CHARACTERIZATION OF SisK AND SisR, AN NtrB/C-TYPE REGULATORY SYSTEM IN THE LYME DISEASE SPIROCHETE, *Borrelia burgdorferi*

by

ANDREA POLACCHINI DE OLIVEIRA
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ABSTRACT

Lyme disease, an inflammatory disease caused by the spirochete *Borrelia burgdorferi*, is transmitted by the *Ixodes* tick. *B. burgdorferi* survives in prolonged stationary phase in the tick midgut and factors enabling *B. burgdorferi* to survive such conditions were investigated here. RpoS (σ^S) is an alternative sigma factor in *B. burgdorferi* that is associated with stress responses and stationary phase survival in other bacteria. Transcription of *rpo*S in *B. burgdorferi* is initiated by σ^{54}-RNA polymerase holoenzyme and requires the activator SisR. Purified SisR-His binds weakly to a DNA fragment located about 1.6 kb downstream of the *rpoS* promoter. SisK, the cognate sensor kinase of SisR, was purified as a maltose-binding protein fusion protein (MBP-SisK) and was shown to phosphorylate itself then transfer the phosphate to SisR. SisK contains a PAS domain that may bind flavin since purified MBP-SisK was faint yellow and displayed a peak absorbance at about 410 nm.

INDEX WORDS: Lyme disease, *Borrelia burgdorferi*, Two-component system, σ^{54}, σ^S, Phosphorylation

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DEDICATION

Mamae querida, for all your hard work, love and care that you have always given me. For every “esfiha”and “torta de frango”that you have ever baked and have sold to get myself and my sisters through school. For every tear you dropped every time I had to return to the US. For every time I couldn’t be in Brazil to hug you and give you the love and the attention that you deserve. For every simple family get together, birthday or holiday that I could not be home to celebrate. Mamae, I dedicate this work to all the wonderful times that we have to look forward to. I love you more than anything, I am so proud of calling you Mom.

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LIST OF ABBREVIATIONS

Abbreviation	Full name
ADP	**adenosine diphosphate**
ARNT	**Aryl hydrocarbon receptor nuclear translocator**
ATP	**adenosine triphosphate**
BCA	**bicinchoninic acid**
BME	**β-mercaptoethanol**
BSKII	**barbour-stoenner-kelly II medium**
CNS	**central nervous system**
DctD	**dicarboxylic acid transport protein D**
DmpR	**phenol catabolic pathway positive regulator**
DTT	**dithiolthreitol**
EDTA	**ethylenediamine tetraacetic acid**
ELISA	**enzyme-linked immunosorbent assay**
EM	**erythema migrans**
EPPS	**N-2-hydroxyethylpiperazine-N'-3-propane-sulfonic acid**
FAD	**flavin adenine dinucleotide**
FhlA	**formate hydrogen-lyase activator**
FlbD	**flagellar transcription activator**
FMN	**flavin mononucleotide**
glnA	**encodes glutamine synthetase**
HEPES	**N-2-hydroxyethylpiperazine-N'-2-ethanesulfonicacid**
IPTG	**isopropyl-β-thiogalactoside**
KSCN	**potassium thiocyanate**

LB	**luria bertani**
LevR	**levanase operon regulator**
NifA	**nitrogen fixation protein A**
NifL	**nitrogen fixation protein L**
Ntr response	**nitrogen regulated response**
NtrB	**nitrogen regulatory protein B**
NtrC	**nitrogen regulatory protein C**
PAS	**PER ARNT SIM**
PCR	**polymerase chain reaction**
PER	***Drosophila* Period clock protein**
PMSF	**phenylmethylsulfonyl fluoride**
PspF	**phage shock protein F**
rpoN	**encodes σ^{54} (also designated *ntrA*, *glnF*)**
rpoS	**encodes σ^{S} (also designated σ^{38})**
SDS-PAGE	**sodium dodecyl sulfate-polyacrylamide gel electrophoresis**
σ factor	**sigma factor**
σ^{54}-holoenzyme	**σ^{54}-RNA polymerase holoenzyme**
σ^{s} - holoenzyme	**σ^{s}-RNA polymerase holoenzyme**
SIM	***Drosophila* Single-minded**
SisR	**sigma s regulatory protein R**
SisK	**sigma s regulatory protein K**
XylR	**xylose repressor**

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Introduction

Lyme disease was first described in Old Lyme, Connecticut in 1977 with a strange outbreak of juvenile arthritis (Steere *et al.*, 1977). The etiology of the disease was confirmed in 1982 when a novel spirochete, now called *Borrelia burgdorferi*, was isolated from Lyme disease patients and from *Ixodes dammini* ticks (Burgdorfer *et al.*, 1982). Lyme disease has a worldwide distribution. In the United States, it is found primarily in the northeast and west (Nocton and Steere, 1995). Lyme disease is currently the most common tick-borne disease in both the United States and Europe (Control, 1997; Schlesinger, 1998).

During the *I. dammini* life cycle, newly hatched larvae take a rodent blood meal, molt into nymphs, and then remain dormant until the following spring. If the rodent is infected, it is during this first blood meal that *B. burgdorferi* can become established in the tick midgut. Transmission of *B. burgdorferi* can occur when the infected nymphs feed on a human host after the dormant period, and if left untreated can result in Lyme disease (Burgdorfer *et al.*, 1982).

Lyme disease is a multi-system inflammatory disease and has two distinct stages, early and late that may be separated by a latent phase. Latency can last for months to years, during which time the spirochetes survive in very low numbers in the host. In early phase patients may develop a rash at the site of the tick bite and flu-like symptoms. Diagnosis and treatment during the early stage with antibiotics such as doxycycline, amoxicillin, penicillin or cefuroxime have high success rate (Hercogova and Brzonova, 2001). Late Lyme disease is hard to diagnose because it mimics other

diseases and cycles through acute symptoms and remission. The alternation of acute symptoms and remission is thought to be due to variation of surface antigens of *B. burgdorferi* and new variants escaping the immune system. Symptoms include central nervous system (CNS) complications and damage to heart and joint tissues (Logigian *et al.*, 1990; Steere *et al.*, 1983). The pathology of late Lyme disease is caused by *B. burgdorferi* lipoproteins triggering the release of host cytokines resulting in inflammation and tissue destruction (Habicht *et al.*, 1991).

Studies that address the regulation of gene expression in *B. burgdorferi* will assist in clarifying Lyme disease transmission and pathogenesis. In particular, identifying genes required for *B. burgdorferi* long-term survival in the tick midgut may lead to new strategies for the prevention of Lyme disease.

In *Escherichia coli* and other bacteria σ^s, encoded by *rpoS*, is the RNA polymerase holoenzyme sigma factor involved in survival during stationary phase (Henaut and Danchin, 1996). *B. burgdorferi rpoS* is regulated by σ^{54}-RNA polymerase holoenzyme (σ^{54}-holoenzyme) (Frye, 2000); Hubner 2001). Transcription initiation with σ^{54}-holoenzyme requires an activator usually part of a two-component regulatory system (Popham *et al.*, 1989). Activators of σ^{54}-holoenzyme generally bind to sites that are located 100-200 bp upstream of the target promoter and contacts σ^{54}-holoenzyme through DNA looping to activate transcription (Kustu *et al.*, 1991; Weiss *et al.*, 1992a). I report here on the preliminary characterization of SisK/SisR two-component regulatory system which we found to be homologous to the *Salmonella enterica* serovar Typhimurium nitrogen regulatory proteins B and C (NtrB/C) two-component regulatory system and suggest a model for how it regulates expression of *rpoS* in *B. burgdorferi*.

Life cycle and transmission of *B. burgdorferi*.

Lyme disease is a zoonotic infection. *Ixodes*, the vector responsible for the transmission of *B. burgdorferi* to humans (Burgdorfer *et al.*, 1982), has a world-wide distribution. In the United States, Lyme disease occurs primarily in midwest, west and northeast and is primarily transmitted by *Ixodes scapularis. Ixodes persucatus* transmits the spirochete in Asia, and *Ixodes ricinus* transmits it in Europe (Burgdorfer and Kierans, 1983; Steere and Malawista, 1979). *Ixodes* ticks feed on mammals, birds and even reptiles, becoming infected with *B. burgdorferi* upon feeding on infected mammals (Benach *et al.*, 1987).

Ixodes species have a two-year life cycle that consists of three stages, larva, nymph and adult, feeding once at each stage of the life cycle. Eggs are laid in late spring and the larvae hatch in the late summer to early fall (Anderson and Magnarelli, 1980). Since there is no trans-ovarian transfer of *B. burgdorferi*, the newly hatched larvae are not infected. Ticks in the larval stage prefer to feed on small rodents, including the white-footed mouse, which is often spirochetemic during the spring, summer and fall (Levine *et al.*, 1985). Over a two- to three-day feeding period, *B. burgdorferi* is transmitted to the larvae via a blood meal and the spirochetes make their way to the tick midgut (Luft and Dattwyler, 1989). After the blood meal is digested, larvae go into a dormant stage during the fall and winter months. In the following spring, the larva undergoes a metamorphosis to the nymph stage of the life cycle. Ingestion of a blood meal into the nymph midgut stimulates the spirochetes to divide and penetrate the midgut wall and eventually migrate to the salivary glands of the nymph (Schwan, 1996). *B. burgdorferi* is probably transmitted to humans by saliva released into the wound by the nymph or through regurgitation of the contents of the nymph midgut (Benach *et al.*, 1987). The nymphs drop off of the host after the

blood meal and molt, entering the adult stage of the *Ixodes* life-cycle in the fall. The nymphs grow to adult ticks, feed on the white-tailed deer, meet the female tick and mate, after which the female drops off, lays her eggs and dies (Luft and Dattwyler, 1989) (Figure 1).

Lyme disease symptoms.

B. burgdorferi enters the host by a bite from an *Ixodes* tick with 60% to 80% of victims developing a red bulls-eye rash at the site of the bite called erythema migrans (EM) (Steere *et al.*, 1983). The EM resolves as the bacteria disseminate to most organ systems of the body, at which time the bacteria can be found in low numbers in the joints and CNS. Additional early symptoms include low-grade fever, malaise, muscle aches, headache, limb pain, chronic cerebrospinal fluid pleocytosis, polyneuropathy, chronic lymphocytic meningitis, and rare CNS involvement. Weeks to months from the early stage, the patient may develop neurological or cardiac abnormalities, and months to years later arthritis (Logigian *et al.*, 1990; Steere *et al.*, 1983). Fibromyalgia can follow infection with *B. burgdorferi* but is unresponsive to antibiotic therapy and is treated with tricyclic antidepressants and an exercise program (Massarotti, 2002).

Diagnosis, Prevention and Treatment.

Diagnosis of early Lyme disease can be difficult if the patient does not notice a tick bite or develop an EM. Laboratory testing for *B. burgdorferi* infection is intended to substantiate a physician's clinical judgment of whether a patient has Lyme disease. Cultivation of *B. burgdorferi* from a patient's skin or blood is the gold standard for demonstration of active infection, but it is expensive and lacks clinical

sensitivity (Berger *et al.*, 1992). Detection of spirochetal DNA in clinical samples by the polymerase chain reaction (PCR) has better sensitivity (Kruger and Pulz, 1991; Moter *et al.*, 1994), but PCR for *B. burgdorferi* has not yet been standardized for routine diagnostic testing. The type of sample, as well as the how the sample was obtained, greatly affect the outcome of the PCR result and the high sensitivity of the assay can lead to false positive results due to contamination of the samples or equipment (Coyle, 1997; Moter *et al.*, 1994)

Detection of antibodies to *B. burgdorferi* is the most practical and common approach for laboratory work-up of a case of suspected Lyme disease. Serologic assays, however, fall short of 100% sensitivity and specificity. Moreover, examination of a single specimen in time does not discriminate between previous and ongoing infections. Because of a background of false positives even among healthy populations of nonendemic regions, serologic testing is recommended only when there is at least a one in five chance in the physician's estimation that the patient has active Lyme disease (Coyle, 1997). The pretest likelihood of the disease is determined by the physician in the context of epidemiologic and clinical facts of the case. This estimate can serve to reassure patients who are at low risk of *B. burgdorferi* infection but are seeking a Lyme test for complaints of a more nonspecific nature. Although new subunit serologic assays based on recombinant proteins are becoming available, the longstanding, two-test approach, in which a positive or indeterminate result with a ELISA test is followed by verification with a more specific Western blot assay, still provides the physician with a reasonably accurate and reliable assessment of the presence of antibodies to *B. burgdorferi* (Coyle, 1997).

