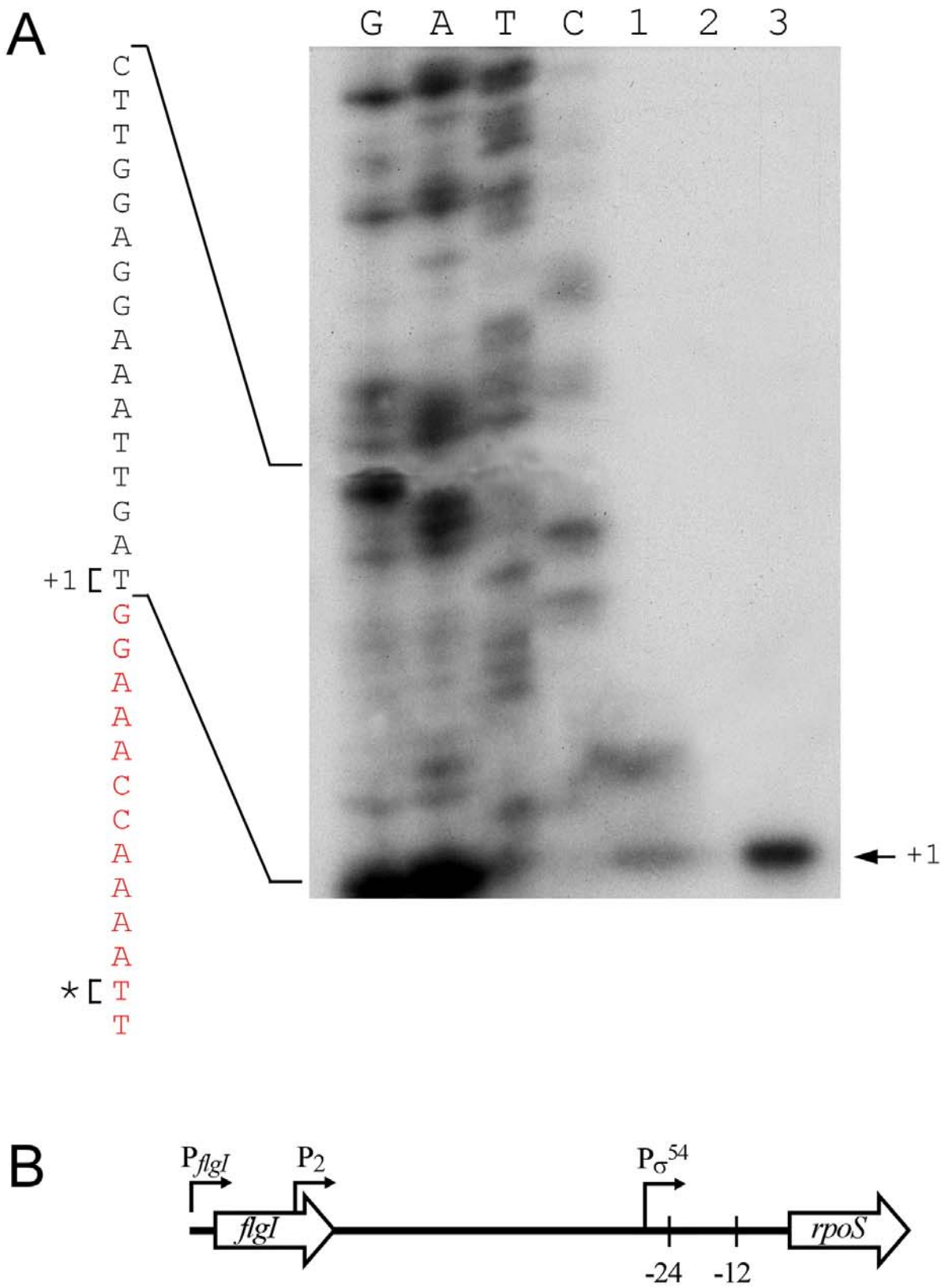

Wild-Type
σ54-
1 2 3 4 5 6 7 8 9 10 11 12
45 kDa-
31 kDa-

Figure 4. Primer extension analysis of the σ^{54}-independent promoter. (A) Primer extension reactions were performed with RNA extracted from *B. burgdorferi* strains B31-A3 (1) and B31-A3RpoN (3) after cells had been diluted into fresh medium and allowed to recover for 16 h. The sequencing ladder was generated with the up-PE primer. No reverse-transcriptase control was performed to confirm that there was no DNA contamination of the RNA (2). The 5' end of the message is indicated by the arrowhead. The sequence in red indicates downstream sequence of the 5' end of the transcript. * indicates the 3' end of *flgI*. (B) Map of the *flgI-rpoS* region indicating the location of the *flgI* promoter (P_{flgI}), σ^{54}-dependent promoter ($P_{\sigma 54}$), and the σ^{54}-independent promoter (P_2)


A
G A T C 1 2 3
C
T
T
G
G
A
G
G
A
A
A
T
T
G
A
+1 T
G
G
A
A
A
C
C
A
A
A
A
* T
T
+1
B
P flgI
P2
P σ54
flgI
rpoS
-24
-12

Chapter 3

Characterization of the SisK-SisR Two-Component System Required for σ^{54}-Dependent Expression in *Borrelia burgdorferi*

Abstract

In *Borrelia burgdorferi* the expression of the sigma factor RpoS (σ^S) is both σ^{54}-dependent and σ^{54}-independent, with expression of *rpoS* from the σ^{54}-dependent promoter occurring as cells enter stationary phase. We investigate here the two-component system that is required for activation of σ^{54}-dependent transcription of *B. burgdorferi rpoS*. *B. burgdorferi* ORF BBO763, designated sigma-s regulatory protein (SisR) has previously been implicated as the σ^{54}-dependent activator (38). Directly upstream of SisR is a putative histidine kinase (ORF BBO764), sigma-s histidine kinase (SisK). Quantitative RT-PCR showed that expression of *rpoS* as cells entered stationary phase was significantly reduced in a strain lacking SisK compared to the wild-type strain. These data confirmed that expression of *rpoS* from the σ^{54}-dependent promoter requires the histidine kinase, SisK. Phosphorylation data demonstrated that SisK has autophosphorylation activity and can transfer the phosphate group to SisR, providing direct evidence that the two proteins are part of the same two-component system. Although gel mobility shift assays were inconclusive in showing that SisR recognized a specific sequence upstream of the *rpoS* σ^{54}-dependent promoter, experiments with *rpoS* promoter-*cat* fusions revealed that upstream sequences were important for maximal SisR-dependent expression of *rpoS*.

Introduction

Borrelia burgdorferi is the causative agent of Lyme disease which is the most common reported arthropod-borne disease in the United States and Europe (1). *B. burgdorferi* is maintained in nature in two very distinct environments, the *Ixodes* tick and mammalian hosts (6, 32). How the spirochete is able to adapt and survive in these very different environments by

altering gene expression has been the subject of many studies (7-9, 12, 13, 17, 24). The ability of bacteria to regulate transcription initiation allows for the coordinated expression of genes at appropriate times. Regulation of gene expression in *B. burgdorferi* is influenced by several factors, including temperature, pH, cell density, oxygen, and host factors (7, 16, 17, 22, 25, 28-30, 33).

Before transcription can occur, core RNA polymerase ($\alpha_2\beta\beta'$) must combine with the dissociable sigma (σ) subunit to form RNA polymerase holoenzyme ($\alpha_2\beta\beta'\sigma$) (5, 26, 34). Recently, the role of σ factors in gene expression was investigated in *B. burgdorferi* (10, 14, 36). The sigma factor σ^S (encoded by *rpoS*) is involved in the regulation of the *B. burgdorferi* stationary phase related response and the expression of membrane lipoproteins such as outer surface protein C (OspC) and decorin-binding protein C (DbpC) (10, 37). RpoS expression during stationary phase is dependent on the sigma factor σ^{54} (14, 36).

A unique characteristic of σ^{54} is the mechanism and specific requirements for transcription initiation with σ^{54}-RNA polymerase holoenzyme (σ^{54}-holoenzyme). σ^{54}-holoenzyme is able to bind promoters and form a stable closed complex, but the closed complex is unable to undergo isomerization to an open complex in the absence of an activator and ATP hydrolysis (3, 5, 26, 31, 34). Approximately half of the σ^{54}-dependent activators in the Pfam database are part of two-component systems, including the well characterized paradigm *Salmonella enterica* serovar Typhimurium nitrogen regulator protein C (NtrC), *Sinorhizobium meliloti* dicarboxylic acid transport protein D (DctD), and *Caulobacter crescentus* flagellar transcription activator D (FlbD) (19, 20, 31).

Typical two-component systems consist of a sensory histidine kinase and a response regulator. The histidine kinase modulates the phosphorylation state of the response regulator, at

a conserved aspartyl residue in the receiver in response to environmental signals or cellular cues (19, 20, 31). Where it has been examined, phosphorylation of the activator stimulates its activity by converting it from a dimer (off-state) to a higher-order oligomeric complex (on-state) which is the form of the protein that is capable of hydrolyzing ATP and activating transcription (21, 23).

A genome search of *B. burgdorferi* indicated that the response regulatory protein, Rrp2 (BBO764) is homologous to activators in the NtrC1 family. Recently, Yang and colleagues showed that a single amino acid change within the ATPase domain of Rrp2 eliminated σ^S-dependent lipoprotein expression by disrupting the cascade involved in the σ^{54}-dependent expression of σ^S (38). Therefore, Rrp2 has been renamed sigma-s regulatory protein (SisR). Directly upstream of SisR is a putative histidine kinase (BBO764). We hypothesized that this is the histidine kinase for the two-component system involved in σ^{54}-dependent activation, and designated this open reading frame (ORF) sigma-s histidine kinase (SisK). To examine the role of SisK in σ^{54}-dependent activation, we inactivated *sisK* and monitored the expression of *rpoS* versus that of *rpoS* in *B. burgdorferi* wild-type and an *rpoN* mutant during the transition from mid-log growth to stationary phase. Levels of *rpoS* mRNA increased significantly in wild-type *B. burgdorferi* cultures during this time, but failed to do so in the *rpoN* mutant strain, as previously reported (36). The *sisK* mutant strain displayed a pattern of *rpoS* expression that was intermediate to that of the wild-type and *rpoN* mutant strains, suggesting that SisK is involved in σ^{54}-dependent expression of *rpoS*, but there was some SisK-independent expression from the σ^{54}-dependent *rpoS* promoter. SisK and SisR were expressed in *Escherichia coli* and purified. The purified SisK autophosphorylated and transferred the phosphate to SisR, but the transfer was not very efficient. The poor phosphate transfer may reflect the need for additional factors from *B. burgdorferi* or could be due to problems associated with expression of SisK and SisR in *E.*

coli. Attempts to isolate *B. burgdorferi sisR* mutants were unsuccessful, suggesting a possible second role for the response regulator that is σ^{54}-independent and essential for cell viability.

Materials & Methods

Bacterial strains and growth conditions. *B. burgdorferi* strains were grown in a modified Barbour-Stoenner-Kelly (BSKII) (2) medium at 34°C under an atmosphere of 5% O_2/5% CO_2/90% N_2. The BSKII medium was supplemented with 200 μg/ml kanamycin (Sigma, St. Louis, MO) or 40 μg/ml gentamycin (Sigma) as indicated. Cultures were inoculated at a density of $5x10^6$ cells/ml and enumerated using dark-field microscopy until the desired cell densities were obtained. *E. coli* strains were grown in LB medium at 37°C and supplemented with 100 μg/ml ampicillin (Sigma), 30 μg/ml chloramphenicol, 5 μg/ml gentamycin, or 30 μg/ml kanamycin or 100 μM isopropyl β-D-thiogalactopyraniside (IPTG, Sigma) as indicated.

Isolation of *rpoN* and *sisK* mutants in *B. burgdorferi*. To remove a section of the multiple cloning site, pCRScript Cam (Stratagene, La Jolla, Ca) was digested with *BamH*I and *Kpn*I, treated with T4 DNA polymerase (Promega, Madison, WI) to create blunt ends, and religated to generate the plasmid pJon. The *rpoN* ORF (BBO450) from *B. burgdorferi* strain B31-A3 was amplified by PCR with primers ntrF3 and ntrR2 (Table 1). The resulting PCR product was cloned into pJon at the *Srf*I site creating the plasmid pJN. To inactivate *rpoN*, a kanamycin resistance cassette was amplified from pJLB12a (4) using primers kMunF and kMunR. The resulting PCR product was cloned into the *Mun*I site of pJN, generating a plasmid carrying *rpoN* interrupted with the kanamycin cassette, pJNK. The resulting plasmid was transformed into *B. burgdorferi* low-passage strain B31-A3 as described by Samuels *et al* (27). After electroporation cells were resuspended in 5 ml BSKII medium, incubated at 34°C for 24 h and plated on BSKII

medium containing agarose and 200 μg/ml kanamycin (27). Chromosomal DNA from kanamycin-resistant colonies was analyzed by PCR using ntrF3 and ntrR2 primers to confirm inactivation of *rpoN*.

To inactivate *sisK* (BBO764), the *sisK* ORF was amplified by PCR from *B. burgdorferi* B31-A3 with primers sisKBF and sisKBR (Table 1). The resulting PCR product was digested with *BamH*I to produce an internal fragment of *sisK*, and cloned into the *BamH*I restriction site of pCRScript Cam (Stratagene), generating the plasmid pSisK. The kanamycin resistance cassette (4) was amplified from pJLB12a using primers kSphF and kSphR generating a *Sph*I restriction site on the ends (4). The PCR product and pSisK were digested with *Sph*I and ligated to generate pSKK. The resulting plasmid was transformed into *B. burgdorferi* low-passage strain B31-A3 as described by Samuels *et al* (27), and kanamycin-resistant colonies were analyzed by PCR using sisKBF and sisKBR primers.