More recent challenges for serologic testing are seropositivity in the population as the result of immunization with the Lyme disease vaccine and the emergence of new *Borrelia* species that cause Lyme disease-like illnesses (Bunikis and Barbour, 2002). Vaccination was the best way of prevention for people living in high-risk areas. LYMErix™, a recombinant vaccine based on OspA for the prevention of Lyme disease was used in persons aged 15-70 (Luke *et al.*, 1997). However, as of February 25, 2002 the manufacturer announced that the LYMErix™ was no longer going to be commercially available. However, treatment with antibiotics during the early stage has a high success rate. Optimal therapy of Lyme borreliosis is still lacking, but doxycycline, amoxicillin, penicillin, and ceftriaxone are recommended most frequently (Hercogova and Brzonova, 2001). Patients whose only symptom is a bulls-eye rash surrounding the tick bite typically receive a 3-week course of doxycycline or amoxicillin. At the other extreme, 25 to 40 percent of patients with joint, neurologic, cardiac, or multiple extracutaneous symptoms or with systemic Lyme disease receive 2 to 3 weeks of intravenous ceftriaxone (Pena *et al.*, 1999). In most patients, antibiotic therapy is curative, but in a smaller percentage of patients, the presence of the HLA-DR beta 1*0401 haplotype can trigger treatment-resistant arthritis in which antibiotic therapy is ineffective. In these instances, hydroxychloroquine and methotrexate are recommended. Arthroscopic synovectomy may be considered when antibiotic therapy is not curative (Massarotti, 2002).

Morphology and Physiology of *B. burgdorferi*.

B. burgdorferi is a member of the spirochetes which form a unique phylogenetic group within the domain bacteria (Johnson *et al.*, 1984). Spirochetes are spiral-shaped, long, slender, and highly motile organisms that move by corkscrew

motility, via seven to eleven periplasmic flagella (Hovind-Hougen, 1984) . *B. burgdorferi* have a type gram-negative cell wall architecture (Holt, 1978). The distinctive helical morphology of *B. burgdorferi* cells consist of a protoplasmic cylinder made of a peptidoglycan layer and cytoplasmic membrane wrapped by endoflagella and surrounded by a multi-layered outer membrane (Johnson, 1977). The periplasmic flagella are attached sub-terminally to each pole of the spirochete with the free end of the flagella extending toward the opposing cell pole (Baranton and Old, 1995; Barbour and Hayes, 1986) (Hovind-Hougen *et al.*, 1986). The outer membrane lacks lipopolysaccharide (LPS) but does contain a number of lipoproteins, some of which are designated Osp for outer surface proteins. OspA and OspB are the two most abundant lipoproteins and have 53% amino acid sequence identity. OspC, OspD, OspE-related proteins (Erps), decorin binding proteins (Dbp), and the antigenically variable proteins known as Vls proteins, have also been identified as outer membrane constituents (Fraser *et al.*, 1997). Expression of OspC and DbpA were shown by Hubner and coworkers to be regulated by *rpoS* (Hubner, 2001).

B. burgdorferi is an aerotolerant anaerobe. This spirochete has extremely limited metabolic capabilities, lacking most genes encoding the enzymes necessary for the synthesis of amino acids, lipids, enzyme cofactors, nucleotides, as well as genes encoding the enzymes necessary for the tricarboxylic acid cycle or oxidative phosphorylation (Fraser *et al.*, 1997). *B. burgdorferi* differs from most bacteria in that it does not require iron for growth or enzymatic activity (Posey and Gherardini, 2000). *B. burgdorferi* produces energy through fermentation of glucose to lactate via the Embden-Myerhof pathway (Johnson, 1976). Fructose, maltose, trehalose, raffinose, glycerol, and N-acetylglucosamine (NAG) may also be used as carbon sources (Barbour and Hayes, 1986; Fraser *et al.*, 1997; Johnson, 1976). *B. burgdorferi*

has genes encoding chitobiase which converts chitobiose to NAG. *B. burgdorferi* must use lipids from the host, or when being cultivated in the laboratory, the media must be supplemented with a source of long-chain fatty acid such as rabbit serum, since this spirochete is incapable of elongating or degrading long-chain fatty acids and incorporates them unaltered into their membranes (Livermore *et al.*, 1978). Bovine serum albumin (BSA) is a suitable lipid carrier and addition of lipids to growth media in the absence of BSA is toxic to the cells (Barbour and Hayes, 1986; Livermore *et al.*, 1978).

The G+C content of the *B. burgdorferi* genome is approximately 25% (Barbour and Hayes, 1986; Fraser *et al.*, 1997). *B. burgdorferi* has a linear chromosome of 950-kb in addition to ~610 kb of DNA within ~23 linear and circular plasmids ranging in sizes from 5 to 56-kb (Casjens *et al.*, 2000). Virulent strains become avirulent after about five to ten *in vitro* passages (Schwan *et al.*, 1988), which correlates with the loss of certain plasmids (Purser and Norris, 2000). The *B. burgdorferi* genome and plasmids have been sequenced, but no genes known to be involved in virulence were identified based on homology searches (Fraser *et al.*, 1997). However, the products of 60% of the genes identified have unknown functions, and many of these may be as yet unrecognized virulence factors (Fraser *et al.*, 1997). Genetic exchange systems for creating mutants or evaluating their impact on virulence are poorly developed, but it is possible to generate mutants in high passage, avirulent *B. burgdorferi* strains (Bono *et al.*, 2000).

Motility may aid movement out of bloodstream and through viscous substances including extracellular matrix and tight intracellular junctions (Barbour *et al.*, 1986). *B. burgdorferi* chromosome devotes about 6% of its genes to motility and chemotaxis, suggesting a significant role for these factors in the life cycle of this

organism (Fraser *et al.*, 1997). Isolation of a non-flagellated, non-motile mutant of *B. burgdorferi* demonstrated the importance of motility in virulence since this mutant showed decreased ability to penetrate a human endothelial cell monolayer and exhibited reduced virulence in experimental animals (Sadziene *et al.*, 1991).

The outer surface lipoproteins elicit the same type of inflammatory response as LPS, and the genes encoding these major outer surface proteins are located on several plasmids (Barbour and Garon, 1987b). For example, *ospA* and *ospB* are located on lp54 (Fraser *et al.*, 1997; Howe *et al.*, 1984), *ospC* is located on lp26 (Sadziene *et al.*, 1993), *ospD* is located on lp38 (Norris *et al.*, 1992), and *ospE* and *ospF* are located on cp32 (Fraser *et al.*, 1997; Lam *et al.*, 1994). *B. burgdorferi* has been shown to adhere to many different host tissue components and it is believed that this attachment may be necessary for long term persistence in an infection (Comstock and Thomas, 1991).

Regulation of stress response genes in *B. burgdorferi*.

Analysis of *B. burgdorferi* genome sequence revealed very few known regulatory proteins, only two complete two-component systems (protein histidine kinase/response regulator), the housekeeping σ^{70}, and the two alternative sigma factors, RpoN (σ^{N}or σ^{54}) (Buck *et al.*, 2000) and RpoS (σ^{s} or σ^{38}) (Fraser *et al.*, 1997). Other regulatory genes included two XylR family members which can both activate and repress gene expression, a metal uptake regulatory protein (Fur), and a handful of other regulatory proteins known to be involved in phosphate and carbon metabolism in other bacteria (Fraser *et al.*, 1997). A *spoT* homolog was also identified in *B. burgdorferi,* suggesting that a stringent response (short-term stress response to amino acid and carbon starvation) is also present in *B. burgdorferi* (Concepcion and Nelson,

2003). Interestingly, no known heat-shock response regulatory factors were identified in the analysis of the *B. burgdorferi* genome.

The sigma subunit is required for promoter-recognition and also plays a role in initiation of transcription by bacterial RNA polymerase (Burgess *et al.*, 1969). Bacteria may contain several alternative sigma subunits with differing sequence specificities that direct the RNA polymerase holoenzyme to different promoters (Helmann and Chamberlin, 1988). One of the two alternative sigma factors in *B. burgdorferi* is σ^S, which is typically associated with general stress responses in bacteria. *B. burgdorferi* cells are exposed to a variety of environmental changes, including temperature, pH, spirochete cell density and different host tissues, which result in differential antigen expression in *B. burgdorferi* (Akins *et al.*, 1995; Carroll *et al.*, 1999; Fikrig *et al.*, 1998; Indest *et al.*, 1997), and σ^s could have a role in regulating expression of genes required for *B. burgdorferi* survival in response to one or more of these environmental stresses.

When infected ticks take a blood meal, *B. burgdorferi* cells migrate from the tick midgut to the salivary glands and are then injected into mammalian dermal tissue (De Silva and Fikrig, 1995; Schwan, 1996). To mimic conditions that change during tick engorgement, virulent *B. burgdorferi* strain 297 was cultivated *in vitro* at elevated temperature (37°C), reduced pH (pH 6.8), and increased spirochete cell density. These conditions, caused an up-regulation of OspC, DpbA, OspF, and Mlp-8 (group I proteins), and a simultaneous down-regulation of OspA, P22 and Lp6.6 (group II proteins) (Yang *et al.*, 2000). Conditions that induced expression of the group I proteins also induced the synthesis of RpoS, which prompted the hypothesis that group I genes in strain 297 may be controlled through RpoS (Yang *et al.*, 2000).

σ^{54} does not share obvious homologies with other sigma factors (Merrick, 1993). This σ factor directs RNA polymerase to a class of distinct promoters that have conserved elements in the -12/-24 regions relative to transcriptional start site. In *B. burgdorferi*, σ^{54} regulates the expression of RpoS (Frye, 2000; Hubner, 2001), which in turn, influences expression of OspC and DbpA (Hubner, 2001). A sequence located approximately 60 bp upstream of the translational start site of *rpoS* matches the consensus sequence for σ^{54}-dependent promoters (Figure 3), and primer extension assays showed that the 5'-end of the *rpoS* mRNA was consistent with this sequence being a σ^{54}-dependent promoter (Frye, 2000). Two-dimensional gel electrophoresis analysis of crude protein extract from a *B. burgdorferi rpoN* mutant strain showed that σ^{54} regulates expression of other genes (Dr. Jonathan Frye, personal communication,). Recent microarray analysis of RNA from *B. burgdorferi rpoN* and *rpoS* mutant strains showed that at least 137 genes were up or down regulated in a *rpoN* mutant strain, 50 of which also displayed altered levels of expression in the *rpoS* mutant strain (Dr. Frank Gherardini, personal communication).

Transcription initiation with σ^{54}-holoenzyme requires an activator (Popham *et al.*, 1989), which generally binds to sites upstream of the promoter and contacts σ^{54}-holoenzyme through DNA looping (Kustu *et al.*, 1991). These activator binding sites can be moved quite far from the promoter in some cases and still function. Hence they are similar to eukaryotic enhancers and are sometimes referred to as bacterial enhancers (Ninfa *et al.*, 1987; Reitzer and Magasanik, 1986). The σ^{54}-dependent activators, also referred to as enhancer-binding proteins, share a highly conserved central domain involved in transcriptional activation and ATP hydrolysis (Popham *et al.*, 1989). Inspection of the *B. burgdorferi* genome reveals a single gene located approximately 8.7 kb downstream of *rpoS* that encodes an enhancer-binding protein

that was designated *sisR* and an adjacent gene encodes the cognate histidine kinase of SisR which was designated as *sisK* (Figure 2) (Dr. Timothy Hoover, personal communication; Fraser *et al.*, 1997).

Two–component regulatory systems.

Signal transduction across biological membranes is used by cells as a means to respond to their environment. Histidine protein kinases and their cognate response regulators are undoubtedly the most widely used signal-transduction enzymes in nature, forming two-component systems that are present in all three major kingdoms of life, Bacteria, Archaea and Eukarya (Wolanin *et al.*, 2002). Analysis of sequenced bacterial genomes reveals that the number of two-component system within an organism range from zero (*Mycoplasma genitalium* and *Mycoplasma pneumonia*) to 70 (*Bacillus subtilus*) (Koretke *et al.*, 2000). This major mechanism of signal transduction has adopted phosphorylation as a means of information transfer (Hoch and Silhavy, 1995)

Bacterial two-component systems mediate adaptive responses to a broad range of environmental stimuli, including starvation for phosphate (Pho) or nitrogen (Ntr), nitrate and nitrite metabolism (Nar), citrate uptake and catabolism (Cit), oxygen limitation (Aer), osmotic stress (EnvZ/OmpR), stress-induced sporulation (Kin/Spo), N-acetylmuramoyl-L-alanine amidase biosynthesis (Lyt), host recognition for pathogen invasion (Vir), and chemotaxis (Che) adaptation to various carbon and nitrogen sources (Hoch and Silhavy, 1995; Stock *et al.*, 1989; Parkinson and Kofoid, 1992). In addition, pathogenic bacteria sense when they need to express virulence factors to survive the variety of environmental changes in the human body (Hoch and Silhavy, 1995)

NtrC/B model system.

The SisK/SisR system is homologous to the well characterized nitrogen regulatory protein B and C (NtrB/C) two-component regulatory system found in *S. enterica* serovar Typhimurium and *Escherichia coli.* Ammonia is the preferred source of nitrogen in *S. enterica* serovar Typhimurium and *E. coli*. The nitrogen regulatory (Ntr) system allows these bacteria to respond to the availability of ammonia in the growth medium and modulate the expression of key proteins involved in ammonia assimilation. Intracellular levels of nitrogen are measured as a ratio of α-ketoglutarate to glutamine and are sensed by uridylyltransferase/uridylyl-removing enzyme (UTase/UR) and the P_{II} protein. Glutamine synthatase (GS), enzyme encoded by *glnA*, catalyzes the synthesis of glutamine from ammonia and glutamate. Under nitrogen poor conditions, GS primarily functions in ammonia assimilation. GS is a dodecamer and its activity is regulated by adenylylation. GS is most active when each subunit is unadenylated. In nitrogen rich conditions, the P_{II}-UMP protein is deuridylylated by UTase/UR. The deuridylylated form of P_{II} stimulates the ATase to adenylylate and thus inactivates GS. Under nitrogen poor conditions the UTase/UR enzyme uridylylates the P_{II} protein, which can then interact with adenylyl transferase (ATase) enzyme to stimulate the deadenylylation of GS-AMP (Reitzer, 1996).

Nitrogen regulatory protein C (NtrC; also called NRII) and nitrogen regulatory protein B (NtrB; also called NRI) are a response regulator and histidine protein kinase, respectively, of a two-component system that activates transcription of *glnA* with a σ^{54}-holoenzyme (Kustu *et al.*, 1989). In nitrogen poor environment as part of the Ntr response, NtrB autophosphorylates and then transfer the phosphoryl group to NtrC. Phosphorylation of NtrC stimulates oligomerization of the protein which activates the ATPase activity of NtrC and allows it to activate transcription. The

deuridylylated form of P_{II} interacts with NtrB to stimulate its phosphatase activity, resulting in the dephosphorylation of NtrC-P (Keener and Kustu, 1988). NtrC-phosphate binds to an enhancer located upstream of the *glnA* promoter and contacts σ^{54}–holoenzyme bound at promoter in closed complex through DNA looping (Kustu *et al.*, 1991; Weiss *et al.*, 1992b). The enhancer can be moved several kilobase away from the *glnA* promoter and still function (Ninfa *et al.*, 1987; Reitzer and Magasanik, 1986). Productive interactions between NtrC-phosphate and σ^{54}–holoenzyme lead to isomerization of the closed complex to an open complex that is competent to initiate transcription. This isomerizaton reaction requires ATP hydrolysis by NtrC-phosphate (Figure 3) (Wedel and Kustu, 1995).

Activators of σ^{54}-holoenzyme.

Bacteria that have σ^{54} usually contain a single copy of *rpoN*, with a few notable exceptions such as *Bradyrhizobium japonicum* which has two copies of *rpoN* (Kullik et al., 1991). However, the number of activators of σ^{54}-holoenzyme within a given bacterial species varies considerably. *E.coli* has eleven potential activators of σ^{54}-holoenzyme, while *Chlamydia trachomatis*, *Helicobacter pylori* and *B. burgdorferi* each have a single potential activator of σ^{54}-holoenzyme. The activator in *B. burgdorferi*, Sigma s regulatory protein R (SisR) is 51.5 KDa and it is highly homologous to activators that belong to the NtrC family (Figure 4) (Fraser *et al.*, 1997).

Activators of σ^{54}-holoenzyme are generally DNA-binding proteins with three functional domains. In most cases the amino-terminal domain is responsible for signal reception (A-domain), the central domain (C-domain) functions in ATP hydrolysis and transcriptional activation, and the carboxy-terminal domain is involved

in binding to the enhancer element (D-domain) (Figure 5) (Morett and Segovia, 1993). Examples of well studied σ^{54} –dependent activators include *S. enterica* serovar Typhimurium NtrC, S*inorhizobium meliloti* dicarboxylic acid transport protein D (DctD), *E. coli* phage shock protein F (PspF), *Pseudomonas putida* xylene catabolism regulator (XylR), *Caulobacter crescentus* flagellar transcription activator (FlbD), *Bacillus subtilis* levanase operon regulator (LevR) and *K. pneumoniae* nitrogen fixation protein A (NifA) (Hoch and Silhavy, 1995; Morett and Segovia, 1993).

Environmental signals are sensed via the A-domain, with activators falling into one of several classes based on the mode of regulation (Shingler, 1996). Activators such as NtrC, DctD and FlbD are response regulators of two-component systems (Benson *et al.*, 1994; Kustu *et al.*, 1989). LevR is both positively and negatively regulated by phosphorylation, but not as part of a two-component system (Debarbouille *et al.*, 1991). XylR, *Pseudomonas putida* phenol catabolic pathway positive regulator (DmpR), and *E. coli* formate hydrogen-lyase transcriptional activator (FhlA), interact directly with an environmental signal through their amino-terminal domains. Binding of an inducer to these proteins stimulates their activities (Perez-Martin and de Lorenzo, 1996) (Maupin and Shanmugam, 1990; Shingler *et al.*, 1993). Nitrogen fixation protein A (NifA) in *Klebsiella pneumoniae* and *Azotobacter vinelandii* is negatively regulated by interactions with nitrogen fixation protein L (NifL) in response to molecular oxygen and fixed nitrogen (Henderson *et al.*, 1989). However, NifA in *S. meliloti*, *Rhizobium trifoli*, and *Bradyrhizobium japonicum* is believed to be under redox control (Fischer *et al.*, 1988; Beynon *et al.*, 1988; Krey *et al.*, 1992; Screen *et al.*, 1994). PspF is unusual in that it lacks an A-domain (Jovanovic *et al.*, 1997). PspF activity is inhibited by interactions with the product of one of the genes that it regulates, *pspA* (Adams *et al.*, 2003).

The N-terminal domain of response regulators is about 120 residues and contains the site of phosphorylation, which is a conserved aspartyl residue located at about position 50. The response regulator domain is joined to the remainder of the protein by a flexible glutamine-rich linker known as the Q-linker (Figure 5) (Morett and Segovia, 1993).

The central activation domain is about 240 residues in length and contains the Walker A (phosphate loop or P-loop) and Walker B ATP binding motifs, as well as determinants for oligomerization and contact with σ^{54}-holoenzyme (Figure 4 and 5) (Morett and Segovia, 1993). This domain is highly conserved among σ^{54}-dependent activators and belongs to the superfamily of AAA^{+} (ATPase associated with diverse cellular activities) ATPases. The AAA^{+} superfamily of ATPases are found in all kingdoms of living organisms where they participate in diverse cellular processes including membrane fusion, proteolysis, DNA replication, recombination, repair and transcription (Ogura and Wilkinson, 2001). This superfamily is defined by their conserved Walker A and Walker B motifis, which mediate NTP-binding and hydrolysis, and the AAA minimum consensus comprised of sensor I and sensor II which are potential γ-phosphate sensor regions (Ogura and Wilkinson, 2001). In general, AAA+ proteins have chaperone-like activity, participating in the assembly or remodeling of DNA-protein or protein-protein complexes (Neuwald *et al.*, 1999; Ogura and Wilkinson, 2001).

The D domain is about 90 residues in length in most activators and contains a helix-turn-helix DNA binding motif (Morett and Segovia, 1993). Activators of σ^{54}-holoenzyme generally bind to sites located 100-200 bp upstream of the target promoter and contact polymerase bound at the promoter to activate transcription (Pelton *et al.*, 1999; Ray *et al.*, 2002). Unlike DctD and PspF, NtrC has major

dimerization determinants present in the D-domain that share homology with the *E. coli* nucleoid-associated protein FIS (Koch *et al.*, 1988; North *et al.*, 1993), a helix-turn-helix regulatory protein of known three-dimensional structure (Kostrewa *et al.*, 1991).

Histidine protein kinases.

The *B. burgdorferi* histidine protein kinase, sigma s regulatory protein K (SisK) is 44 KDa, and is highly homologous to NtrB (Figure 6). In contrast to most histidine protein kinases, SisK appears to be a soluble, cytoplasmic protein, and in that regard is similar to NtrB.

Histidine protein kinases or signal sensors are part of a superfamily of proteins consisting of 11 subfamilies with conserved domains of approximately 200 residues (Hoch and Silhavy, 1995). Histidine protein kinases generally function as membrane receptors with sensory domains at the external surface that serve to regulate the kinase domain at the membrane-cytoplasm interface. All histidine protein kinases have a conserved ATP-binding catalytic domain that is required for kinase activity. This catalytic domain, together with a dimerization domain, forms the kinase core. The classification into the 11 subfamilies is based on the sequence of these two core domains. The dimerization domain includes a motif known as the H-box, which contains the site of autophosphorylation (Grebe and Stock, 1999). The histidine protein kinase catalytic domain contains four additional conserved motifs, the N, D, F, and G boxes (Figure 7) which, are also involved in ATP binding as well as catalysis and phosphotransfer (Bilwes *et al.*, 2001; Marina *et al.*, 2001; Wolanin *et al.*, 2002). This domain binds ATP and catalyzes the phosphorylation of a histidine side chain (Hoch and Silhavy, 1995).