Purification of *B. burgdorferi* SisR and SisK. The *sisR* ORF (BBO763) was amplified by PCR from *B. burgdorferi* B31-A3 with primers act-exp and act-2 (Table 1). The resulting PCR product was digested with *Nde*I and *BamH*I and cloned in the T7 expression vector pT7-7 (35) to generate pAPO1. The *Nde*I-*Sal*I fragment containing SisR from pAPO1 was cloned into pET28a+ (Novagen, Madison, WI) generating the plasmid pAPO3. The pAPO3 plasmid was introduced into *E. coli* strain Rosetta [BL21 (DE3) pLysS] (Novagen) with selection for resistance to kanamycin. For overexpression, *E. coli* strain Rosetta [BL21 (DE3) pLysS] cells containing pAPO3 were grown in 1 L of LB with kanamycin at 37^{o}C with shaking. When cells reached A_{600} of 0.5-0.8, expression of the hexahisitidine-tag SisR (His-SisR) was induced with 1 mM IPTG for 4 h. The cells were harvested by centrifugation at 12,000 x g for 20 min at 4^{o}C, resuspended in lysis buffer (20 mM phosphate buffer, pH 7.4, 500 mM NaCl, 10 mM imidazole,

75 mM KSCN), and lysed by two passages through a chilled French Press cell at 10,000 psi. Unlysed cells were removed by centrifugation (10,000 x g, 20 min, 4^{o}C). To solublize His-SisR the cell pellet was resuspended in lysis buffer with 0.5% sarkosyl, incubated at 4^{o}C for 1 h with shaking, and cell lysate harvested by centrifugation (30,000 x g, 30 min, 4^{o}C). The cell lysate was applied to a 5 ml HiTrap chelating HP column (Amersham Pharmacia, Piscataway, NJ) equilibrated with nickel sulphate according to manufacturer's suggestions. The proteins were eluted by using a linear 0-1.0 M imidazole gradient. Two ml fractions containing His-SisR, as determined by SDS-PAGE, were pooled, dialyzed against 3 L buffer containing 20 mM Tris, pH 8.0, 75 mM KSCN and 5 % glycerol overnight at 4^{o}C, and the purified protein was stored at -20^{o}C until needed. Purified His-SisR was used to raise polyclonal antiserum in a New Zealand white rabbit (Rocky Mountain Labs, Hamilton, MT).

Recombinant SisR was overexpressed from pAPO1 and harvested from *E. coli* strain Rosetta [BL21 (DE3) pLysS] as described above. The cell pellet was suspended in lysis buffer (20 mM Hepes buffer, pH 8.0, 75 mM KSCN, 0.5 mM DTT, 5% glycerol), lysed by two passages through a chilled French press cell at 8,000 psi, and centrifuged at 9,000 x g for 30 min at 4^{o}C. To precipitate SisR, 40% ammonium sulfate was added to 40% saturation while slowly stirring the cell lysate, incubated for 30 min at 4^{o}C, and the protein harvested by centrifugation (9,000 x g, 20 min, 4^{o}C). The precipitated protein pellet was resuspended in lysis buffer and dialyzed overnight against 3 L of lysis buffer at 4^{o}C. The dialyzed protein was applied to a 5 ml heparin-agarose column (Amersham) equilibrated with lysis buffer at 4^{o}C, and proteins were eluted by using a linear 0-1.0 M KCL gradient. Two ml fractions containing SisR, as determined by SDS-PAGE were pooled, dialyzed overnight against lysis buffer at 4^{o}C, and purified protein

was stored at -20°C until needed. Purified SisR was sent to MSCAN (West Chester, PN) for amino-terminal sequencing.

The *sisK* gene was amplified from *B. burgdorferi* B31-A3 by PCR with primers sisKF and sisKR (Table 1). The resulting PCR product was digested with *Nde*I and *Sal*I and cloned into pET21b (Novagen) generating the plasmid pAPOk. To fuse SisK to a maltose binding protein pAPOk was digested with *Nde*I, blunt-ended with T4 DNA polymerase, and then digested with *Sal*I. The resulting fragment was introduced into the pMAL-C expression vector that had been digested with *EcoR*I, blunt-ended, and digested with *Sal*I. The resulting plasmid, pAPOxu, was introduced into *E. coli* strain Rosetta [BL21 (DE3) pLysS] (Novagen). For overexpression, *E. coli* strain Rosetta [BL21 (DE3) pLysS] cells containing pAPOxu were grown in 1 L of LB containing ampicillin at 37°C with shaking. When cells reached A_{600} of 0.5-0.8, expression of the MBP-SisK was induced with 1 mM IPTG for 4 h. Cells were harvested as described above, resuspended in lysis buffer (50 mM Tris-acetate, pH 8.2, 200 mM NaCl, 5 mM imidazole, 1 mM dithiothreitol (DTT) and 1 mM EDTA), and lysed by two passages through a chilled French press cell at 15,000 psi. Unlysed cells were removed by centrifugation (15,000 x g, 15 min, 4°C). The cell lysate was applied to a 5 ml amylose affinity column (New England Biolabs, Beverly, MA), equilibrated with column buffer (20 mM Tris-HCl pH 7.4, 200 mM KCl, 5% glycerol, 1 mM DTT, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride), and proteins were eluted with column buffer containing 10 mM maltose. Two ml fractions containing MBP-SisK, as determined by SDS-PAGE, were pooled, and purified protein was stored at -20°C until needed. Purified MBP-SisK was used to raise polyclonal antiserum in a New Zealand white rabbit (Rocky Mountain Labs).

Expression of *rpoS* in *rpoN* and *sisK* mutants. *B. burgdorferi* B31-A3 4-1 (*rpoN* mutant) and B31-A3 2-1 (*sisK* mutant) strains were diluted to $5x10^6$ cells/ml and allowed to grow to cells densities of $5x10^7$ and $2x10^8$ cells/ml. RNA was isolated by a phenol extraction method using TRI-Reagent (Sigma) as described by the manufacturer, and the RNA quantified by measuring absorbance at 260 nm. RT-PCR was used to synthesize first strand cDNA by using SuperScript II (Invitrogen, Carlsbad, CA) and following manufacturer's protocols. The final concentration of reaction components were: 1.5 μg of RNA, 25 ng of random 10-mer primers, 1 mM dNTP, 1 X first strand buffer, 10 mM DTT, and 40 units Rnasin (Promega).

Quantitative RT-PCR (QRT-PCR) primers and probes specific for *rpoS*, *cat*, and *flaB* (Table 1) were designed using the Primer Express 1.0 program (PE Applied BioSystems) and synthesized by Sigma Genosys. All components were supplied from Invitrogen, and the final concentration of the reaction components used for analysis were: 30 ng first strand cDNA, 200 μM dNTP, 6 mM $MgCl_2$, 1 X DNA polymerase buffer, 0.4 μM each primer and probe, and 1 U Platinum *Taq* (Invitrogen). All reactions were carried out on the ABI PRISM 7700HT Sequence Detection System (PE Applied BioSystems) using a PCR cycle of 2 min at 95°C, and cycled for 40 times at 95°C for 30 sec and 60°C for 45 seconds. Each transcript was normalized by comparison to the constant, internal control *flaB*. Assays were done in triplicate, and each quantitation was performed in quadruplicate.

Phosphorylation assays of MBP-SisK and His-SisR *in vitro*. Autophosphorylation of 5 μg of MBP-SisK was performed at 37°C for 1-5 min in binding buffer (20 mM Tris-acteate, pH 8.0, 5 mM $MgCl_2$) (15). To start the reaction, 2.2 pmol of $^{32}P(\gamma)$-ATP (3000 Ci/mMol, Perkin Elmer, Shelton, CT) was added to the MBP-SisK mixture and incubated for 1, 2, and 5 min. The reaction was stopped by adding 5 X SDS sample buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 14.4

mM β-mercaptoethanol, 0.1% bromophenol blue, 25% glycerol) to a final concentration of 1X SDS sample buffer. Samples were separated by electrophoresis on a 6% SDS-PAGE gels and visualized by autoradiography. To observe the transfer of the phosphoryl group from MBP-SisK to His-SisR, the ^{32}P(γ)-ATP and MBP-SisK reaction was incubated for 5 min, His-SisR or SisR (5 μg) was added, and the incubation continued for 1, 2, and 5min. The reactions were stopped as described above.

***rpoS* promoter reporter constructs**. Primers rpoS-S and rpoS-L (Table 1) were designed to clone fragments extending from the translational start of *rpoS* from *B. burgdorferi* to either 150 bp (RS) or 300 bp (RL) upstream (Figure 1, panel A). The RS and RL PCR products were TA cloned into pCR2.1 (Invitrogen) to generate the plasmids p2RS and p2RL, respectively. The chloramphenicol resistance gene was amplified from pCR ScriptCam (Stratagene) using primers cat-NF and cat-KR (Table 1) and digested with *Nde*I and *Kpn*I (Figure 1, panel B). The resulting fragment was fused to the short (RS) and long (RL) *rpoS* promoter by inserting the PCR product into the *Nde*I and *Kpn*I sites of p2RS and p2RL to generate p2RSC and p2RLC, respectively. The *rpoS* promoter-*cat* fusions (P_{rpoS}-*cat*) were digested with *Hind*III and *KpnI* (RSC) or *BamH*I and *Kpn*I (RLC) and inserted into the appropriate restriction sites of pBSV2gent (11) to generate the plasmids pBRSC and pBRLC. All plasmid sequences were confirmed by sequencing reaction. The resulting plasmids were transformed into *B. burgdorferi* low-passage strain B31-A3, B31-A3 4-1, and B31-A3 2-1 as described above. Gentamycin resistant colonies were analyzed by PCR to confirm the presence of the reporter constructs. As a control, the promoterless *cat* was cloned into pBSV2gent at the *Nde*I and *Kpn*I restriction sites, to generate the plasmid pBCAT. Expression of chloramphenicol acetyltransferase in these strains was examined by QRT-PCR as described above.

Results

Expression of *rpoS* in *sisK* and *rpoN* mutants. We found previously that mutations in *rpoN* resulted in reduced levels of *rpoS* transcript as cultures enter stationary phase (36). σ^{54}-holoenzyme requires an activator protein to initiate transcription, and Yang and coworkers identified SisR as the probable activator in *B. burgdorferi* (38). SisR is predicted to be a response regulator of a two-component system, and we predicted that a protein encoded immediately upstream of *sisR* was the cognate protein histidine kinase of SisR, which we refer to as SisK. To test this hypothesis, a *sisK* mutant strain was generated to examine how this mutation would influence the expression of *rpoS*.

Internal fragments of *sisK* and *rpoN* were interrupted by a kanamycin resistance cassette (4) and cloned into pCR Script Cam, which is unable to replicate in *B. burgdorferi*. The resulting suicide vectors (pJNK and pSKK) were introduced into *B. burgdorferi* low-passage B31-A3 cells. The transformants were plated on BSKII medium containing kanamycin to select for mutant strains in which allelic exchange between the suicide vector and the appropriate target gene had taken place and colonies were screened by PCR. Two colonies out of ~> 400 transformants from the *sisK* transformations and four colonies out of ~ 112 transformants from the *rpoN* transformations yielded a PCR product that was the expected size for insertion of the kanamycin resistance cassette into *sisK* and *rpoN*. Each product was sequenced to confirm inactivation of the target gene (data not shown). We also tried to inactivate *sisR* using the same procedure, but no mutants were isolated despite numerous attempts. Similarly, Yang and colleagues reported on the inability to inactivate *sisR* (38). Our failure to inactivate *sisR* does not appear to be due to polar effects on downstream genes because *sisR* is predicted to be the last

gene within the operon. We postulate that SisR has an additional unknown function, such as repression of one or more genes that is essential for viability of the cell.

QRT-PCR was used to determine the effect of *sisK* mutation on transcription of *rpoS*. Expression levels of *rpoS* in the *sisK* mutant (B31-A3 2-1) were compared to wild-type (B31-A3) and the *rpoN* mutant (B31-A3 4-1). All reactions were normalized to *flaB* mRNA. Figure 2 demonstrates that *rpoS* mRNA was comparable for the B31-A3, B31-A3 4-1, and B31-A3 2-1 strains from mid-log phase ($5x10^7$ cells/ml). In contrast, *rpoS* mRNA was significantly reduced (~100-fold), in RNA samples prepared from the *rpoN* mutant strain in stationary phase when compared to wild-type (Figure 2). An intermediate amount of *rpoS* mRNA was present in the *sisK* mutant compared to B31-A3 (4.4-fold less) and B31-A3 4-1 (22-fold more) (Figure 2).

***In vitro* phosphorylation of SisK and SisR.** Our attempts to purify a histidine-tagged version of SisK were unsuccessful due to insolubility of the protein. When fused to other recombinant proteins, *E. coli* maltose-binding protein (MBP) often enhances the solubility of these proteins (18). Joining the *E. coli* MBP to the amino-terminus of SisK improved the solubility of the protein to allow purification. MBP-SisK, His-SisR and recombinant SisR were purified from *E. coli* Rosetta [BL21 (DE3) pLysS] strain, which contains some of the tRNAs for rare codons that are often present in bacteria with high A+T content in their genomic DNA. Expression in strains without the rare codons did not overexpress the *B. burgdorferi* proteins.

Phosphorylation assays performed with purified MBP-SisK and ^{32}P-(γ)-ATP demonstrated that SisK could autophosphorylate (Figure 3, lanes 1-3). To determine if SisK can transfer the ^{32}P to SisR, His-SisR was added to the MBP-SisK-ATP reaction mixture at a 1:1 ratio. After 5 min phosphorylated His-SisR appeared, demonstrating that MBP-SisK is able to transfer the phosphate to the His-SisR (Figure 3, lane 9). Complete transfer of the phosphate

from SisK to SisR was never observed, despite longer incubations or varying protein concentrations (data not shown). This suggests that either another component is needed for efficient transfer or the recombinant proteins may not be assembled correctly. To determine if hexahistidine-tag affected the phosphate transfer, purified recombinant SisR was added to the reaction as described (Figure 3, lanes 4-6). While transfer was observed from SisK to the recombinant SisR, the transfer appeared less efficient when compared to the transfer to His-SisR. These experiments show that SisK and SisR interact and are part of the same two-component system.

***B. burgdorferi rpoS* promoter-*cat* reporter constructs.** Activators of σ^{54}-holoenzyme generally bind to sites located 100-200 bp upstream of the promoter and contact the closed complex through DNA looping to activate transcription. To identify the SisR-binding site, mobility gel shift assays were performed with varying concentrations of SisR or His-SisR, as well as different binding buffers. None of these conditions, however, resulted in consistent binding of SisR to DNA fragments corresponding to regions upstream or downstream of the σ^{54}-dependent *rpoS* promoter (data not shown).

To verify that SisR required an upstream activation sequence to function efficiently at the *rpoS* promoter, two reporter constructs were made by fusing the chloramphenicol acetyltransferase gene from pCRScript Cam to either a short (~150 bp) or a long (~300 bp) intergenic region upstream of the σ^{54}-dependent *B. burgdorferi rpoS* promoter. These constructs were transformed into *B. burgdorferi* B31-A3, B31-A3 4-1, and B31-A3 2-1. As a control, a promoterless *cat* was transformed into *B. burgdorferi* B31-A3, B31-A3 4-1, and B31-A3 2-1. RNA was extracted from the transformants at mid-log (5×10^7 cells/ml) and stationary phase (2×10^8 cells/ml), and expression of the *cat* mRNA was followed by QRT-PCR. The short and

long constructs were expressed in *B. burgdorferi* B31-A3, and expression from this construct increased as the cultures entered stationary phase (Figure 4). To verify that *cat* expression was dependent on σ^{54} and SisK, both constructs were transformed into the *sisK* and *rpoN* mutant strains. As cultures of the mutant strains entered stationary phase expression of *cat* mRNA from the long promoter construct did not increase as it did with the wild-type strain (Figure 4), suggesting an upstream activation sequence was required for expression from the σ^{54}-dependent *rpoS* promoter.

Discussion

Unlike other forms of RNA polymerase holoenzyme, σ^{54}-holoenzyme has an absolute dependence on activator proteins for transcription initiation. σ^{54}-holoenzyme binds the promoter to form a stable closed complex that is unable to undergo isomerization to an open complex in the absence of an activator. Productive interactions between the activator and σ^{54}-holoenzyme lead to isomerization of the closed complex to a transcriptionally active open complex in a reaction that is coupled to ATP hydrolysis by the activator (3, 5, 26, 31, 34).

Inspection of the *B. burgdorferi* genome revealed a single open reading frame (*sisR*) that could encode a σ^{54}-dependent activator, and this protein was also predicted to be a response regulator of a two-component system. Directly upstream of *sisR* is a gene encoding a protein that appeared to be the most likely candidate for the cognate histidine kinase of SisR. We present here genetic and biochemical evidence, which confirms that SisK is the cognate histidine kinase of SisR. Interestingly, when compared to the σ^{54} mutant, expression of *rpoS* mRNA was higher in the *sisK* mutant. We postulate that this was due to SisR being phosphorylated by either another protein histidine kinase or small phospho donors such as acetyl phosphate in the cell.

Phosphorylation assays demonstrated that MBP-SisK is able to undergo autophosphorylation and pass the phosphate group to His-SisR or recombinant SisR, providing evidence for the interaction between SisK and SisR, despite the inability of SisK to completely transfer the phosphate to SisR. This incapacity could be caused by interference of the maltose binding protein bound to SisK or the histidine tag on SisR, although there is evidence against this. These experiments suggest that SisR and SisK are part of the two-component regulatory system involved in the activation of σ^{54}-dependent promoters.

Despite the fact that the region located 100-300 bp upstream of the σ^{54}-dependent *rpoS* promoter appeared to be required for σ^{54}- and SisK-dependent expression of the *rpoS* promoter-*cat* fusions, we could not demonstrate the binding of SisR to this region. This could be due to one of several reasons, including inactive protein, the need to phosphorylate SisR, suboptimal assay conditions, or the methylation state of the DNA probe. Another possible explanation for the failure to observe DNA-binding activity with the purified SisR is that the *sisR* transcript was not translated correctly in *E. coli*.

Analysis of SisR indicated that approximately 37% of the codons are rare codons not used in *E. coli* with multiple consecutive rare codons near the amino-terminus of the protein (Figure 5). Although SisR was overexpressed and purified from *E. coli* Rosetta [BL21 (DE3) pLysS], which contains a number of tRNAs for rare codons, only about half of the all of the needed codons are repaired in this strain. Amino-terminal protein sequencing demonstrated that the protein purified from *E.coli* did not match exactly with the deduced amino acid of the *sisR* sequence (Table 2). The reaction for the apparent misincorporation of amino acids into SisR is not clear, but it raises the possibility that the recombinant SisR protein expressed in *E. coli* has alterations within it that impact the DNA-binding activity of the protein. These experiments

illustrate the need to develop a system to express and purify proteins directly from *B. burgdorferi* to ensure their correct synthesis and assembly.

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Table 1. Oligonucleotide primers used in this study

Primer	Primer Sequence (5'-3')
ntrF3	TACAAACAATAAAAATATTAAGCC
ntrR3	ATAACAGAAATCTCTTTGTCTGAC
kMunF	CCCCAATTGCCCGTCAAGTCAGCGTAATGC
kMunR	GCCCAATTGAGCAAGAGATTCGCGCAGAC
sisKBF	GTGGGATCCATCGCCCCTATAATCAAAAT
sisKBR	GTGGGATCCAGCTTCTTCTTCGTCACTA
kSphF	GTGGCATGCAGTGTTACAACCAATTAACC
kSphR	GTGGCATGCAATCTCTGATGTTACATTGC
act-exp	TATTGCATATGAGCAAAATACTTGTAG
act-2	GGTCAGGCTCTGGATCCAGCTAAATAT
sisKF	GCTAAATTATGAGGAGATATGAATAATT
sisKR	CTTCTCCGTCGACAGCAGTGAAAAC
rpoS-S	TAAAAGCTTCAAAAAATACTCCCCCT
rpoS-L	AAGCGGATCCTTGAAAGCATGAAAAAA
cat-NF	CTAAGGAAGCTCATATGGAGAAAAAA
cat-KR	AGCGGTACCAGCACCTTGTC
rpoS-215F	AAGAAGGCAACTTGGGATTAATAAGA
rpoS-308R	TGCTTAATCCAAAATGATGCATAAG
rpoS-242T	**FAM**[1]-CTGCTGAAAAATATGACCCGAATAAAAATACCAAATT-**BHQ**[2]
flaB-586F	AATCTTTTCTCTGGTGAGGGAGCT
flaB-657R	TCCTTCCTGTTGAACACCCTCT
flaB-611T	**FAM**[1]-AAACTGCTCAGGCTGCACCGGTTC-**BHQ**[2]
cat-284F	ACAAGGGTGAACACTATCCCATATC
cat-256R	GAATGCTCATCCGGAATTACG
cat-310T	**FAM**[1]-CCAGCTCACCGTCTTTCATTGCCA-**BHQ**[2]

[1]-FAM = 6-carboxyflourescein [2]-BHQ = Black hole quencher 1

Table 2. Amino acid sequencing of purified SisR

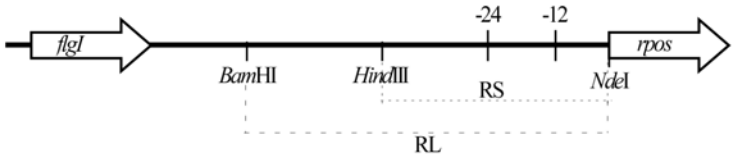
Expected sequence	Amino acid detected*
S	S, A, M
K	K, E, Q
I	I, N
L	L, Y
V	V, N
A	A, K
D	D
D	G, V **
E	E, N
K	K
N	N, V
I	I, D
R	R, L
E	E, Y
G	G
I	I, K
A	A
T	T, V
Y	Y, G
L	L

* Residues with multiple amino acids detected were found in proportional amounts

** Required for magnesium binding for correct phosphorylation

Figure 1. Construction of the *rpoS* promoter-*cat* reporter constructs. (A) Diagram shows location of the PCR products for 150 bp region (RS) and 300 bp region (RL) upstream of the *rpoS* start codon to the translational start of *rpoS*. (B) Schematic diagram of how the *rpoS* long promoter-*cat* fusion was generated on a *B. burgdorferi* shuttle vector bearing a gentamycin resistance marker. pBRSC was generated using the restrictions sites *Hind*III and *Nde*I and the same steps as pBRLC were followed. Plasmids are not drawn to scale.

A.


flgI
-24
-12
rpoS
BamHI
HindIII
RS
NdeI
RL

B.

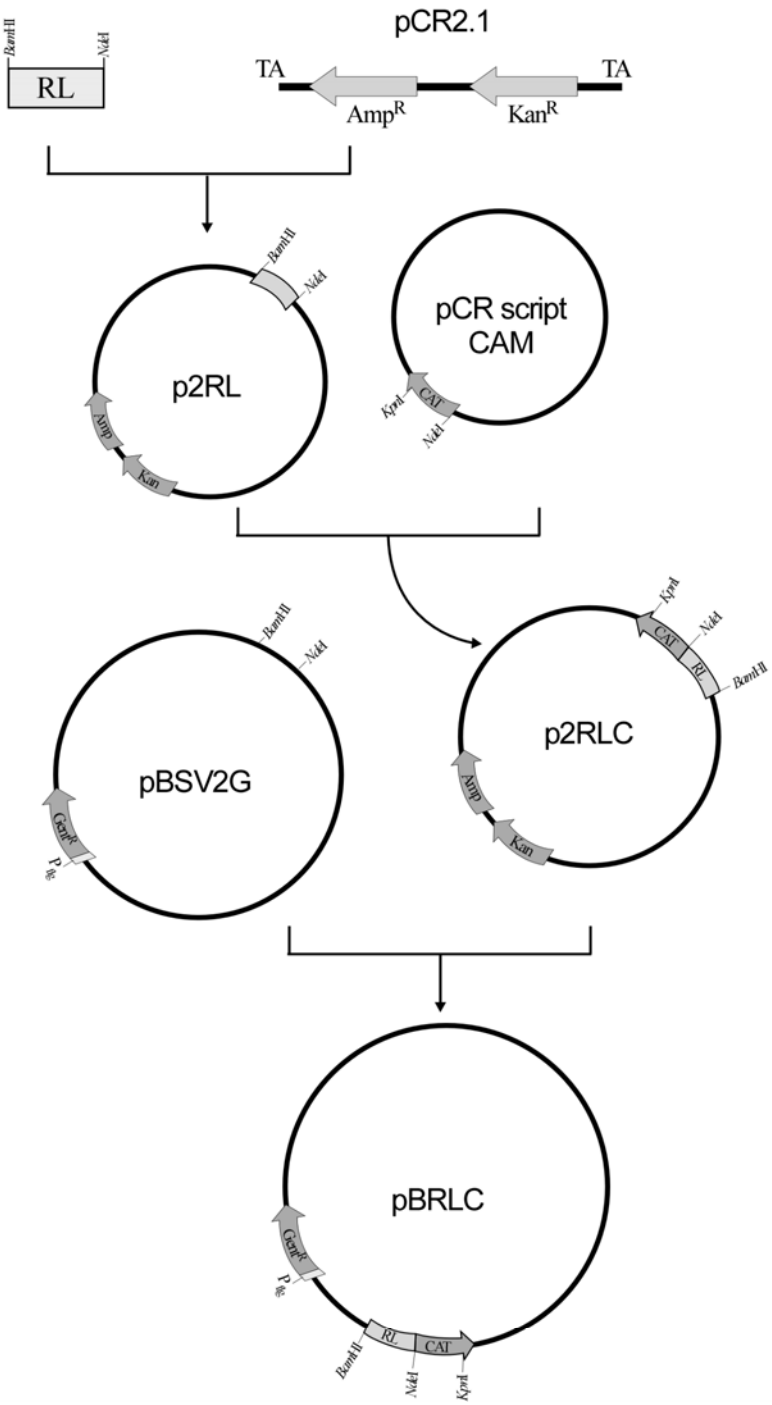

BamHI
NdeI
RL
pCR2.1
TA
TA
AmpR
KanR
p2RL
pCR script
CAM
KpnI
CAT
NdeI
Amp
Kan
pBSV2G
GentR
P
pp
p2RLC
pBRLC

Figure 2. Quantitative RT-PCR analysis of *rpoS* transcripts in *sisK* and *rpoN* mutants. RNA was extracted from *B. burgdorferi* B31-A3, B31-A3 2-1, and B31-A3 4-1 strains at mid-log phase (5×10^7 cells/ml) and stationary phase (2×10^8 cells/ml) and quantitated using specific primers and the Taqman system. Numbers indicate cell densities per ml of culture.

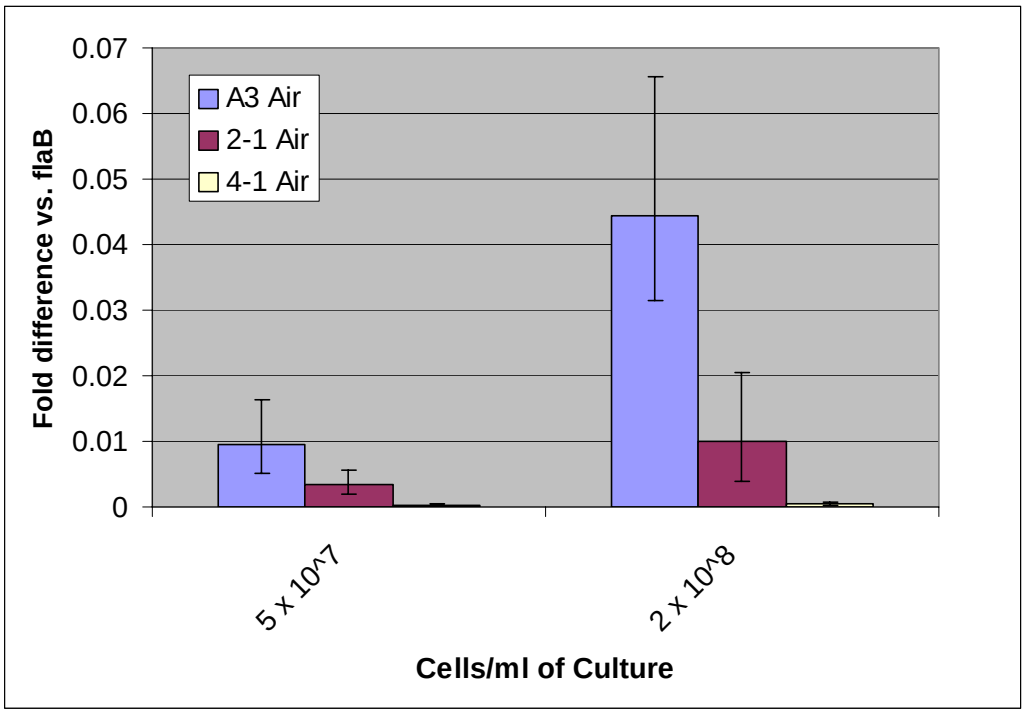

0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
A3 Air
2-1 Air
4-1 Air
Fold difference vs. flaB
5 x 10^7
2 x 10^8
Cells/ml of Culture

Figure 3. *In vitro* phosphorylation of SisK and SisR. MBP-SisK (5 μg) was incubated with 2.2 pmol of $^{32}P(\gamma)$-ATP, and the accumulation of MBP-SisK-phosphate was followed for 1 min (lane 1), 2 min (lane 2) or 5 min (lane 3). To examine any transfer of the phosphate group from MBP-SisK to SisR or His-SisR, MBP-SisK was incubated with 2.2 pmol of $^{32}P(\gamma)$-ATP for 5 min. Either 5 μg of SisR (lane 4-6) or 5 μg of His-SisR (lane 7-9) was added and incubated with MBP-SisK-phosphate for 1 min (lane 4 & 7), 2 min (lane 5 & 8), or 5 min (lane 6 & 9). The arrows at the right mark the protein bands, top arrow is MBP-SisK-phosphate, middle arrow is His-SisR-phosphate and the lowest arrow is SisR-phosphate.