One structural feature commonly found in the N-terminal domain of signal sensors is the PAS domain. The PAS acronym was coined originally to describe the ~270-residue region encompassing two direct sequence repeats (PAS-A and PAS-B) of ~50 residues each that had been identified in the *Drosophila* Period clock protein (PER), the vertebrate Aryl hydrocarbon receptor nuclear translocator (ARNT), and the *Drosophila* Single-minded (SIM) (Hogenesch *et al.*, 1998; Nambu *et al.*, 1991). These three proteins are involved in regulation of circadian rhythms, activation of the xenobiotic response, and cell fate determination, respectively. More recently, PAS domains have been found in many other proteins, including histidine protein kinases (Figure 8), light receptor and regulator proteins, clock proteins, sensor proteins (oxygen/redox sensors), ion channels, and a Ser/Thr kinase with a putative redox-sensing or flavin-binding domain, in which PAS regions are named "LOV" (light, oxygen, or voltage) (Pellequer *et al.*, 1998). These PAS-containing proteins occur in a wide range of living organisms including eubacteria, archaea, cyanobacteria, fungi, plants, insects and mammals (Taylor and Zhulin, 1999).

PAS domains contain a small number of conserved amino acids within a sequence of about 90 residues (Gong *et al.*, 1998). PAS-containing proteins have been categorized into three functional subgroups: (*i*) transcription activators [DNA-binding proteins with both basic helix-loop-helix (bHLH) and PAS sequence motifs], (*ii*) sensor modules of two-component regulatory systems (oxygen sensor, nitrogen fixation, sensor kinase, etc.), and (*iii*) ion channels in eucaryotes (Pellequer *et al.*, 1998). One function of the PAS domain is to mediate protein-protein interactions.

Dimerization has been demonstrated for many transcriptional activators such as the aryl hydrocarbon receptor (AHR), ARNT, SIM, hypoxia-inducible factor 1 (HIF-1), member of the PAS superfamily, and the trachealess protein. In transcriptional

activators, dimerization is mediated by both the bHLH region and by the PAS repeats. Some PAS-containing proteins lack a bHLH region (PER) but can still either homodimerize or heterodimerize with other bHLH-PAS-containing proteins through their PAS domains *in vitro*. A second function for PAS domains is ligand and/or cofactor binding, as is the case for AHR and for the heme-binding bacterial O_2-sensing protein FixL (Pellequer *et al.*, 1998). Several PAS-domain proteins sense redox potential by recognizing signals such as oxygen, redox or light (Gong *et al.*, 1998) via an associated cofactor such as heme, flavin or a 4-hydroxycinnamoyl chromophore (Taylor and Zhulin, 1999).

Many bacterial two-component systems are bifunctional, having both kinase activity (acting on histidine) and phosphatase activity (acting on phophoaspartate). (Klumpp S. and Krieglstein, 2002). In many histidine protein kinases the regulatory effects of the phosphatase activity dominate (Hoch and Silhavy, 1995; Zhu *et al.*, 2000). The dominant phenotype of a mutant lacking a particular receptor-histidine protein kinase is often low, constitutive activity of the response regulator under conditions that normally stimulate phosphatase activity of the histidine protein kinase and lead to response regulator inactivation. Regulation of these dual-function receptor-histidine protein kinases appears to involve modulation of a balance between two distinct states, namely kinase on and phosphatase off, or kinase off and phosphatase on (Hsing *et al.*, 1998). The phosphatase activity is not simply a reverse phosphotransfer. Phosphatase activity is retained in some mutant histidine protein kinases lacking the H-box histidine (Hsing and Silhavy, 1997). In the case of EnvZ, the osmosensor in *E. coli* and its cognate response regulator OmpR (Zhu *et al.*, 2000), it has been suggested that OmpR-phosphate is regulated by the OmpR-phosphate autophosphatase activity, whereas the kinase activity of OmpR is maintained at a

constant level. Similarly, in the CheA chemotaxis system, dephosphorylation of the phospho-CheY response regulator is modulated by the CheZ phosphatase (Klumpp S. and Krieglstein, 2002).

Figure 1. Life cycle and transmission. *B. burgdorferi* larvae hatch in the spring and feed on infected mice at which point they become infected since there is no trans-ovarian transmission from the female to her eggs. The following spring, the larvae molt into nymphs and feed again. Mice, the reservoir for *B. burgdorferi* remain bacterimic through out the spring and summer. *Ixodes* feed on the mice, keeping them infected or other mammals such as humans. The nymphs grow to adults, which feed and mate on deer, or other large animals. The female then drops off the deer and lays her eggs in the leaf litter before dying. Life cycle picture presented here was modified from http://www.kwic.com/~pagodavista/schoolhouse/species/insects/tick.htm.

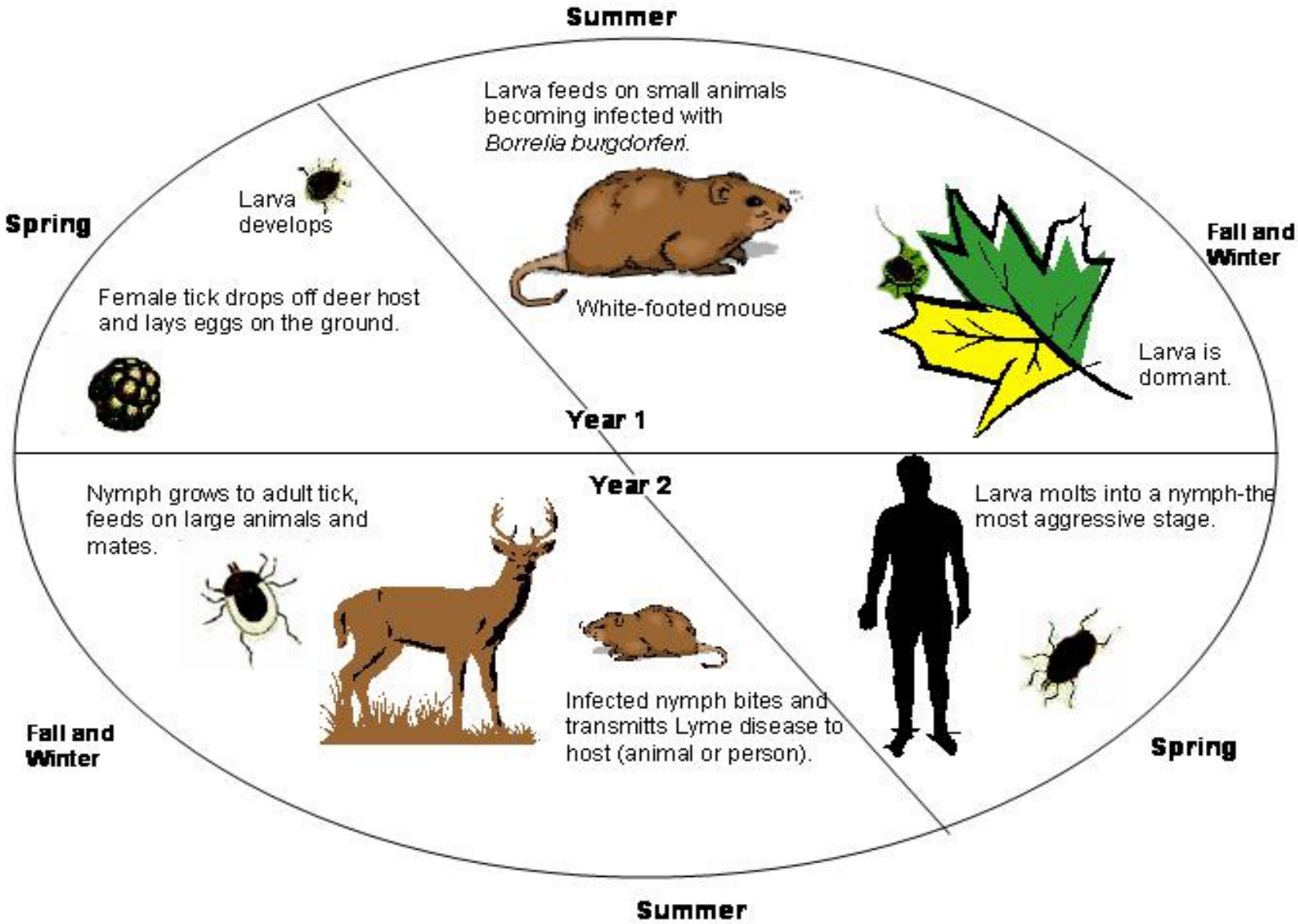
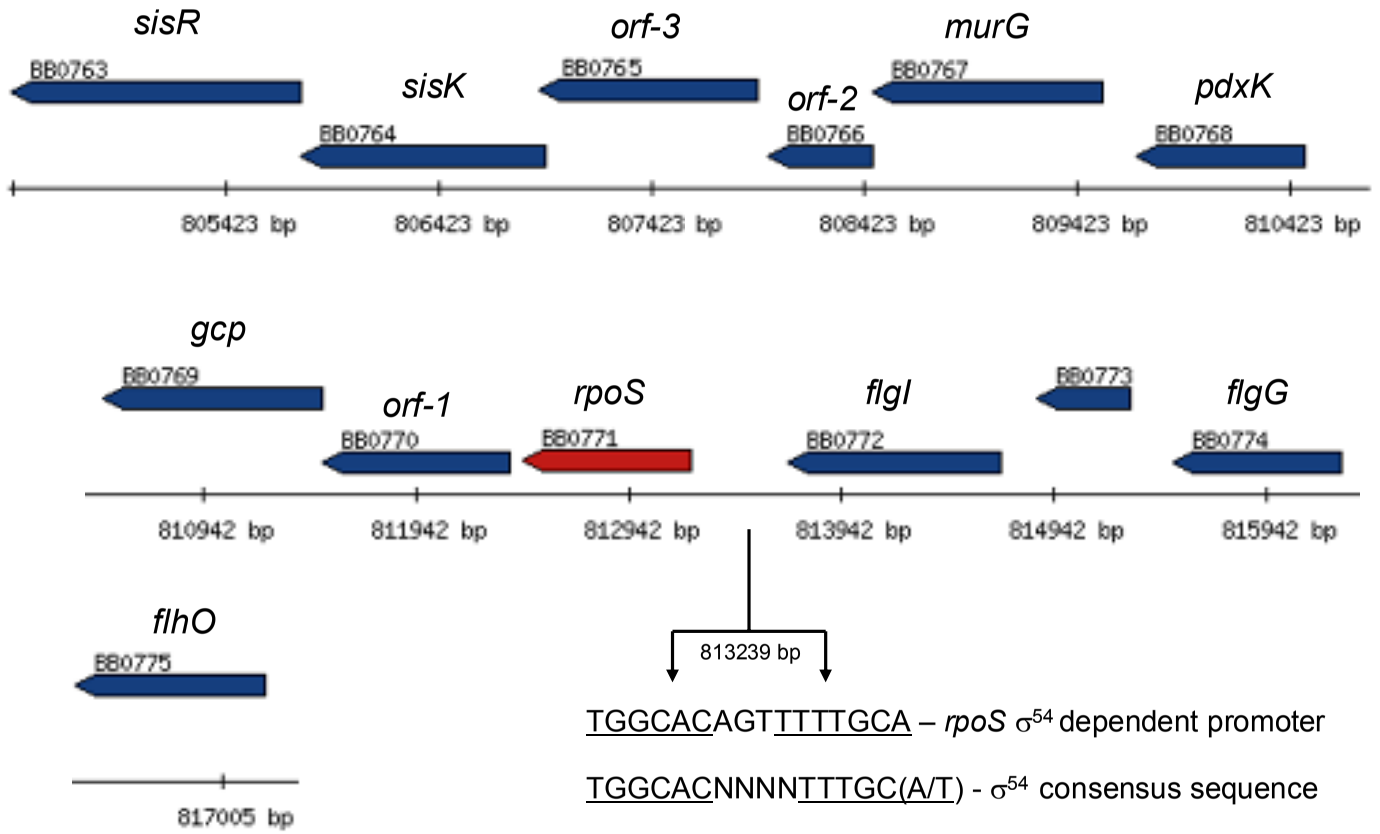