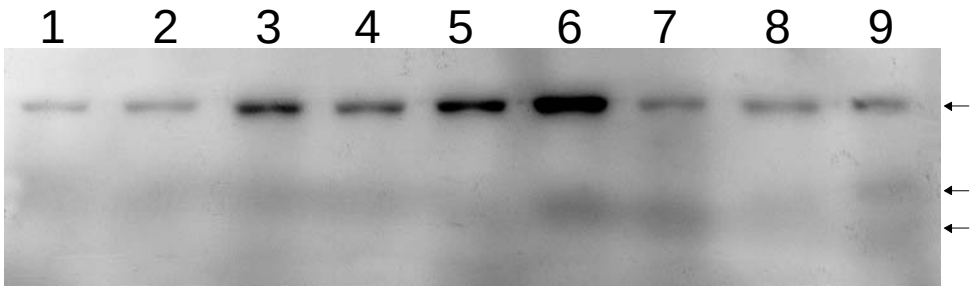

1
2
3
4
5
6
7
8
9

Figure 4. Quantitative RT-PCR analysis of *cat*. RNA extracted from *B. burgdorferi* strains B31-A3, harboring the reporter constructs (pBCAT, pRSC and pRLC) and B31-A3 2-1 pRLC and B31-A3 4-1 pRLC was quantitated using specific primers and the Taqman system. Values have been normalized to the internal control, *flaB,* and are in arbitrary units. Numbers indicate cell densities per ml of culture.

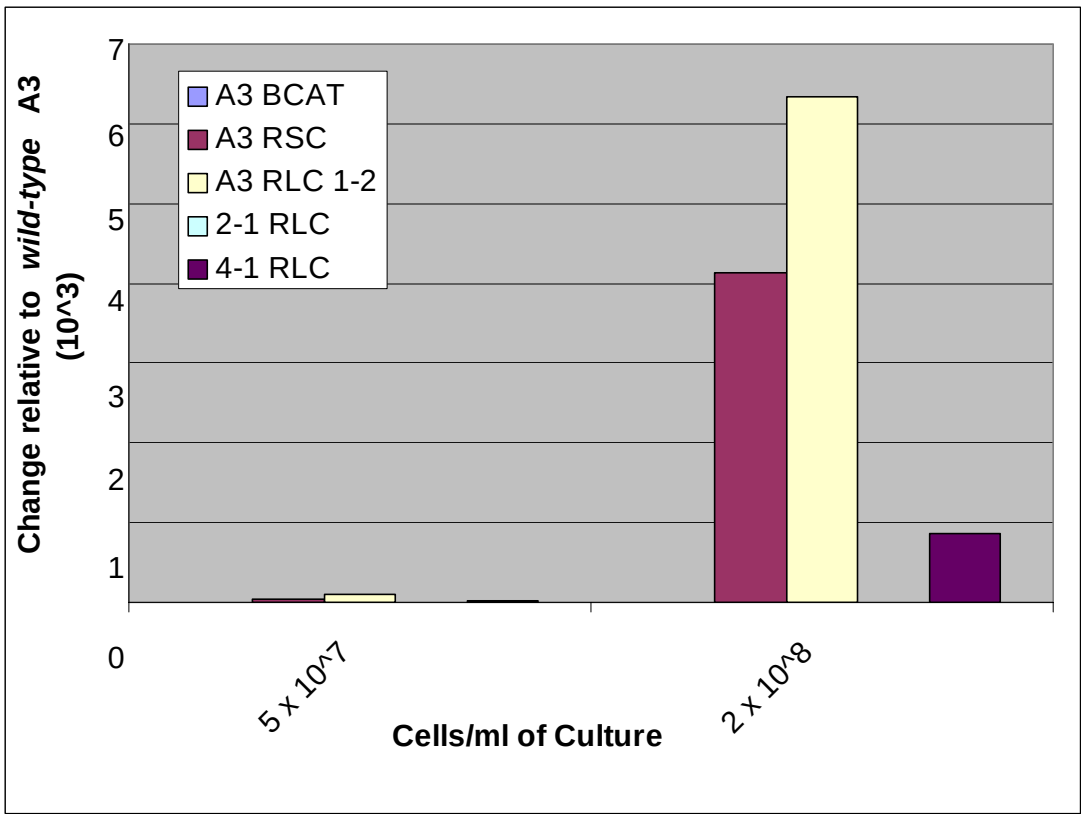
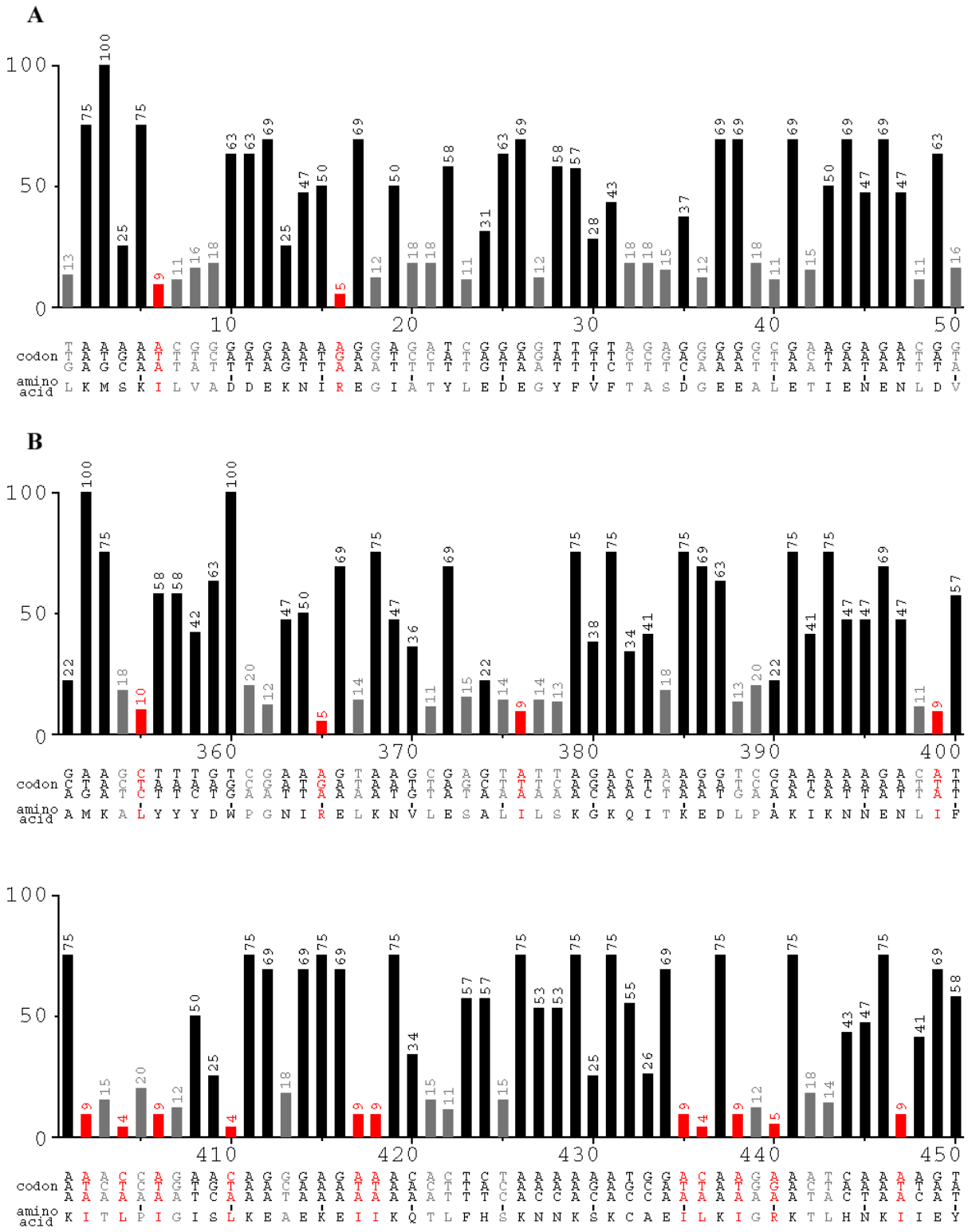

Change relative to wild-type A3 (10^3)
7
6
5
4
3
2
1
0
A3 BCAT
A3 RSC
A3 RLC 1-2
2-1 RLC
4-1 RLC
5 x 10^7
2 x 10^8
Cells/ml of Culture

Figure 5. Codon usage table for SisR from *B. burgdorferi* expressed in *E. coli*. The nucleotide and amino acid sequences are represented along the X-axis. The black bars represent codons that are above the threshold (> 20%) for recognition in *E. coli*. The gray bars represent the codons that *E. coli* will recognize 10-20% of the time. The red bars represent the codons that are correctly recognized < 10% of the time. The numbers above each bar represent the actual percentage of recognition in *E. coli*. Panel A represents the first 50 amino acids of SisR that were sequenced in table 2. Panel B contains the C-terminal end of SisR that includes the DNA binding domains. (Figure generated with assistance of codon usage program www.gcua.de)


A
B
codon
amino acid

Chapter 4

Preliminary Characterization of Oxidative Damage and Protection in *Borrelia burgdorferi*

Abstract

Preliminary experiments demonstrated that *B. burgdorferi* has: 1) a unique enzyme for protecting the bacterium from reactive oxygen species (ROS) and 2) polyunsaturated membrane lipids and lipoproteins which, unlike most bacteria, can undergo peroxidation. BB0690, designated NapA, encodes a Dps/Dpr-like protein that, unlike its homologs, has no associated metal cofactors and was unable to bind DNA. Interestingly, NapA complements *E. coli* TA4351 (Δ*ahpCF*) suggesting that the protein had alkyl hydroperoxide reductase activity. Deletion of 18 amino acids from the C-terminal end of the protein or changing C172, C173 and C175 to serines abolished the complementing activity. *In vitro* enzyme assays with the purified protein using *t*-butyl hydrogen peroxide or linoleic acid hydroperoxide as a substrate suggested a peroxiredoxin activity, although the low levels of enzyme activity indicated that this was not its primary function. Analysis of *t*-butyl hydrogen peroxide treated *B. burgdorferi* cells by electron microscopy showed significant " blebbing" indicative of membrane damage. Fatty acid analysis of similarly treated cells indicated that host-derived linoleic acid had been dramatically reduced in these cells. This suggested that *B. burgdorferi* membrane lipids and lipoproteins were potential targets for attack by ROS encountered in the various stages of the infective cycle.

Introduction

B. burgdorferi, the causative agent of Lyme disease, has been shown to adapt to environmental changes as the bacteria cycles between tick and mammalian hosts. Specific adaptations include altered protein expression in response to reactive oxygen species (ROS), dissolved oxygen, pH, and temperature (8, 18, 22, 27-30). For example, the initiation of feeding of infected ticks on a new mammalian host results in a corresponding increase in temperature

from 23°C to 34°C, which alters gene expression by up-regulating the expression of OspC and down-regulating OspA (27, 28, 30). This shift in the expression of key surface proteins appears to be necessary for successful transmission. Another environmental conditions to which *B. burgdorferi* is exposed during colonization of a mammalian host is ROS generated by the host innate immune response (19, 33). Adaptation to this hostile environmental challenge is essential for successful colonization of the new host. It has been shown recently that dissolved O_2 and ROS affect the expression of proteins essential for transmission (e.g., VlsE, OspC, DbpA and B) (29) and those potentially involved in the reduction/detoxification of ROS (SOD, NapA), suggesting, that these adaptations are very important for the transmission and survival of virulent *B. burgdorferi*.

The affects of ROS, such as superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxide radicals (OH^-), on bacterial cells is thought to involve damage to DNA, RNA, proteins and lipids (17, 31). Much of the cellular damage in bacteria is due to OH^- generated from H_2O_2 reacting with Fe^{2+} (free intracellular Fe) via the Fenton reaction (17, 31, 36). Subsequent reactions of OH^- with DNA cause the most significant damage and pose the greatest threat to cell survival. Other targets of ROS in bacteria include enzymes, such as aconitases and fumerases, that contain an "accessible" iron-sulfur (Fe-S) center and, to a lesser extent, proteins that contain critical cysteine residues. Peroxidation of lipids in bacterial membranes is not considered a problem since most bacterial membranes contain saturated and monounsaturated fatty acids rather than "reactive" polyunsaturated lipids (16).

To survive the challenge of ROS, bacteria have developed numerous and often redundant pathways to detoxify the environment by converting O_2^- to H_2O_2 and finally to H_2O. These systems include catalases, peroxidases, alkyl hydroperoxide reductase (AhpR), glutathione

peroxidases, DNA binding protein induced in stationary phase (dps) homologs, superoxide dismutases (SODs), and superoxide reductases (5, 16, 17, 21, 31). These ROS defense enzymes commonly contain a catalytic metal cofactor (e.g., Fe, Mn, or Cu/Zn containing SODs, Fe containing peroxidases etc.) or key reactive cysteine residues (e.g., AhpR). The later peroxiredoxin family of proteins is fairly promiscuous, low efficiency peroxidases, that use thiols as reductants. These proteins have one or two well conserved, redox-active cysteine residues, which allows them to reduce hydroperoxide and alkyl hydroperoxides at the expense of thiols (23, 24). One of the best characterized AhpC/AhpF enzymes from *Salmonella typhimurium* has been shown to reduce lipid hydroperoxides and other alkyl hydroperoxides directly to the corresponding alcohols (12, 23, 24).

In *B. burgdorferi,* there seems to be relatively few ROS scavenging enzymes. Whitehouse *et al*. cloned the gene encoding a SOD from *B. burgdorferi* but no other functional ROS defense enzymes have been described (37). Analysis of the *B. burgdorferi* genome provides only marginal help in identifying these types of enzymes. The only other identifiable ROS protection/detoxifying enzyme is encoded by BB0690. Identified as a homolog to the neutrophil activating protein (NapA) from *Helicobacter pylori,* BB0690 belongs to the large family of Dps/Dpr proteins. These proteins are induced during stationary phase and oxidative stress, and are thought to have two major functions: 1) bind free Fe to prevent Fenton chemistry and 2) physically bind to and protect DNA from oxidative damage (1, 39, 40). In this chapter, we describe experiments that suggest: 1) that the Dps/Dpr homolog, BB0690 (*napA*), functions as a peroxiredoxin not as a metal-dependent, DNA-binding Dps/Dpr enzyme and 2) that ROS damage to *B. burgdorferi* can occur to host-derived membrane lipids.