Summer
Larva feeds on small animals becoming infected with *Borrelia burgdorferi*.
Spring
Larva develops
White-footed mouse
Fall and Winter
Female tick drops off deer host and lays eggs on the ground.
Larva is dormant.
Year 1
Year 2
Nymph grows to adult tick, feeds on large animals and mates.
Larva molts into a nymph-the most aggressive stage.
Infected nymph bites and transmitts Lyme disease to host (animal or person).
Fall and Winter
Spring
Summer

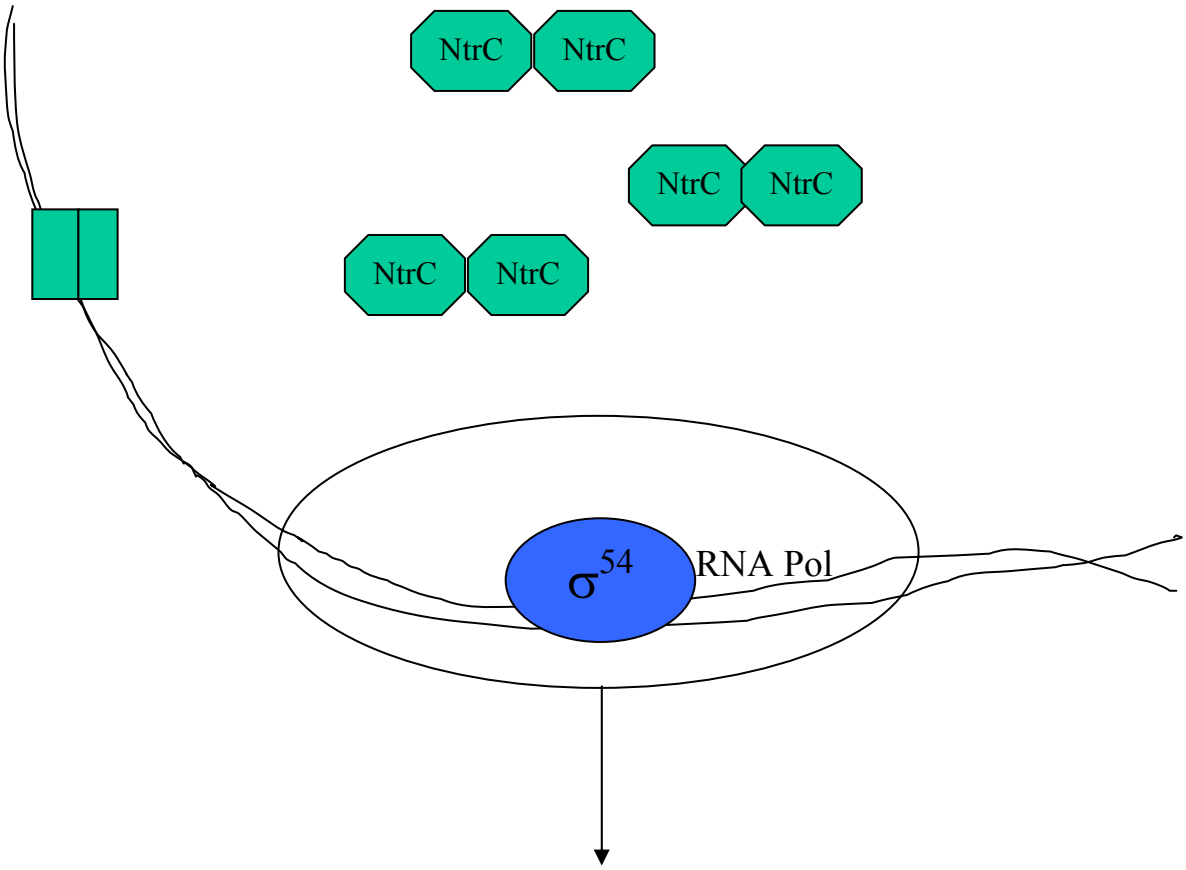
Figure 2. The *B. burgdorferi rpoS* operon and σ^{54}- dependent *rpoS* promoter upstream of the operon. The *rpoS* gene (red) is 54 bp upstream of an open reading frame (*orf-1*) encoding a product that shares homology with a hypothetical protein from *Haemophilus influenzae*. Downstream of *orf-1* are open reading frames that encode products with homology to a *H. influenzae* sialoglycoprotease (encoded by *gcp*), a *Salmonella choleraesuis* pyridoxal kinase (encoded by *pdxK*), *Bacillus subtilis* acetylglucosamine transferase (*murG*), the σ^{54}-dependent activator (*sisR*) and the protein histidine kinase (*sisK*). Several flagellar genes are located upstream of rpoS, including *flhO*, *flgG*, and *flgI*. The sequence of the σ^{54}-dependent *rpoS* promoter (upper sequence) is compared with the consensus sequence for σ^{54}-dependent promoters (lower sequence). "N" refers to any base. The conserved -24 and -12 promoter elements are underlined.



TGGCACAGTTTTTGCA – *rpoS* σ^{54} dependent promoter

TGGCACNNNNTTTGC(A/T) - σ^{54} consensus sequence

Figure 3. Mechanism of σ^{54} –dependent activation (NtrC/B model system). σ^{54} – holoenzyme (blue) binds the *glnA* promoter to form closed complex in which the DNA remains double-stranded. NtrC-P (green) binds to the enhancer (-108 and -140, green squares), upstream of the promoter and contacts σ^{54} –holoenzyme by means of DNA loop formation. To activate transcription, NtrC-P couples ATP hydrolysis to the isomerization of closed complexes to an open complex in which a region of localized strand denaturation occurs around the transcriptional start site.


NtrC
NtrC
NtrC
NtrC
NtrC
NtrC
σ54
RNA Pol

NtrB~P

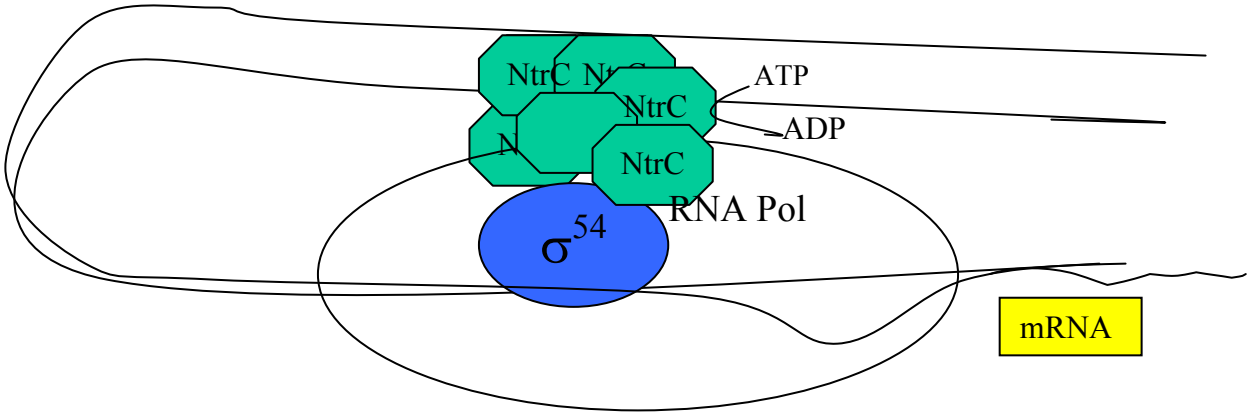

NtrC
NtrC
NtrC
ATP
ADP
σ54
RNA Pol
mRNA

Figure 4. Sequence comparison of NtrC and SisR representatives of RpoN-dependent activator of a two-component system. The boxed sequences identify the three (A, C, and D) domains typically found in this family of proteins. Residues outside the boxes identify the linker sequences between domains. Aspartate (D, in blue) residues within the A domain identify the potential phosphoryl group binding site. Green shaded residues within the C domain identify the consensus ATP binding motif (also known as the P loop) G--G-GK (Gao *et al.*, 1998) and the red shaded residues are unique to the σ^{54}-dependent enhancer binding proteins and appears to have a role in interactions with the σ^{54} protein (Zhang *et al.*, 2002). Underlined sequences labeled C1 through C7 represent highly conserved residues (Morett and Segovia, 1993). Orange shaded, boldface residues in the D domain identify the helix-turn-helix motif needed to bind DNA. The sequence found in the D-domain of NtrC that is absent in SisR (hyphens) corresponds to the region with homology to FIS protein. An asterisk (*) denotes a conserved residue; a colon (:) and a period (.) identify strongly and weakly conserved residues, respectively. This alignment was performed using program CLUSTAL (version 3.2). NtrC, from *S. enterica* serovar Typhimurium (accession no. CAA59425). SisR, from *B. burgdorferi* (accession no. BB0763).