Materials & Methods

Strains, growth conditions and reagents. *B. burgdorferi* strain B31 was grown in modified Barbour-Stoenner-Kelly (BSK-II) (2) medium at 34°C under concentrations of O_2 from 0-20% with 5% CO_2 and the balance N_2. *Escherichia coli* strains XL1 Blue MRF' (Stratagene, La Jolla, CA), BL21 GOLD (DE3) (Novagen, San Diego, CA) and TA4315 (kindly provided Dr. G. Storz, NCI, NIH, Bethesda, MD) (32) were grown at 37°C in LB broth supplemented with ampicillin (100 μg/ml) and isopropyl−β-D-thiogalactopyranoside (IPTG) (1.0 mM) when appropriate. All reagents were purchased from Sigma Chemicals, St. Louis, MO unless stated otherwise.

DNA manipulations. Chromosomal DNA from *B. burgdorferi* strain B31 was isolated as described previously (26). PCR primers were designed to incorporate either *Nde*I and *BamH*I or *EcoR*I and *Hind*III sites into PCR products and used to amplify *napA* from 25 ng of *B. burgdorferi* chromosomal DNA. The NapAF (GGAGATACATATGGAAAAGTATTTAAGC) (underlined sequence denotes restriction site) and NapAR (GCCCGGATCCC CAACAACTACCACAGCCTC) primers were designed to amplify *napA*. The PCR product was cloned into the *Nde*I and *BamH*I sites of pT7-7 (34) generating pJT3. The NapA5 (GGCGAATTC TATGGAAAAGTATTTAAGC) and NapA3 (GCCCAAGCTTCCAACAACTACCACAGCCTC) primers were used to amplify *napA*. The resulting PCR product was digested with *EcoR*I and *Hind*III and cloned into the *EcoR*I and *Hind*III sites of pKK223-2 (Amersham Pharmacia, Piscataway, NJ) to generate pJT4. The NapA5 and ΔNapA*Hind* (GGCAAGCTTCTAATTTTCAAGCAATGCC) primers were used to amplify a truncated *napA*. ΔNapA*Hind* introduced a stop codon after amino acid residue 159 deleting the last 18 amino acids of NapA (Figure 3, panel E). The PCR product generated using NapA5 and ΔNapA*Hind* was cloned into pKK223-3 generating pJT5. The primers NapA5 and MutNapA*Hind* (CCAAGCTT TATTTTGCATCAGACTCGGAAGATTTGCTTTCATTC) were used to amplify *napA* and change

the codons for the last three cysteine residues to serines (C172S, C173S, and C175S) (Figure 3, panel E). The PCR products generated using NapA5 and MutNapA*Hind* were cloned into pKK223-3 as described above to generate pJT6. All constructs were sequenced (LHBP, NIAID, NIH Rocky Mountain Labs, Hamilton, MT) to confirm the sequence of the various constructs.

Primer extension and reverse transcriptase-polymerase chain reaction (RT-PCR). *B. burgdorferi* strain B31 cells were grown to different cell densities in BSK-II medium (2) as described above. RNA was extracted from cells harvested at 9 x 10^7 or 2 x 10^8 cells/ml using Tri Reagent according to manufacturer's technical bulletin. Primer extension was performed using the Primer Extension System-AMV Reverse Transcriptase kit (Promega, Madison, WI) following the manufacturer's technical bulletin. Briefly, 100 fmoles of ^{32}P(γ)-ATP end labeled NapAPE (AATTGTATTGCGTCTAAATCATCC) primer was mixed with 1 or 5 μg of RNA in 1 x AMV Primer Extension Buffer in a final volume of 11 μl. The samples were heated at 58^{o}C for 20 min and then placed at room temperature for 10 min to allow the primer to anneal to the RNA. Nine μl of a master mix containing 5 μl of 2 x AMV Primer Extension Buffer, 1.4 μl of 40 mM sodium pyrophosphate, 1 μl of AMV RT, and 1.6 μl of nuclease-free water per reaction was added to each reaction and incubated at 42^{o}C for 30 min. Twenty μl of loading dye were added to each reaction and heated at 90^{o}C for 10 min before loading 4-5 μl onto a precast sequencing gel (Stratagene). The NapAPE primer was also used to perform a sequencing reaction using the *fmol* DNA Cycle Sequencing System (Promega) following the manufacturer's recommended protocol. The products were visualized by autoradiography.

RT-PCR was done using the Access RT-PCR System (Promega) on 1 μg of RNA with NapART5 (GGATGATTTAGACGCAATACAATT) and NapART3 (TTAGAAGTTGATTCA ATATCAAGCTCC) primers according to the manufacturer's technical manual. Reactions with

no reverse transcriptase were included to ensure that all contaminating DNA was removed from each sample. RT-PCR products were analyzed on a 2% agarose gel, and DNA was visualized by staining with ethidium bromide.

Purification of recombinant and native NapA. Recombinant NapA was expressed in *E. coli* BL21 GOLD (DE3) harboring pJT3. A 500 ml culture of cells was grow in LB at 37^{o}C until cells reached an OD_{600} of 0.8, IPTG was added and incubation was continued for 3h. After induction, the cells were harvested by centrifugation (5,000 x *g*, 10 min, 4^{o}C), and either used immediately or stored at -20^{o}C until needed. The cell pellet was suspended in lysis buffer (50 mM NaCl / 5% glycerol / 20 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), pH 7.6) and the cells were lysed by three passages through a chilled French Press (Thermo Spectronic, Waltham, MA) cell at 10,000 psi. Cell debris was removed by centrifugation (12,000 x *g*, 30 min, 4^{o}C). The cleared cell lysate was applied to a 5 ml HiTrap Q HP (Amersham Pharmacia) column that was equilibrated with lysis buffer, proteins were eluted with a linear 0-1.0 M NaCl gradient, and fractions were collected. Fractions were analyzed by SDS-PAGE and those containing NapA were pooled, desalted on PD-10 columns (Amersham) equilibrated and eluted with lysis buffer. Partially purified NapA was applied to a CHT-11 (BioRad, Hercules, CA) column that was equilibrated with lysis buffer, and the proteins were eluted with a linear 0-0.5 M Potassium phosphate, pH7.0 gradient. Fractions were collected and assayed as above. The fractions containing NapA were pooled and dialyzed against 4 L of dialysis buffer (50 mM NaCl, 5% glycerol, 0.5 mM GSH, 20 mM HEPES, pH 7.6) for 12 h at 4^{o}C. The identity of purified NapA was confirmed by immunoblot and N-terminal sequencing (Molecular Genetics Sequencing Facility, University of Georgia, Athens, GA), and 3 mg of purified NapA was used to generate polyclonal antiserum in a female New Zealand White rabbit

(Cocalico, Reamstown, PA) (7). Purification of NapDel was done as described above for recombinant NapA.

Native NapA was purified from a 1 L culture of *B. burgdorferi* strain B31A grown in BSK-II medium under 20% O_2. Once the cells reached a cell density of approximately 1 x 10^8 cells/ml, the cells were harvested by centrifugation and washed two times with wash buffer (100 mM NaCl / 20 mM HEPES, pH7.6). The pellet was suspended in lysis buffer and lysed by two passages through a chilled French press (16,000 psi). NapA was purified as indicated above except the hydroxy apatite column was not necessary due to the purity of NapA after the HiTrap Q column. The fractions containing NapA were assayed by SDS-PAGE and immunoblot. Lysis buffer was used to lower the salt concentration and concentrate the protein in Amicon Centricon Plus-20 (Millipore, Bedford, MA) with a 10,000 MW cutoff. The purified NapA was stored at -80^{o}C until needed.

SDS-PAGE and immunoblotting. Proteins (20 μg) were separated on a 12.5% SDS polyacrylamide gel and visualized as previously described (9). For immunoblots, proteins were separated on SDS-PAGE gels, transferred to nitrocellulose, probed using anti-NapA serum (1:2000), and α-rabbit antisera conjugated to horseradish peroxidase (Sigma) was used as secondary antibody (7). The immunoreactive proteins were detected with ECL reagents (Amersham Biosciences) according to manufacturer's recommended protocol.

Complementation and enzyme assays. *E. coli TA4315* (Δ*ahpCF*) (32) with no plasmid or harboring pKK223-3, pJT4, pJT5, or pJT6 were grown in 10 ml of LB supplemented with ampicillin (except for cells only) at 37°C for 12 h. After diluting the cultures 1:10 into fresh medium, IPTG was added to a final concentration of 1 mM, and the cultures were incubated at 37°C for 4 h. At this point, 200 μl of each culture (~ 2 x 10^8 cells) was added to 3.5 ml of top

agar (0.8%) and spread onto LB plates supplemented with ampicillin (50 μg/ml) and IPTG (500 μg/ml). However, cells only were spread onto LB plates containing only IPTG. A sterile paper disc wet with either 10 μl of DMSO or 10 μl of 100 mM t-butyl hydrogen peroxide in DMSO was placed onto the center of the plate. The zones of inhibition were measured after incubating the plates at 37°C for 12 h. These experiments were performed in triplicate in two independent experiments.

Peroxiredoxin activity was determined as described by Cha *et al* (10). Briefly, purified NapA (2-10 μM) in reaction buffer (50 mM -NaOH, pH 7.0) was incubated with 25-100 μM *t*-butyl hydrogen peroxide or 75- 100 μM linoleic acid hydroperoxide at 37°C, and samples were collected at 0, 2.5, 5, and 10 min. The amount of peroxide remaining in each sample was determined by adding 1 ml of 1X Fox 1 reagent and after 30 min, measuring the absorbance at 560 nm (38). In some cases, the reactions were initiated by adding 0.5 mM DTT or 0.5 mM reduced glutathione (GSH) or a mixture of 2.5 μM *E. coli* thioredoxin (Trx1), 2.5 μM *Spirulina* thioredoxin reductase (TrxR) and 25 μM NADPH (or NADH). A standard curve was generated using 0 - 100 μM *t*-butyl hydrogen peroxide using the same assay. One unit of NapA activity was defined as the amount of enzyme (mg protein) that catalyzed the reduction of 1 μmol *t*-butyl hydrogen peroxide per min at 37°C.

Differential expression of NapA. To determine if NapA is expressed under normal growth conditions and if the amount of oxygen present had an effect on the amount of protein expressed, four 500 ml cultures of *B. burgdorferi* strain B31A were grown in BSK-II medium under 2, 5, 8 and 12% O_2. Once the cells reached a concentration of approximately 1 x 10^8 cells/ml, the cells were harvested by centrifugation (5,000 x *g*, 10 min, 4°C) and washed two times with HEPES

buffer (50 mM NaCl, 20 mM HEPES, pH7.6). Cell pellets were resupsended in 2 X SDS sample buffer, and proteins were analyzed by SDS-PAGE and immunoblotting.

Localization of NapA in *B. burgdorferi*. *B. burgdorferi* strain B31 A3 was grown in BSK-II under atmospheric oxygen or under anaerobic conditions (4% H_2, 5% CO_2, 91% N_2). Once the cultures reached a density of ~2 x 10^8 cells/ml, cells were harvested by centrifugation (5,000 x *g*, 4°C, 15 min) and washed three times with 20 mM, 50 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, pH 7.6 (HEPES-II buffer) and resuspended in ice cold HEPES-II buffer. Cells were lysed by two passages through a chilled French press (14,000 psi), and unlysed cells were removed by centrifugation (10,000 x *g*, 20 min, 4°C). The soluble and membrane fractions were separated by centrifugation (125,000 x *g*, 2 h, 4°C). The membrane fraction was washed twice and total membranes were resuspended in HEPES buffer (6). The soluble and membrane fractions were stored at -80°C.

NapA was also localized by immunoelectron microscopy. *B. burgdorferi* cells were grown under atmospheric oxygen or under anaerobic conditions to approximately 5 x 10^7 cells/ml. The anaerobic cultures were divided and one of the cultures was exposed to 1 mM *t*-butyl hydrogen peroxide for 12 h. Cells were harvested by centrifugation (5,000 x *g*, 10 min, 4°C) and resuspended in 100 µl Tyrodes buffer (14). 3-5 µl aliquots of each sample were applied to parlodion coated nickel grids and adsorption was allowed to take place for 30 min at room temperature. Excess supernatant was removed, and grids were treated with blocking solution (BS) (3 % BSA (globulin free/), 20 % FCS (Hyclone, Logan, UT) in 0.1 M PBS) for 15 min at room temperature (RT). Polyclonal antisera (α-NapA, 5 µl) was applied to each grid and allowed to incubate for 1 h at RT. Grids were washed once for 10 min with 0.05 M PBS/BSA/FCS and then rinsed four additional times with 0.05 M PBS/BSA/FCS for 5 min each.

Gold conjugate (Goat anti-rabbit/BB International/ Ted Pella, Inc., Redding, CA) secondary antibody was applied to the grid for 1 h. Samples were washed with 2.5 M NaCl for 5 min, followed by four additional water washes of 5 min. The grids were stained for 30 seconds with 1 % ammonium molybdate, excess stain was removed and the samples were allowed to air dry. The samples were embedded in Medium hardness LR white resin (Ted Pella, Inc., Redding CA) following fixation in PLP fixative (0.1 M Polylysine/2.0 % Paraformaldehyde/0.05 M sodium meta-periodate) and 50 through 100 % ethanol dehydration. Thin sections were subjected to the same procedures outlined above. Thin sections were stained with uranyl acetate and lead citrate for contrast. Samples were examined and photographed using a Hitachi H-7600 electron microscope (Hitachi High Technologies America, Pleasanton, CA) operated at 80 kV, fitted with a Hammamotsu CCD camera. Captured image were converted to digital files using Advanced Microscopy Techniques Corporation imaging software (Danvers, MA).

Lipid analysis of *B. burgdorferi.* A 2 L culture of *B. burgdorferi* strain B31 A3 was grown under anaerobic conditions to a cell density of 5 x 10^7 cells/ml. The culture was split into 500 ml aliquots and one was treated with 1 mM *t*-butyl hydrogen peroxide, a second with 0.25 mg lipoxidase (17,700 units) and a third was untreated. All samples were incubated for 12 h at 34°C. Cells were harvested by centrifugation (5,000 x *g*, 4°C, 15 min) and washed three times with HEPES buffer, and cell pellets were analyzed by Industrial Laboratory (Wheat ridge, CO). Fatty acids were reported as percentage of total cell mass.