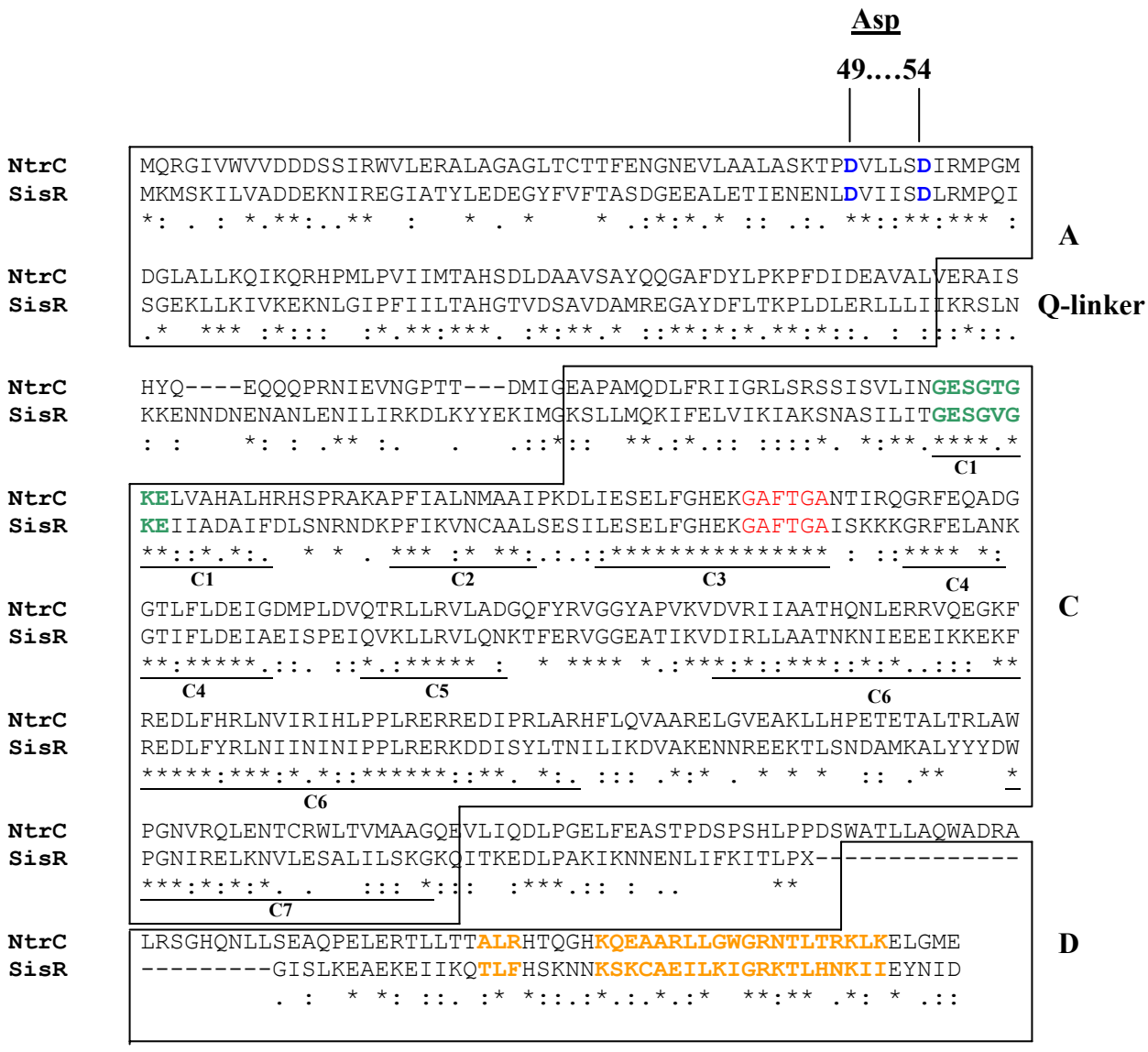

Asp
49....54
NtrC MQRGIVWVVDDDSSIRWVLERALAGAGLTCTTFENGNEVLAALASKTPDVLLSDIRMPGM
SisR MKMSKILVADDEKNIREGIATYLEDEGYFVFTASDGEEALETIENENLDVIISDLRMPQI
A
NtrC DGLALLKQIKQRHPMLPVIIMTAHSDLDAAVSAYQQGAFDYLPKPFDIDEAVALVERAIS
SisR SGEKLLKIVKEKNLGIPFIILTAHGTVDSAVDAMREGAYDFLTKPLDLERLLLIIKRSLN
Q-linker
NtrC HYQ----EQQQPRNIEVNGPTT---DMIGEAPAMQDLFRIIGRLSRSSISVLINGESGTG
SisR KKENNDNENANLENILIRKDLKYYEKIMGKSLLMQKIFELVIKIAKSNASILITGESGVG
C1
NtrC KELVAHALHRHSPRAKAPFIALNMAAIPKDLIESELFGHEKGAFTGANTIRQGRFEQADG
SisR KEIIADAIFDLSNRNDKPFIKVNCAALSESILESELFGHEKGAFTGAISKKKGRFELANK
C1 C2 C3 C4
NtrC GTLFLDEIGDMPLDVQTRLLRVLADGQFYRVGGYAPVKVDVRIIAATHQNLERRVQEGKF
SisR GTIFLDEIAEISPEIQVKLLRVLQNKTFERVGGEATIKVDIRLLAATNKNIEEEIKKEKF
C
C4 C5 C6
NtrC REDLFHRLNVIRIHLPPLRERREDIPRLARHFLQVAARELGVEAKLLHPETETALTRLAW
SisR REDLFYRLNIININIPPLRERKDDISYLTNILIKDVAKENNREEKTLSNDAMKALYYYDW
C6
NtrC PGNVRQLENTCRWLTVMAAGQEVLIQDLPGELFEASTPDSPSHLPPDSWATLLAQWADRA
SisR PGNIRELKNVLESALILSKGKQITKEDLPAKIKNNENLIFKITLPX----------
C7
NtrC LRSGHQNLLSEAQPELERTLLTTALRHTQGHKQEAARLLGWGRNTLTRKLKELGME
SisR ---------GISLKEAEKEIIKQTLFHSKNNKSKCAEILKIGRKTLHNKIIEYNID
D

Figure 5. Organization of sequence motifs in SisR. The colored boxed sequences identify the three domain structure of SisR and NtrC (A, C, and D) typically found in this family of proteins. In yellow is the A domain, that contains the conserved aspartate residue that is phosphorylated. In green is the highly conserved C domain that contains the consensus ATP-binding motifs, and is sufficient for transcriptional activation. In blue is the D domain, which contains the helix-turn-helix motif that recognizes the enhancer (Morett and Segovia, 1993)

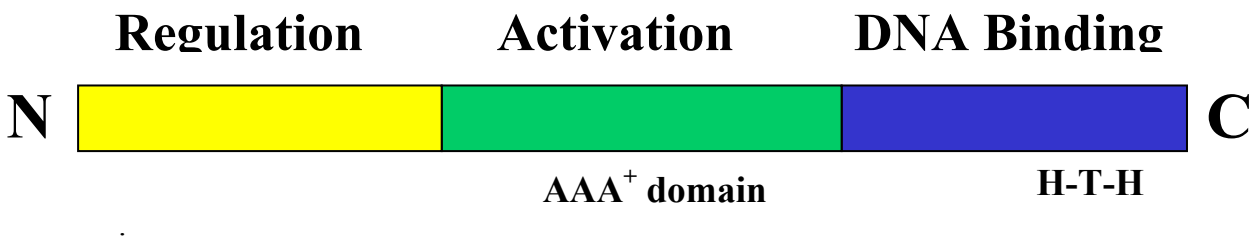

Regulation
Activation
DNA Binding
N
C
AAA+ domain
H-T-H

Figure 6. Sequence comparison of NtrB and SisK, representatives of RpoN-dependent protein histidine kinases of a two-component system. The PAS domain designated as PAS in red, the central domain designated as the H-box in blue, the catalytic domain contains four conserved motifs, the N, D, F, and G boxes lavender, green/green and burgundy, respectively. Identical (*), strongly conserved (:), weakly conserved (.), residues are indicated in the figure. This alignment was performed using program CLUSTAL (version 3.2). NtrB, nitrogen regulatory protein B (accession no. P06712) and SisK, Sigma s regulatory protein K (accession no. BB0764)


NtrB ----------MATGTQPDAGQILNSLINSILLIDDNLAIHYANPA
SisK MNNFFKKALTKLNKLSNEQKTKFIEQIYKKIEIYDGIFASINEGIIVLDKQNNIIYANKI
PAS
NtrB AQQLLAQSSRKLFGTPLPELLSYFSLNIELMQESLEAGQGFTDNEVTLVIDGRSHILSVT
SisK LYQILALTSKSKIEILDDIQIPNLINLIKELVRTEDKIIGLEVPISNGIYIKISFMPYVK
NtrB AQRMPDGMILLEMAPMDNQRRLSQEQLQHAQQVAAR-DLVRGLAHEIKNPLGGLRGAAQL
SisK EKKLEGNIILIEDIKEKKKK---EELFRRVEALASFTRHARNIAHEIKNPLGAIDINLQL
H
NtrB LSKALP-----DPSLLEYTKVIIEQADRLRNLVDRLLGPQLPGTR--VTESIHKVAERVV
SisK LKKEIEKQKMKNGKAENYFKVIKEEINRVDKIVTEFLLTVRPIKINLQEKDIKQVIGSVC
NtrB TLVSMELP-DNVRLIRDYDPSLPELAHDPDQIEQVLLNIVRNALQALGPEGGEIILRTRT
SisK ELLNPGLENKHIKLLLNLN-KISNILIDEKLLKQVIINIVKNAEEALLETKKEIKKIEIF
N
NtrB AFQLTLHGERYRLAARIDVEDNGPGIPPHLQDTLFYPMVSGREGGTGLGLSIARNLIDQH
SisK LFEKDNK-------IHINIKDNGNGIKDGVKEEIFKPQFSTKEKGSGIGLTISYKIIKEL
D/F
NtrB SGKIEFTSWPG-HTEFSVYLPIRK---------
SisK GGEIFVESKEGKGTIFTITLPKLNKKNILIEGY
G

Figure 7. Organization of sequence motifs in SisK, Sigma s regulatory protein *K*. SisK N-terminal end has the designated PAS domain, responsible for sensing (Gong *et al.*, 1998). The central domain designated as the H-box, contains the site of autophosphorylation and dimerization (Grebe and Stock, 1999). The catalytic domain contains four conserved motifs, the N, D, F, and G boxes involved in ATP binding as well as catalysis and phosphotransfer (Bilwes *et al.*, 2001; Marina *et al.*, 2001; Wolanin *et al.*, 2002)

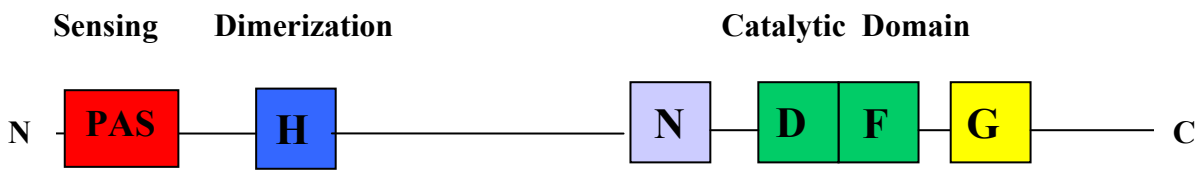

Sensing
Dimerization
Catalytic Domain
N
PAS
H
N
D
F
G
C

Figure 8. PAS domain in SisK. A. PAS domain consensus sequences **.** Consensus/80% represent the consensus sequence present in 80% of PAS domains, with the abbreviations for amino acid residues indicated in the following key:

Class	*Key*	*Residues*
alcohol	o	S,T
aliphatic	l	I,L,V
any	.	A,C,D,E,F,G,H,I,K,L,M,N,P,Q,R,S,T,V,W,Y
aromatic	a	F,H,W,Y
charged	c	D,E,H,K,R
hydrophobic	h	A,C,F,G,H,I,K,L,M,R,T,V,W,Y
negative	-	D,E
polar	p	C,D,E,H,K,N,Q,R,S,T
positive	+	H,K,R
small	s	A,C,D,G,N,P,S,T,V
tiny	u	A,G,S
turnlike	t	A,C,D,E,G,H,K,N,Q,R,S,T

B. SMART alignment of the PAS domain from *B. burgdorferi* SisK (SM00091) with consensus/80%, *E. coli* NtrB and *Azospirillum brasilense* NtrB;

(http://smart.embl-heidelberg.de/smart/do_annotation.pl?BLAST=DUMMY&DOMAIN=PAS)

SisK residues in red match with the consensus/80% residues which are also in red. SisK residues in black are those that deviate from the consensus/80% sequence. SisK residues that are not part of the consensus/80% sequence are indicated in green.