Results

Expression of *napA* in *B. burgdorferi*. The open reading frame (ORF) BBO690 (*napA*) of the *B. burgdorferi* chromosome (TIGR database) encodes a protein with homology to bacterial

Dps/Dpr proteins. Dps forms a multimer (decamer to dodecamer), usually binds divalent metals (mostly iron) and protects DNA from damage during oxidative stress and starvation (1). Based on homology, we began to characterize NapA. To determine if this ORF is expressed under normal growth conditions. RT-PCR was performed on RNA isolated from *B. burgdorferi* cells using primers specific for BBO690. A 273 bp RT-PCR that corresponds to the expected size for NapA was produced (data not shown), while no product was observed in the negative control. This demonstrated that NapA is expressed in *B. burgdorferi* when cultured under normal *in vitro* growth conditions.

Within the TIGR database, the translational start site of NapA was predicted to start at nucleotide 731,151 of the linear chromosome. Upon further evaluation, a putative ribosome binding site (RBS) was not identified immediately upstream of this translational start site. However, the next methionine located 33 bp downstream of the predicted start site contained a putative RBS 5 bp upstream. To identify the transcriptional and translational start sites, primer extension was performed on RNA isolated from *B. burgdorferi*. The 5' end of the message was mapped to nucleotide 731,170 (T) of the linear chromosome (Figure 1). Based upon this data, we predict that the actual translational start site is located at nucleotide 731,184 of the chromosome.

Recombinant and native NapA purification and expression under different oxygen concentrations. The ORF initiating at the corrected translational start site was amplified by PCR and cloned into the expression vectors pT7-7 and pKK223-3 to generate pJT3 and pJT4, respectively. The pJT3 plasmid was transformed into *E. coli* BL21 (DE3), and expression of NapA was induced. After induction, a major band was observed at 21 kDa, which is slightly smaller than the predicted size of NapA (22.4 kDa) on the TIGR database, which could be due to

the misidentification of the translational start of the protein (Figure 2). The recombinant NapA was purified from the soluble fraction after lysis of the cells as described above. Based on the homology of NapA with Dps, we initially tried to purify NapA using a heparin column. However, NapA did not bind the column under these conditions. This was the first indication that NapA may not be functioning as Dps homolog.

To confirm the correct translational start site, native NapA was purified from *B. burgdorferi* strain B31A at 12 % O_2. The native protein was subjected to N-terminal sequencing to confirm the translational start site of NapA. The translational start site predicted by the primer extension (located at 731,170) was confirmed by the N-terminal sequence of the purified native protein. Additionally, native NapA was run unheated on an SDS-PAGE gel and high molecular weight oligomers were observed as seen with other Dps/Dpr homologs (Figure 2).

Characterization of NapA as a possible Dps homolog. As mentioned earlier, Dps binds DNA nonspecifically and protects it from oxidative damage during stress and starvation (1, 39, 40). Based upon homology, NapA is similar to Dps homologs of other bacteria. To determine the ability of NapA to bind DNA in a nonspecific manner electrophoretic mobility shift assays were performed. NapA was assayed for DNA binding activity under various conditions including 1) presence and absence of divalent metals or metal chelators 2) varying NaCl concentrations. Various DNA templates including lamda DNA, plasmids from *E. coli* (linear and circular) and chromosomal and plasmid DNA isolated from *B. burgdorferi* were used. Under these conditions, we were not able to detect any DNA binding by NapA (data not shown).

Typically, Dps homologs form decamers or dodecamers and usually bind 400-500 molecules of iron per complex (39, 40). To determine if NapA has any associated metals, *B. burgdorferi* NapA purified from an aerobically grown B31A culture. Protein samples were

analyzed by inductively coupled plasma emission mass spectroscopy (ICP-MS) at the Chemical Analysis Laboratory (University of Georgia, Athens, GA). ICP-MS results showed no metals associated with NapA in *B. burgdorferi* (data not shown). The DNA binding assays and ICP-MS are further evidence that NapA may not function as a Dps homolog in *B. burgdorferi*.

Complementation of an *ahpCF* mutant with *B. burgdorferi* NapA. To determine if NapA from *B. burgdorferi* has alkyl hydroperoxidase activity, complementation assays were performed. For these assays an *E. coli* mutant TA4315 ($\Delta ahpCF^-$) that lacks the *ahpCF* operon and is sensitive to alkyl hydroperoxide was used (32). To determine if *napA* could complement the *ahpCF* mutant, TA4315 was transformed with pJT4. Bacteria were spread in top agar on to LB plates. Sterile filter discs were placed on the agar plates and spotted with 10 μl of 100 mM *t*-butyl hydrogen peroxide in DMSO (Figure 3, panel A). As a positive control, wild-type bacteria *E. coli* MC4100 that produces alkyl hydroperoxide reductase was used. The diameter of the zone of inhibition in the wild type *E. coli* MC4100 was 0.33 ($\pm$ 0.33) mm (data not shown) and 1.3 ($\pm$ 0.06) mm for the mutant *E. coli* TA4315 (Figure 3, panel B). However, the *ahpCF* mutant strain harboring *napA* (pJT4) exhibited a zone of inhibition (0.16 $\pm$ 0.09 mm) equivalent to that of the wild-type (Figure 3, panel C) (Mean values from three experiments). The plasmid vector alone was transformed into the mutant and no reduction in *t*-butyl hydrogen peroxide sensitivity was observed. DMSO alone did not produce a zone of inhibition. The ability of NapA to restore resistance to other ROS (including, hydrogen peroxide, cumene peroxide, and peroxynitrite) was tested. Results of this complementation assays indicate, indirectly, the *B. burgdorferi* NapA has alkyl hydroperoxide reductase activity.

To determine if the six cysteine residues located at the C-terminal end of NapA in *B. burgdorferi* are required for this enzymatic activity, two constructs were generated that alter the

C-terminal end of NapA. The truncated NapA (NapADel) lacks the last 19 amino acids from the C-terminus, and the mutated NapA (NapAMut) changes the last three cysteine residues to serines (C172S, C173S, and C175S). The plasmid pJT5 or pJT6 was transformed into the mutant *E. coli* TA4315. The diameter of the zone of inhibition in the *E. coli* TA4315 harboring either NapAMut or NapADel was equivalent to that of the mutant alone (Figure 3, panel D). This data demonstrates that the C-terminal cysteine residues are required for NapA activity.

Enzyme assays. The complementation results demonstrated that NapA was able to restore resistance to ROS of an *E. coli aphCF* mutant only when exposed to *t*-butyl hydrogen peroxide. To examine this unusual activity further, *in vitro* enzyme assays were performed to determine NapA activity in the presence of lipid peroxides. Peroxiredoxin activity was determined following the method described by Cha *et al* (10). When NapA was incubated with 2.5 μM *E. coli* Trx1, 2.5 μM *Spirulina* TrxR, 25 μM NADH and 50 μM *t*-butyl hydrogen peroxide 4-220 units of activity/mg of NapA was observed (data not shown). The reaction did not appear to consume all of the substrate and the enzyme was not recycled as seen in assays described by Cha *et al* (10). However, this is a hybrid reaction system therefore the enzymes might not be interacting as well as they would with *B. burgdorferi* specific proteins also, the reactions could be missing some unknown factor from *B. burgdorferi* that is necessary for the optimal activity of NapA. These data and the complementation data suggest that although NapA has some alkyl hydroperoxidases activity, it may not be the primary function.

Effect of oxygen on NapA expression. Previously, Boylan and coworkers reported that NapA expression was induced when exposed to increasing levels of ROS (7). To determine if the concentration of oxygen in the environment plays a role in the expression level of NapA, *B. burgdorferi* strain B31A was grown to ~8 x 10^7 cells/ml under various concentrations of oxygen.

Figure 4 shows that as the amount of oxygen in the media increases the expression of NapA in *B. burgdorferi* increases, suggesting that NapA has a role in detoxifying the cells in the presence of oxygen.

Localization of NapA in *B. burgdorferi*. To determine where NapA localizes in *B. burgdorferi* the membrane and soluble fractions were isolated from B31 A3 cultures grown under aerobic and anaerobic conditions. In cells grown under anaerobic conditions, NapA is associated with both the soluble and membrane fractions of the cell (Figure 5 panel A). In contrast, under aerobic conditions NapA is associated with the membranes of *B. burgdorferi* (Figure 5, panel A). As a control to demonstrate the membrane and soluble fractions were not contaminated, the location of OspA was followed using α-OspA antibody (generously provided by T. Schwan, Hamilton, MT) (Figure 5, panel A).

Immunoelectron microscopy (IEM) was used to confirm the location of NapA in *B. burgdorferi*. In anaerobically grown cultures, there are few gold particles associated with NapA, and when present, the protein appeared to be associated with the membrane 50% of the time (Figure 5, panel B). Cultures were grown under aerobic conditions, an increase in NapA was observed, and >95% of the protein was found to be associated with the membranes. When the anaerobic cultures were exposed to *t*-butyl hydrogen peroxide for 12 h the IEM demonstrates increased expression of NapA in *B. burgdorferi* as compared to untreated anaerobic cells. In these cultures, NapA localized to the membranes (Figure 5, panel C & D), while in the untreated anaerobic cultures it seemed to be evenly distributed between the cytosol and membrane fractions (Figure 5, panel B). Unexpectedly, as compared to the aerobic and anaerobic cultures, membrane blebs formed around the spirochete when exposed to *t*-butyl hydrogen peroxide, suggesting possible lipid peroxidation (Figure 6).

Polyunsaturated fatty acids of *B. burgdorferi*. Others have reported significant levels of polyunsaturated fatty acids in *B. burgdorferi* cells (3, 4, 15, 20). To further examine if the membrane blebs could be due to chemical attack on the polyunsaturated fatty acids, cultures were treated with either *t*-butyl hydrogen peroxide or lipoxidase and analyzed for fatty acid composition. As a control, untreated cells were also analyzed. Cell pellets were weighed and values were reported as percentage of the total mass of the cells. Table 1 shows that in all samples oleic acid and pentadecanoic acid levels were comparable. Linoleic acid was altered in the various samples. Untreated cells contained 0.03%, while cells treated with *t*-butyl hydrogen peroxide contained 0.004% (Table 1). Cells treated with lipoxidase had no detectable linoleic acid in the sample. These data show that > 90% of the linoleic acid in *B. burgdorferi* cells can be attacked by chemical treatment.

Discussion

Bacteria have adopted several strategies to deal with ROS. Whatever the source of ROS, the biochemical approach cells employ to neutralize these incomplete reduction products is accomplished in two steps. First, singlet oxygen radicals (O_2^-) are reduced by SODs or superoxide reductases (SOR) to H_2O_2 (5, 15, 16, 30). Three major classes of SODs have been described (Fe, Mn, and Cu/Zn types) and while these enzymes are widely distributed among aerobic and microaerophilic bacteria, it should be noted that some bacteria, particularly anaerobes, do not necessarily contain SODs (5, 15, 16, 20, 30). These bacteria can contain SOR to begin the reduction of O_2^- (20). To complete the detoxification of ROS, H_2O_2, the end-product of SODs and SORs, is reduced to H_2O by other enzymes such as catalases, peroxidases,

AhpR, and glutathione peroxidases (15, 16, 30). The exact role/function of other putative ROS protective enzymes, such as the Dps proteins, remains to be determined.

The *B. burgdorferi* genome contains the genes encoding few ROS protective enzymes. To date, only a putative Mn-dependent SOD has been identified and partially characterized (12). Initial studies in *B. burgdorferi* of the effects of oxygen concentration and ROS on protein expression showed that the most dramatic increase was observed in the levels of a 21-kDa protein (7, 28). This protein, identified as NapA in the TIGR database, has homology with the neutrophil activating protein (NapA) from *H. pylori* (51.8% similarity) and Dps proteins, such as MrgA from *B. subtilis*, (52.4% similarity). Dps proteins are typically expressed as cells enter stationary phase, during metal starvation or when cells are challenged with ROS. They usually form decamers or dodecamers and bind 400-500 molecules of iron per complex (37, 38). Because of their Fe-binding capacity, they were thought to be primarily Fe storage proteins and were originally called "ferritins (non-heme Fe)" or "bacterioferritins (heme Fe)" (1). More recent experimental data suggest that this may not be their only function. Biochemical and genetic analysis of Dps proteins indicate that they are ROS defense proteins that bind "free" Fe to prevent Fenton chemistry (37, 38).

The increased expression of NapA in response to O_2 and ROS, and its homology to Dps proteins suggested a possible role for NapA in ROS defense in *B. burgdorferi.* Therefore, the properties of purified NapA were assayed for similarities with those of Dps proteins. Purified native and recombinant NapA did form a large molecular weight oligomers similar to those reported for Dps proteins but that was where the similarity ended. Importantly, metal analysis by ICP-MS demonstrated that no Fe or other metals co-purified with native NapA. This was consistent with the previous report that *B. burgdorferi* does not contain any intracellular Fe (24).

Since another characteristic of most Dps homologs is their ability to bind DNA (e.g., MrgA), purified native and recombinant NapA were assayed for binding to *B. burgdorferi* or *E. coli* DNA. No DNA binding activity has been observed with either purified NapA indicating that DNA binding/protection was not a function of the protein. Dissimilarities between Dps proteins and NapA from *B. burgdorferi* suggest a different role for the protein in ROS defense.

An interesting feature of NapA that distinguishes it from other Dps homologs is the very unusual C-terminal end. Amino acid alignments of NapA with several Dps proteins indicated that NapA was 17-20 amino acids longer than any of the homologs. Interestingly, this C-terminal "tail" contains 6 cysteine residues at positions 161, 163, 165, 172, 173, and 175. Since the oxidation/reduction of cysteine residues is critical for the function of important redox/ROS enzymes such glutathione reductase (Gor), thioredoxin reductase (TrxR), and AhpR, it seemed possible that these residues where critical for the function of NapA. To test this hypothesis, *napA* was cloned into *E. coli* mutants (e.g., Gor^-, and $AhpR^-$) and tested for the ability to complement these mutations. NapA was able to complement the AhpR mutant strain but only when challenged with *t*-butyl hydrogen peroxide. *In vitro* enzyme assays confirmed that NapA had activity against *t*-butyl hydrogen peroxide but that activity was very low. While it was possible that the assay conditions were not optimal (i.e., NapA required *B. burgdorferi* Trx and TrxR or some other unidentified electron donor), it seems more likely that NapA has a different enzymatic function in *B. burgdorferi*.