A

```
CONSENSUS/80%
t.ht.hhpt.......hhhhhs.......ptthh.hs.th.thhs...h..tph.tt...
CONSENSUS/65%
tthpthhps....hssshhhhs.......psplhhhNtshtphhu...hs.pchhsp...
CONSENSUS/50%    pphpsllcs....sssslhllD......tcGplhhsNsspppllG...hspc-
llGp...


CONSENSUS/80%    .h.phh..tt.....h.t.h.t..t
CONSENSUS/65%    shhphht.pph..tthtpthpphhp
CONSENSUS/50%    sltcllpssct..pphppplppshs
```

B

```
CONSENSUS/80%            t.ht.hhpt.......hhhhhs.......ptthh.hs.th.thhs
SisK                  ...EIYDGIFAS....INEGIIVLD......KQNNIIYANKILYQILA..
NTRB_ECOLI            ...PDAGQILNS....LINSILLID......DNLAIHYANPAAQQLLA..
NTRB_AZOBR            ...IDPSVMLNA....LPDPVLVVD......GSGDIRYVNLEAQEFFG

CONSENSUS/80%         ...h..tph.tt.. ..h.phh..t  t.....h.t.h.t..t
SisK                  ..LTSKSKIEI...-LDDIQIPNLI--NLIKELVRTEDK...
NTRB_ECOLI            .QSSRKLFGT...PLPELLSYFSLNIELMQESLEAGQG...
NTRB_AZOBR            .LSAAMMEGM...PLAELLPPNSP----VSQLIEQVQQGR.
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Overview of thesis work.

I report here on the preliminary characterization of SisK/SisR two-component regulatory system and suggest a model for how it regulates expression of *rpoS* in *B. burgdorferi*. A series of overlapping PCR products spanning 2 kb on either side of the *rpoS* σ^{54}- dependent promoter was examined for the presence of an enhancer in a gel mobility shift assay. Identification of the *rpoS* enhancer would provide important information about the regulation of *rpoS* and could lead to the identification of other σ^{54}-regulated genes in *B. burgdorferi*. SisR bound weakly to a DNA fragment located about 1.6 kb downstream of the promoter. Unfortunately, the poor binding of SisR to this site prevented the identification of the enhancer sequence.

SisK is the cognate sensor kinase for SisR. A maltose-binding protein-SisK fusion protein (MBP-SisK) was purified and shown to phosphorylate itself and transfer the phosphate to SisR. Inspection of the SisK amino acid sequence suggests that it contains PAS domain that could bind a cofactor such as heme or flavin. Purified MBP-SisK was faint yellow, and an absorption spectrum of the purified protein revealed a peak at about 410 nm suggesting the presence of a flavin cofactor. Thus, SisK appears to be a flavoprotein, and we postulate that the flavin is involved in signal sensing of oxygen, or other reactive oxygen species. Consistent with this hypothesis, a *B. burgdorferi rpoN* mutant strain was found to have increased sensitivity to tertiary-butyl peroxide (Dr. Frank Gherardini, personal communication).

CHAPTER 2

MATERIALS AND METHODS

Bacterial strains, growth conditions, and plasmids. *Escherichia coli* and high passage, avirulent *Borrelia burgdorferi* strains and plasmids are described in Table 1**.** *E. coli* strains were cultured in Luria Bertani broth (LB) at 37°C and either supplemented with 100 μg/ml ampicillin, 40 μg/ml kanamycin and/or 34 μg/ml chloramphenicol. *B. burgdorferi* strains were grown in Barbour-Stoenner-Kelly II (BSKII) medium (Barbour *et al.*, 1984) at 34°C under an atmosphere of 5% O_2/5%CO_2/90%N_2. Cells were examined and enumerated using dark-field microscopy until desired cell densities were obtained for chromosomal isolation.

B. burgdorferi **DNA isolation.** Chromosomal and plasmid DNA were isolated from *B. burgdorferi* strain B31A (Table 1) as previously described (Barbour, 1984; Barbour and Garon, 1987a; Barbour, 1988).

Cloning and overexpression of recombinant *sisR* in *E. coli*. *B. burgdorferi sisR* was amplified by PCR with primers act-exp and act2 (Table 2) using the Easy Start 100 kit (Molecular Bio-Products, Inc., San Diego, CA) with a PTC-100 thermocycler (MJ Research, Inc., Watertown, MA). The temperature regime used for PCR was 2 min at 94°C, 30 sec at 55 °C, followed by 2 min at 72°C for 30 cycles. The resulting PCR product was cloned into *Nde*I and *Bam*HI restriction sites of pT7-7 (Tabor, 1990), resulting in plasmid pAPO1. The correct clone was confirmed by digestion

with restriction enzymes (Promega, Madison, WI) and DNA sequencing (done at Molecular Genetics Instrumentation Facility, University of Georgia). Plasmid pAPO1 was further digested with *Nde*I and *Sal*I and subcloned into pET28a+ expression vector (Novagen, Madison, WI), to generate plasmid pAPO3 which introduced a sequence encoding a hexahistidine tag at the 5'-end of *sisR*. Plasmid pAPO1 was transformed into *E. coli* strains DH5α, *recA*⁻ DH5α, YMC11, JM105, Topp1,2,4,5,6, BL21 (DE3), (DE3) pLysS, CodonPlus (DE3)-RIL or Rosetta pLysS. Plasmid pAPO3 was transformed into *E.coli* strain Rosetta pLysS. Transformants were grown to OD_{600} of 0.5-0.8 at 37°C with vigorous shaking and aeration and induced overnight (10-14 h) by including 1 mM isopropyl-β-thiogalactoside (IPTG) in the growth medium. Overexpression was analyzed by 12.5% sodium-dodecyl sulfate polyacrylamide gel (SDS-PAGE) (Sambrook *et al.*, 1989).

Purification of histidine-tagged *B. burgdorferi* SisR (SisR-His). One-liter cultures of *E. coli* expressing SisR-His (from plasmid pAPO3) were divided into 100 ml batches that were harvested by centrifugation at 7,000 x g for 10 min at 4°C. After removal of the supernatant fluid, cell pellets were suspended in 10 ml of ice cold breakage buffer (50 mM Tris-acetate, pH 8.2, 200 mM NaCl, 5 mM imidazole, 1 mM dithiolthreitol (DTT), and 1 mM ethylenediamine tetraacetic acid (EDTA)). Cells were lysed by two passages through a chilled French pressure cell at 15,000 p.s.i. (103.5 MPa). The resulting lysates were centrifuged at 15,000 x g for 15 min at 4°C and the resulting supernatant liquids and pellets were analyzed by 12.5% SDS-PAGE (Sambrook *et al.*, 1989). Clarified cell lysates were loaded onto a 5 ml nickel-nitrilotriacetic acid affinity column (Qiagen, Valencia, CA) that had been equilibrated previously with 30 ml sonication buffer (300 mM NaCl, 50 mM Na_2PO_4, pH 7.8). The

column was washed with 20 ml sonication buffer containing 25 mM imidazole, and the SisR-His was eluted with sonication buffer containing 250 mM imidazole. Fractions containing SisR-His were collected, pooled, and dialyzed against buffer A1 (20 mM N-2-hydroxyethylpiperazine-N'-3-propane-sulfonic acid (EPPS), pH 8.0, 75 mM potassium thiocyanate, 5% glycerol, 0.5 mM DTT) overnight. The protein preparation was then loaded onto a 5 ml HI-TRAP heparin column (Amersham-Pharmacia) using FPLC system from Amersham-Pharmacia that had been equilibrated previously with 25 ml buffer A1. Protein was eluted from the column at a flow rate of 1 ml per min over a linear gradient to buffer B (buffer A1 + 1 M KCl). Fractions containing SisR-His (300-350 mM KCl) were pooled following analysis of column fractions by 12.5% SDS-PAGE. Following dialysis of protein against buffer A1 samples were stored in 20 ul aliquots at -80°C at a final concentration of 0.5-1.0 mg protein per ml. Protein concentrations were determined by bicinchoninic acid method (Pierce, Rockfort, IL). N-terminal sequence was performed (done at National Institute of Health, Rocky Mountain Labs, Hamilton, Montana) to confirm protein identity. Antibodies were generated against the purified SisR-His in New Zealand White rabbits at NIH Rocky Mountain Labs.

Purification of native *B. burgdorferi* SisR. Native SisR was a gift from Dr. Frank Gherardini (National Institute of Health, Rocky Mountain Labs, Hamilton, Montana). Plasmid pAPO1 encoding native SisR expression system was constructed in this study, as well as optimization of conditions for the expression and purification of SisR but large scale purification was performed at the Rocky Mountain Labs. One-liter culture of *E. coli* expressing native SisR (from pAPO1) was divided into 100 ml batches that were harvested by centrifugation at 7,000 x g for 10 min at 4°C. After

removal of the supernatant fluid, cell pellets containing SisR were suspended in 10 ml of ice cold breakage buffer. Cells were lysed by two passages through chilled French pressure cell at 15,000 p.s.i. (103.5 MPa). The resulting lysate was centrifuged at 15,000 x g for 15 min at 4°C and the resulting supernatant liquid and pellet were analyzed by 12.5% SDS-PAGE and western blotting (Sambrook *et al.*, 1989). Ammonium sulfate was added to 35% saturation to the clarified cell extracts and the solution was incubated for 1 h at 4°C (Wood, 1976). Following centrifugation at 9,000 x g for 15 min at 4°C, protein pellets were resuspended in 10 ml buffer A2 (20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), pH 8.0, 100 mM potassium thiocyanate, 5% glycerol, 0.5 mM DTT), dialyzed overnight against buffer A2 and loaded onto a 5 ml HI-TRAP heparin column (Amersham-Pharmacia) using FPLC system from Amersham-Pharmacia that had been equilibrated previously with 25 ml buffer A2. Protein was eluted from the column at a flow rate of 1 ml per min over a linear gradient of buffer B2 (buffer A2 + 1 M KCl). Fractions containing native SisR (300-350 mM KCl) were pooled following analysis of column fractions by 12.5% SDS-PAGE. Following dialysis of protein against buffer A2 samples were stored in 20 ul aliquots at -80°C at a final concentration of 0.5-1.0 mg per ml.