Possible targets for ROS damage in bacteria include DNA, RNA, proteins, and lipids. The most extensive damage is thought to be due to hydroxyl radicals (OH^-) generated when H_2O_2 reacts with iron via the Fenton reaction (16, 30, 34). Preliminary analyses of the internal chemistry and chemical composition of *B. burgdorferi* strongly suggest that the targets of ROS

are very different in this bacterium. *B. burgdorferi* does not contain any intracellular iron, thus minimizing the possibility of DNA being a major target for damage via the Fenton reaction (24). This seems to be the case since high concentrations of ROS (50 mM H_2O_2 or *t*-butyl hydrogen peroxide) have little to no effect on the mutation rate in *B. burgdorferi* strain B31 (unpublished data). Additionally, while damage to cysteine residues in proteins most likely occurs, ROS attack on iron-sulfur proteins seems unlikely since none have been identified in the genome of *B. burgdorferi* (12). In most bacteria ROS mediated damage to phospholipids, lipoproteins or glycolipids is very unlikely due to the lack of polyunsaturated fatty acids (e.g., linoleic acid) in bacteria such as *E. coli* (15, 16).

In contrast to most bacteria, *B. burgdorferi* phospholipids, lipoproteins and glycolipids contain unsaturated fatty acids, such as linoleic and linolenic acids, which are derived from the host (3, 4, 14, 19). Lipid peroxidation initiated by the attack of free radicals on these polyunsaturated fatty acids could decrease the membrane fluidity and**,** if these reactions propagate, lipid peroxides and their degradation products (e.g., aldehydes) in turn could damage proteins (15). This would dramatically affect the function of transmembrane proteins and membrane bound lipoproteins involved in the maintenance of membrane potential and solute transport, decreasing cell survivability. Thus, it seems most likely that lipids and proteins rather than DNA are the primary targets of ROS in *B. burgdorferi.*

Preliminary analysis by negative stained immunoelectron microscopy of *B. burgdorferi* cultures exposed to *t*-butyl hydrogen peroxide revealed membrane blebs developed around the spirochete (Figure 6, panel C). These blebs were not observed in the electron micrographs of the aerobic or untreated anaerobic cultures (Figure 6, A & B), suggesting the membrane blebs were developing in response to ROS treatment. The potential effect of chemical treatment on *B.*

burgdorferi lipids and lipoproteins was determined by analyzing the fatty acid profiles of anaerobically grown cultures treated with *t*-butyl hydrogen peroxide or lipoxidase. The decrease in linoleic acid in treated cultures versus untreated cultures suggested that this polyunsaturated fatty acid was either no longer being incorporated into the membrane or was destroyed. Therefore, there is the possibility that the polyunsaturated fatty acids present in *B. burgdorferi* membranes are targets to lipid peroxidation.

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Table 1. Fatty acid analysis of anaerobically grown *B. burgdorferi* cultures.

Fatty acid Distribution as Triglycerides		Cells Only % of total mass	Cells + t-butyl % of total mass	Cell + lipoxidase % of total mass
Linoleic Acid	(c18:2n6)	0.0393	0.0044	0.0000
Oleic Acid	(c18:1n9)	0.3264	0.3165	0.2469
Palmitic Acid	(c16:0)	0.9808	0.8846	0.7000
Pentadecanoic Acid	(c15:0)	0.0344	0.0332	0.0262

Figure 1. Primer extension analysis of *napA* transcripts. Primer extension was performed with RNA extracted from *B. burgdorferi* strain B31. The sequencing ladder was generated with the napAPE primer. Lane 1:1 μg RNA, Lane 2: 5 μg RNA. The 5' end on the transcript is indicated by the arrowhead. The nucleotide sequence is located on the left. The predicted -10, -35, and ribosome binding site (RBS) are indicated. * denotes the TIGR translational start site. TRNSL indicated the putative translations start site.

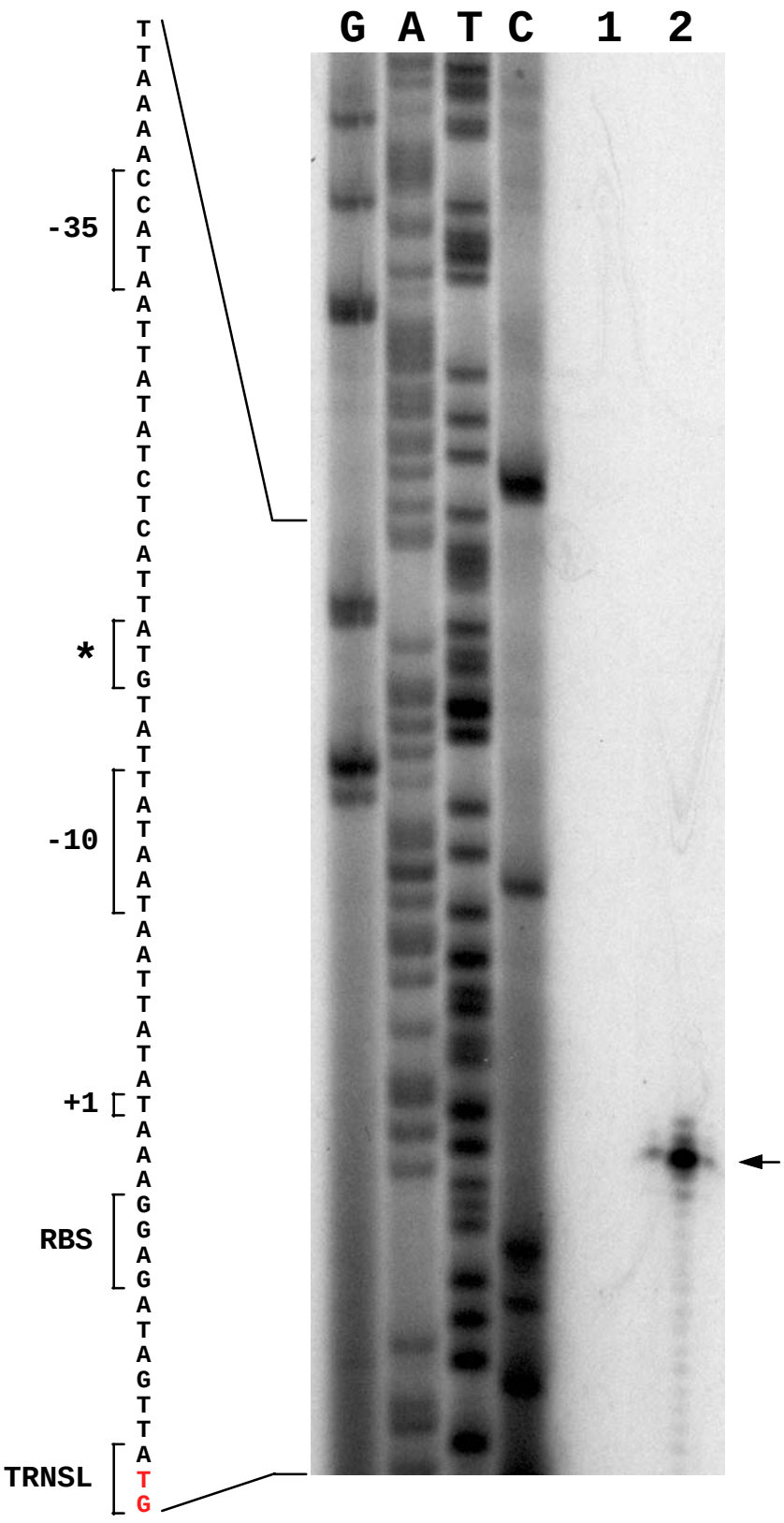

G A T C 1 2
-35
*
-10
+1
RBS
TRNSL
T T A A A A C C A T A A A T T A T A T C C T C A T T A T G T A T T A T A A T T A A T T A T T A T A T T A A A A G G A G A G A T A G T T A T G

Figure 2. Electrophoretic mobility of purified recombinant and native NapA. Purified protein was isolated from *E. coli* (lane A, heated) and *B. burgdorferi* (lane B, unheated) and separated by electrophoresis on an SDS-PAGE gel. Size standards, in kDa, are indicated to the left.

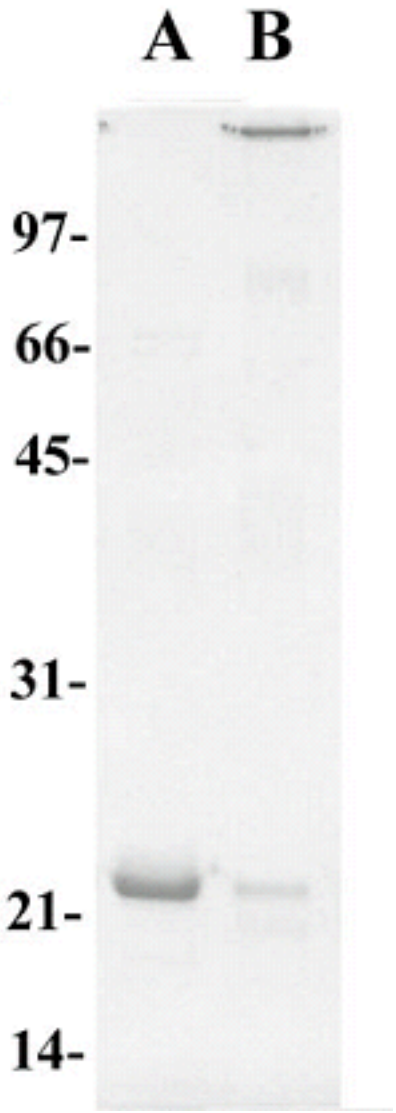
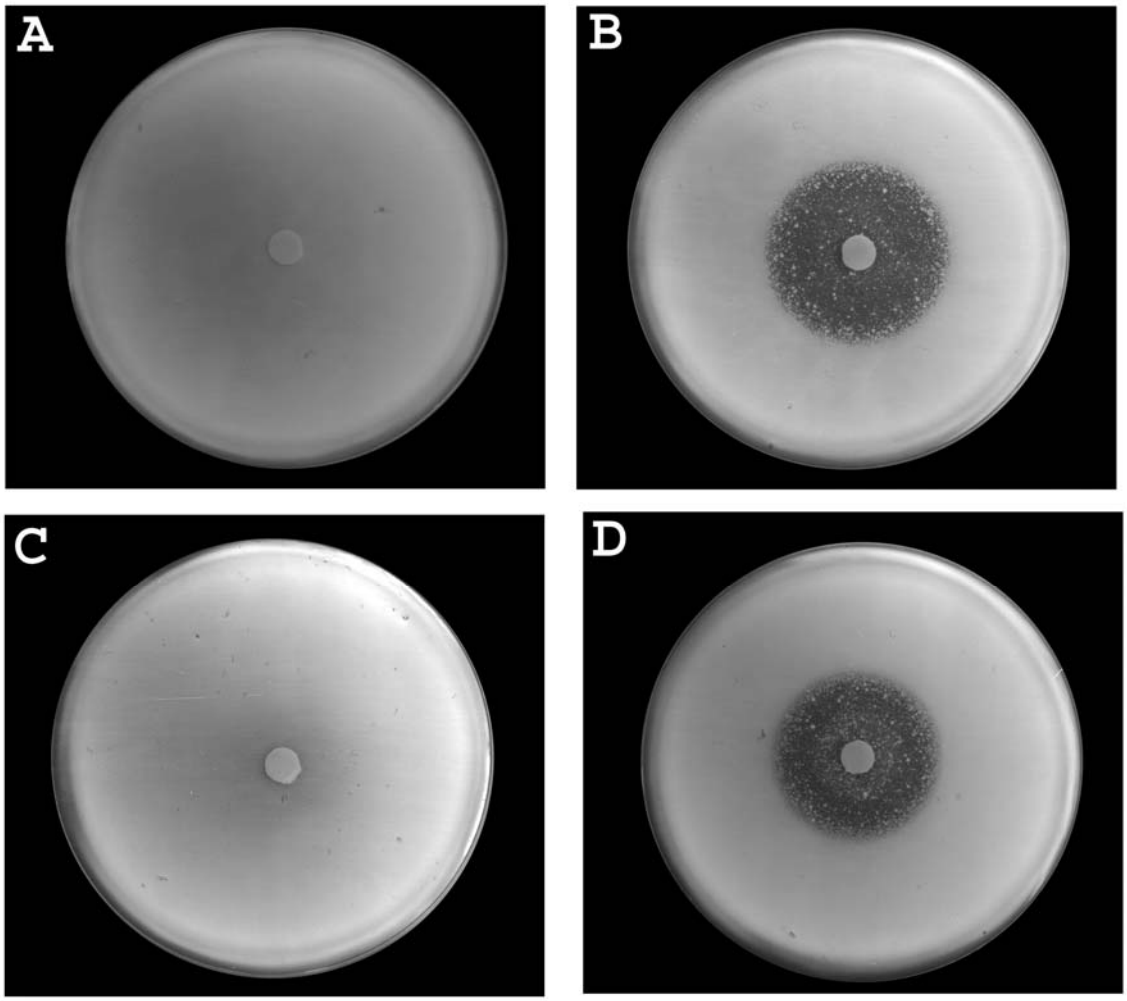

A B
97-
66-
45-
31-
21-
14-

Figure 3. Complementation of *E. coli* mutant TA4315 (Δ*aphCF*). *E. coli* TA4315 harboring pKK223-3, pJT4, or pJT5 was plated in top agar, sterile filter discs placed on the top agar were spotted with 10 μl of 100 mM *t*-butyl hydroperoxide in DMSO. (A) TA4315 cells alone exposed to DMSO. (B) TA4315 + pKK223-3 exposed to *t*-butyl hydrogen peroxide. (C) TA4315 + pJT4 exposed to *t*-butyl hydrogen peroxide. (D) TA4315 + pJT5 exposed to *t*-butyl hydrogen peroxide. (E) Amino acid sequence of NapA. Indicated are the sites of premature termination (Term) to produce NapADel and the mutation sites of the three C-terminal cysteines to serines to produce NapAMut..



E

Amino Acid Sequence

MEKYLSYIKKDDLDAIQLKLQELLASLHIFYSNLRGIHWNIKDTNFFVIH
KKTQKLYEYIEKIIDIVAERSRMLGYDSEFRYSEFMKKSFIKELDIESTSN
FLPSMESIVCSLTEILKNIFGMRKLIDTAGDYGTANIMDDIMSDLEKHLW
MHKALLENCDCFCHDENESKCCECDAK

Term S S S

Figure 4. Effect of oxygen concentration on expression of NapA. *B. burgdorferi* B31 A cultures were grown to 1 x 10^8 cells/ml under different 2, 5, 8, and 12 % oxygen. Total proteins were separated by SDS-PAGE and probed with α-NapA antisera. Purified NapA was loaded as a control. Size in kDa, are indicated to the left.

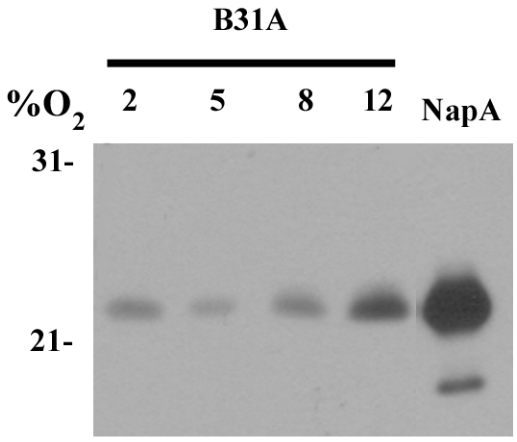
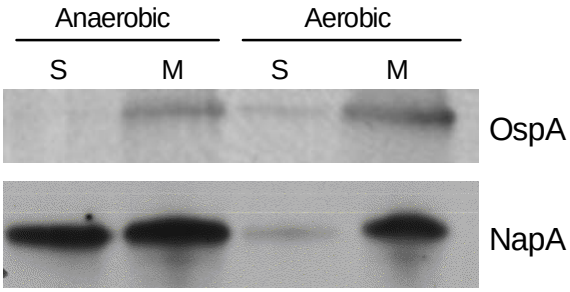
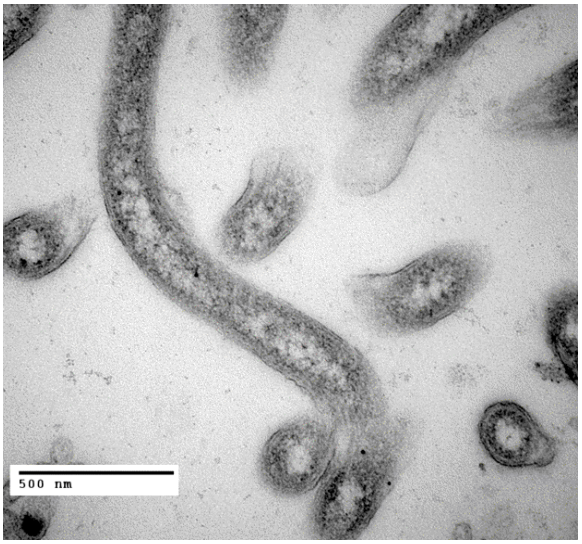

B31A
%O2
2
5
8
12
NapA
31-
21-

Figure 5. Effects of *t*-butyl hydrogen peroxide on the localization of NapA in *B. burgdorferi*. (A) *B. burgdorferi* B31 A3 cultures grown either in aerobic (lanes 1 & 2) or anaerobic (lanes 3 & 4) conditions were harvested at 5 x 10^7 cells/ml**.** Twenty-five μg of soluble (lanes designated S) and membrane (lanes designated M) proteins was separated by SDS-PAGE, transferred, and probed with α-NapA antisera or α-OspA antisera. Thin section immuno electron micrographs of *B. burgdorferi* cells probed with α-NapA and gold-labeled. Cultures were grown to 5 x 10^7 cells/ml under anaerobic (B) or aerobic (C) conditions. The anaerobic cultures were split and half was treated with 1 mM *t*-butyl hydrogen peroxide (D).

A

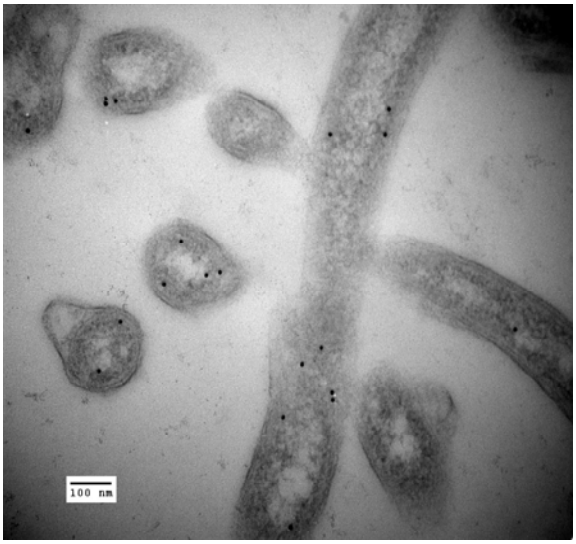


B



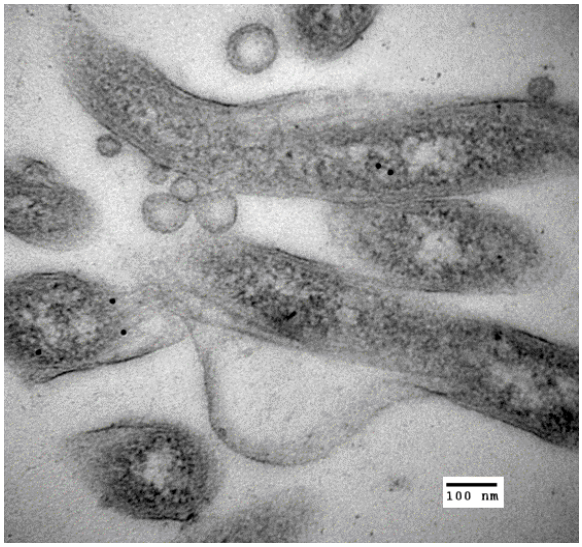
Anaerobic

C



Aerobic

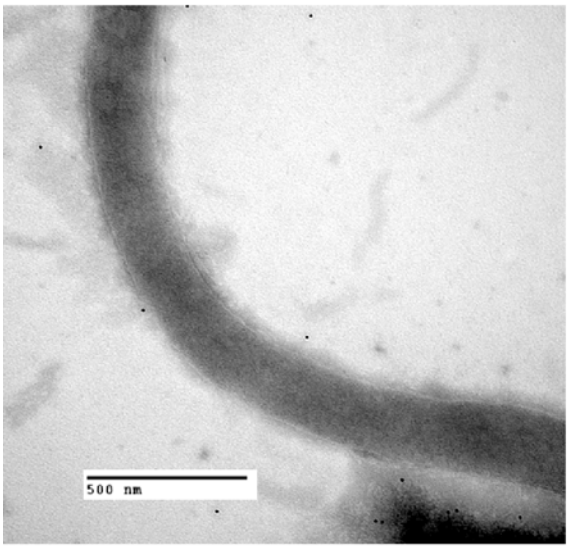
D



Anaerobic + *t*-butyl peroxide

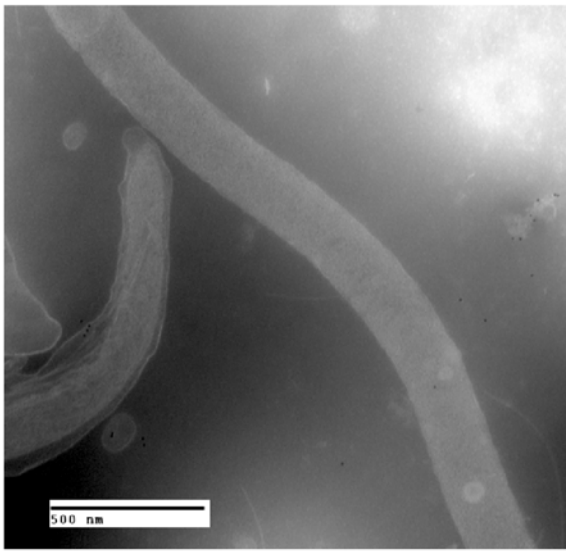
Figure 6. Whole cell immunoelectron micrographs of *B. burgdorferi* cells probed with α-NapA and gold-labeled secondary antibody. Cultures were grown under anaerobic (A) or aerobic (B) conditions and half of the anaerobic culture was treated with 1 mM *t*-butyl hydrogen peroxide (C).

A



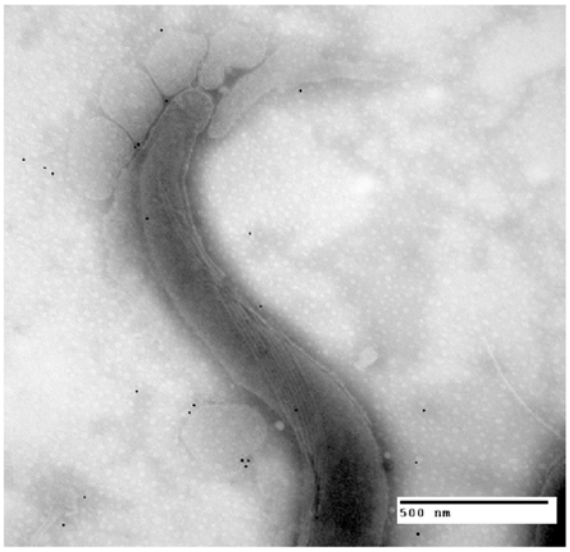
Anaerobic

B



Aerobic

C



Anaerobic + *t*-butyl peroxide

Chapter 5

Dissertation Summary

Lyme disease is a multi-system disorder caused by the spirochete *B. burgdorferi* and is transmitted by the bite of an infected *Ixodes* tick (1). The transmission of the disease is preventable if ticks are removed quickly before the bacteria can be transmitted. Early symptoms include EMs, limb pain, flu-like symptoms, swollen lymph nodes, peripheral neuropathy and lymphocytic meningitis (7, 11). If untreated, these early symptoms resolve and late Lyme disease, which is defined as the onset of chronic arthritis and infection of the central nervous system, begins after a latent period (5, 6). *B. burgdorferi* must survive long term in nutrient deplete environments in both the mammalian hosts and tick vectors for survival. For long-term survival in various environments, *B. burgdorferi* must regulate coordinated gene expression.

The genome sequence for *B. burgdorferi* was released in 1997 and revealed few known regulatory and virulence factors (4). Due to the lack of genetic tools to study the spirochete, very little was known about the virulence factors and how *B. burgdorferi* regulates gene expression. Recent advances in mutagenesis techniques and the development of shuttle vectors have opened up the genetic studies in *B. burgdorferi* (2, 8-10, 13). However, the regulation of gene expression in response to various environments and conditions in *B. burgdorferi* is just starting to be investigated.

The regulatory proteins identified in *B. burgdorferi* include three sigma factors, σ^{70}, σ^{S}, and σ^{54}, and an oxidative stress response regulator, BosR, (3, 4). Two complete histidine-kinase/response regulators, two-component systems, were also identified, and one of these systems is suggested to be required for σ^{54}-dependent gene expression (14). This work investigates the role of σ^{54} in expression of *rpoS*, the two-component system involved in σ^{54}-dependent activation and how *B. burgdorferi* responds to ROS.

Genome sequence analysis identified a putative σ^{54}-dependent promoter upstream of the *rpoS* promoter in *B. burgdorferi*. Primer extension mapped the translational start site downstream of the -24 and -12 GG and GC doublets found in σ^{54}-dependent promoter. By isolating mutations in *rpoN*, QRT-PCR analysis was used to demonstrate that as *B. burgdorferi* cultures enter stationary phase σ^{54} is required for maximum expression of *rpoS*. However, both immunoblots and QRT-PCR showed that *rpoS* expression increased when cultures were diluted into fresh BSKII medium. This phenomenon was observed in both wild-type and *rpoN* mutant strains of *B. burgdorferi* suggesting a second promoter for *rpoS* that was independent of σ^{54}. 5'RACE and primer extension mapped this second promoter to 13 bp inside of the 3' end of the upstream gene, *flgI*. These data confirm that *rpoS* expression as cultures enter stationery phase is σ^{54}-dependent and there is additional expression of *rpoS* from a σ^{54}-independent promoter when cultures are diluted into fresh medium.

σ^{54}-holoenzyme, unlike other sigma factor-holoenzyme complexes, is capable of binding DNA in a stable closed complex. For transcription initiation, an activator protein is required to hydrolyze ATP to aide in isomerization of the DNA and form the transcriptional active open complex. Approximately half of the σ^{54}-dependent activators in the Pfam database are part of two-component systems and by searching the *B. burgdorferi* genome a two-component system

was identified by homology. The goal of this project was to provide evidence to connect this two-component system to σ^{54}-dependent activation. QRT-PCR on mutants in the putative histidine kinase (SisK) demonstrated that as cultures enter stationary phase SisK is required for optimal expression of *rpoS* compared. To investigate the interactions of the histidine kinase and the putative response regulator (SisR) phosphorylation assays were performed. SisK demonstrated the ability to autophosphorylates and transfer the phosphate to SisR. However, the reaction never went to completion and analysis of the codon usage of SisR revealed that the recombinant SisR is not correctly assembled, which could be interfering with the phosphate transfer. Due to this, binding of SisR to enhancer binding site was not confirmed. To demonstrate that the enhancer-binding site for SisR is located 100-300 bp upstream of the σ^{54}-dependent promoter, *rpoS* promoter-*cat* fusions were constructed. QRT-PCR demonstrated that in *B. burgdorferi* cultures harboring these fusions, expression of *cat* increased as the cells entered stationary phase. These data support the hypothesis that the SisK and SisR are part of the two-component system involved in activation of σ^{54}.

B. burgfdorferi must not only survive long term in various host but must develop methods to survive other stresses encountered. Recently, BosR was shown to regulate the oxidative stress response in *B. burgdorferi* (3). One of the proteins determined to be regulated by BosR was a Dps/Dpr homolog, NapA (3, 12). To begin to investigate NapA, the correct translational start site was mapped and revealed that the +1 was located 33 bp downstream of the predicted start site and contained a putative RBS 5 bp upstream. Expression of NapA has previously been described as increasing when *B. burgdorferi* cells were exposed to ROS (3). Therefore, we investigated the effect of dissolved oxygen on NapA expression and found that as oxygen concentrations increased the levels of NapA were upregulated. NapA was found to

complement an *E. coli aphCF* mutant that is sensitive to alkyl hydroperoxides, when exposed to *t*-butyl hydrogen peroxide. *In vitro* enzyme assays showed that although NapA has some alkyl hydroperoxidases activity, that is most likely not the proteins primary function.

The streamlined *B. burgdorferi* genome sequence revealed no genes encoding proteins involved in cellular biosynthetic reactions for the synthesis of amino acids, fatty acids, lipids, enzyme cofactors, or nucleotides (4). Therefore, *B. burgdorferi* membranes are host derived, and unlike most bacteria, have been shown to contain polyunsaturated fatty acids. While investigating the expression of NapA by immunoelectron microscopy in aerobic and anaerobic cultures, we found that in anaerobic cultures treated with ROS formed blebs around the spirochete. The effect of chemical treatment on the *B. burgdorferi* cells was determined further by analyzing the fatty acid profiles of these cultures. Linoleic acid amounts decreased in treated cultures versus untreated cultures, suggesting that the linoleic acid is either no longer being incorporated into the membrane or being destroyed. Therefore, the possibility exists that the polyunsaturated fatty acids present in *B. burgdorferi* membranes are susceptible to lipid peroxidation.

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