Cloning and overexpression of recombinant *sisK* in *E. coli.* *B. burgdorferi sisK* was amplified by PCR with primers act-1 and SisKR (Table2) using the Easy Start 100 kit (Molecular Bio-Products, Inc., San Diego, CA) with a PTC-100 thermocycler (MJ Research, Inc., Watertown, MA). The temperature regime used for PCR was 2 min at 94°C, 30 sec at 55 °C, followed by 2 min at 72°C for 30 cycles. The resulting PCR product was cloned into *Nde*I and *Sal*I restriction sites of the pET21b+ expression vector (Novagen, Madison, WI), resulting in the plasmid pAPOk, which

introduced a sequence encoding a hexahistidine tag at the 5'-end of *sisK*. The correct clone was confirmed by DNA sequencing. Plasmid pAPOk was further digested with *Nde*I, blunt-ended with T4 DNA polymerase, and then digested with *Sal*I. The resulting fragment was ligated into the pMAL-C expression vector (New England Biolabs) that had been digested previously with *Eco*RI, blunt-ended with T4 DNA polymerase, then digested with *Sal*I. The resulting plasmid, pAPOxu, introduced a sequence encoding the maltose binding protein at the 5'-end of *sisK*. Plasmid pAPOk was transformed into *E. coli* strains BL21(DE3) containing pLysE, Topp 1,2,4,5,6, BL21 Codon +, or Rosetta pLysS. Plasmid pAPOxu was transformed into *E.coli* strain DH5α and Rosetta pLysS. Transformants were grown to OD_{600} of 0.5-0.8 at 37°C with vigorous shacking and aeration, and expression of the SisK proteins was achieved with 1 mM IPTG at 37°C for 3 hours or 15°C overnight. Overexpression was analyzed by 12.5% SDS-PAGE (Sambrook *et al.*, 1989).

Purification of histidine-tagged *B. burgdorferi* SisK (SisK-His) and maltose binding protein-SisK (MBP-SisK) fusion protein. One-liter cultures of *E. coli* expressing histidine-tagged SisK or MBP-SisK was divided into 100 ml batches that were harvested by centrifugation at 7,000 x g for 10 min at 4°C. After removal of the supernatant fluid, cell pellets containing SisK-His or MBP-SisK were suspended in 10 ml of ice cold breakage buffer. Cells were lysed by two passages through a chilled French pressure cell at 15,000 p.s.i. (103.5 MPa). The resulting lysate was centrifuged at 15,000 x g for 15 min at 4°C and the resulting supernatant liquid and pellet were analyzed by 12.5% SDS-PAGE (Sambrook *et al.*, 1989). SisK-His was determined to be in the pellet and attempts to solubilize it with sarkosyl (Posey *et al.*, 1999) were unsuccessful. However, SisK-MBP was soluble and then was loaded onto

a 5 ml amylose affinity column (New England Biolabs) that had been equilibrated previously with 30 ml column buffer (20 mM Tris-HCl, pH 7.4, 200 mM KCl, 5% glycerol, 1 mM DTT, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride (PMSF)). The column was washed with 20 ml column buffer and eluted with column buffer plus 10 mM maltose. Fractions containing MBP-SisK fusion protein were collected and pooled following analysis of the column fractions by 12.5% SDS-PAGE (Sambrook *et al.*, 1989). Pooled fractions were stored in 20 ul aliquots at -80°C at a final concentration of 0.5-1.0 mg per ml.

Characterization of the flavin associated with MBP-SisK. Purified MPB-SisK was analyzed on a Shimadzu UV 160U, UV visible scanning spectrophotometer from 350 to 750 nm wavelength light. The flavin cofactor was extracted from MPB-SisK by boiling the purified protein for 10 min. Precipitated protein was removed by size exclusion ultrafiltration using an Amicon column. The flavin cofactor in the yellow filtrate was spotted onto a silica gel thin layer chromatography plate along with flavine adenine dinucleotide (FAD) and flavin mononucleotide (FMN) standards. The plates were developed by ascending chromatography with 4 n-butanol:1 acetic acid : 4 water (v/v/v) as the mobile phase. Spots were visualized by UV light. as described (Fetzner *et al.*, 1992).

***In vitro* phosphorylation assays.** Autophosphorylation of MPB-SisK was performed at 37 °C, 30°C or 4°C for 2-45 minutes in binding buffer (20 mM Tris-acetate, pH 8.0, and 5 mM $MgCl_2$). A total of 5 μg MPB-SisK was used for each reaction with reaction volumes ranging from 20 μl to 30 μl. The reaction was started by adding ^{32}P-(γ)-ATP (30 Ci/mmol, Amersham) to a final concentration of 33 μM and was stopped

at time intervals 2, 5, 10, 15, 30 or 45 minutes by adding 5x SDS sample buffer (60 mM Tris HCl, pH 6.8, 2% SDS, 14.4 mM BME, 0.1% bromophenol blue, 25% glycerol) to obtain 1X SDS sample buffer final concentration. Samples were subjected to SDS-PAGE and the resulting gels were exposed to X-ray film. Phosphoryl group transfer from MBP-SisK to active SisR and histidine-tagged SisR was assayed by including 5 μg of these proteins in the reaction mix prior to the addition of ^{32}P-(γ)-ATP. Alternatively, SisR proteins also at 5 μg were added to the reaction mix following incubation of MBP-SisK with ^{32}P-(γ)-ATP.

Gel mobility shift assays. A series of 18 overlapping 337-541 bp DNA target sequences spanning 2 kbp on either side of the *rpoS* promoter were amplified by PCR using the Easy Start 100 kit (Molecular Bio-Products, Inc., San Diego, CA) with a PTC-100 thermocycler (MJ Research, Inc., Watertown, MA). Thirty cycles of the temperature regimes that included 30 sec at 94°C for denaturation, 30 sec at 46°C for primers fu1-fu14 and 50°C for uy1-uy4 for annealing, followed by 1 min at 72°C for elongation were used for PCR (Table 2). PCR products (regions uy1-uy4, fu1-fu14; see Figure 9) were dephosphorylated using calf intestine phosphatase (Boheringer Mannheim, Milan, Italy) then labeled at their 5'-ends with ^{32}P-(γ)-ATP and T4 polynucleotide kinase (Promega, Madison, WI). Each binding reaction contained 25,000-100,000 cpm of the ^{32}P-labelled DNA probe. Reactions were incubated at 37 °C, 30°C or 4°C in the presence and absence of purified SisR-His (0.1μ M-1 μM) or native SisR (0.1μ M-1 μM). For some experiments 1-10 μl of *E. coli* cell extracts containing either SisR or histidine-tagged SisR were included in the reaction mixtures. Proteins and DNA targets were incubated together for 2-15 min under conditions previously described (Romero-Arroyo *et al.*, 1995) with the exception that

the buffer used was 50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 1 mM EDTA, 40-100 mM KCl, 4% glycerol, 1 mM β-mercaptoethanol (BME), 0.05 mg/ml bovine serum albumin, and 0.50 mg/ml sonicated salmon sperm DNA (41% G+C). DNA-protein complexes were analyzed on a 5% non-denaturing polyacrylamide gel as described by Parsek *et al.* (1994). Bands for free DNA, and shifted species for the DNA binding reactions were quantitated with PhosphorImager.SI (Molecular Dynamics) along with scanner control SI and ImageQuant 1.0 software.

Figure 9. Diagram of the *B. burgdorferi rpoS* region with location of primers used for generating PCR products used for gel mobility shift assays. *B. burgdorferi rpoS* (in red) with its σ^{54}- dependent promoter located at 813239 bp (arrows) aligned with σ^{54}- consensus sequence. Genes located downstream of *rpoS* include an open reading frame (*orf-1*) encoding a product that shares homology with a hypothetical protein from *Haemophilus influenzae*; open reading frames that encode products with homology to *H. influenzae* sialoglycoprotease (encoded by *gcp*), *Salmonella choleraesuis* pyridoxal kinase (encoded by *pdxK*), *Bacillus subtilis* acetylglucosamine transferase (*murG*); and a σ54-dependent activator (*sisR*) and a protein histidine kinase (*sisK*). Several flagellar genes are located upstream of rpoS, including *flhO*, *flgG*, and *flgI*. The sequence of the σ^{54}-dependent *rpoS* promoter (upper sequence) is compared with the consensus sequence for σ^{54}-dependent promoters (lower sequence). "N" refers to any base. The conserved -24 and -12 promoter elements are underlined. The relative positions of the primers used to amplify DNA sequences for gel mobility shift assays are indicated in yellow.

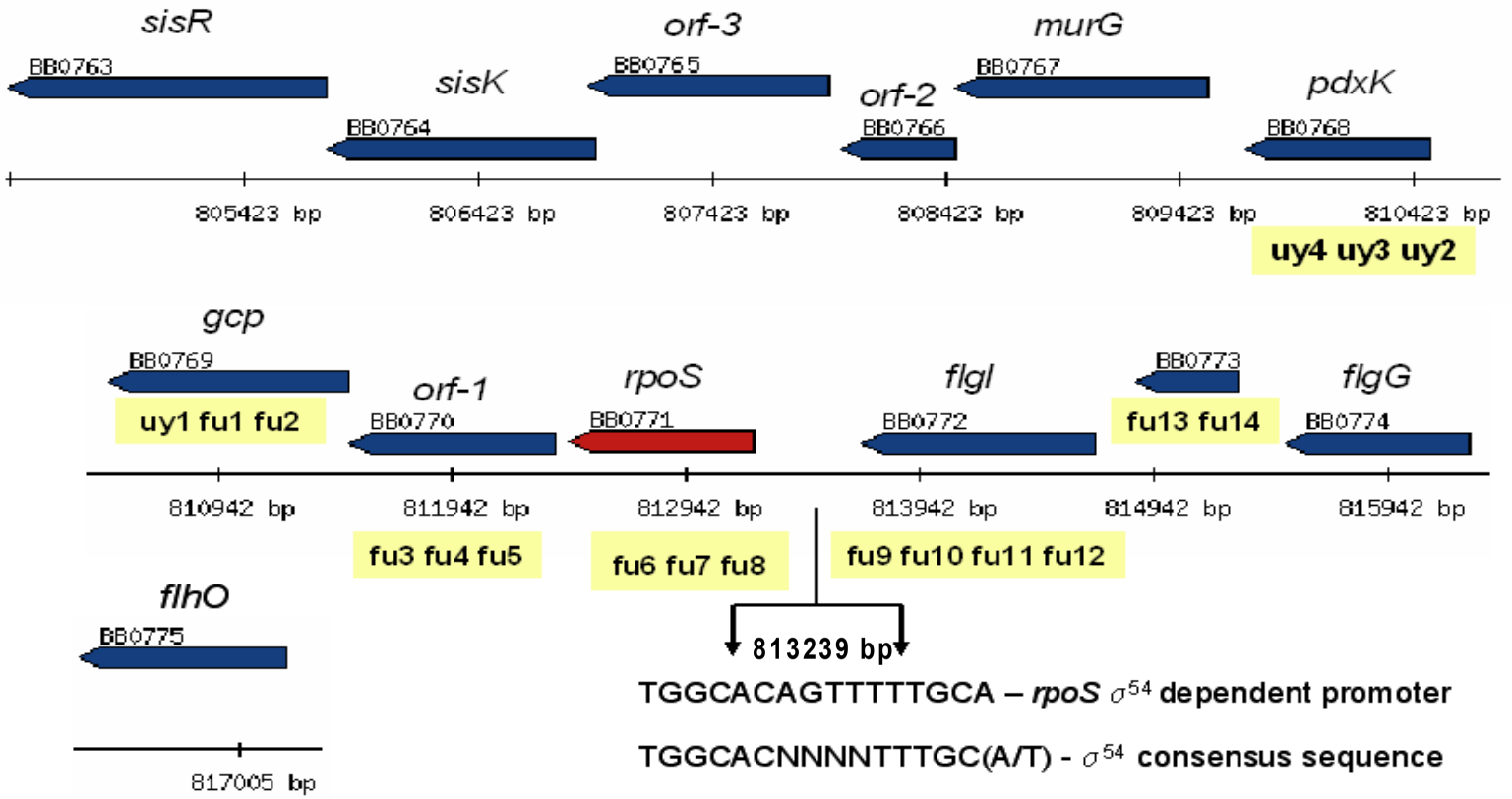

sisR
BB0763
sisK
BB0764
orf-3
BB0765
orf-2
BB0766
murG
BB0767
pdxK
BB0768
805423 bp
806423 bp
807423 bp
808423 bp
809423 bp
810423 bp
uy4 uy3 uy2
gcp
BB0769
uy1 fu1 fu2
orf-1
BB0770
rpoS
BB0771
flgI
BB0772
BB0773
fu13 fu14
flgG
BB0774
810942 bp
811942 bp
812942 bp
813942 bp
814942 bp
815942 bp
fu3 fu4 fu5
fu6 fu7 fu8
fu9 fu10 fu11 fu12
flhO
BB0775
817005 bp
813239 bp
TGGCACAGTTTTTGCA – rpoS σ54 dependent promoter
TGGCACNNNNTTTGC(A/T) - σ54 consensus sequence

Table 1. Strains, plasmids used in this study

Strain or plasmid	Relevant characteristics	Source
Strains:		
B. burgdorferi		
B31A	B31 high passage avirulent	Lab strain
E. coli		
BL21	F$^-$ *dcm* Hte *ompT hsdS* ($r_B^-m_B^+$) *endA* Tc^r	Stratagene
BL21(DE3) pLysS	F$^-$ *dcm* Hte *ompT hsdS* ($r_B^-m_B^+$) *galλ* (DE3) *endA* pLysS	Stratagene
BL21(DE3)	F$^-$ *dcm* Hte *ompT hsdS* ($r_B^-m_B^+$) *galλ* (DE3) *endA*	Stratagene
BL21 CodonPlus (DE3)-RIL	F$^-$ *ompT hsdS* ($r_B^-m_B^-$) *dcm*$^+$ *galλ* (DE3) Tc^r *end*A Hte (*argU ileY leuW* Cam^r)	Stratagene
DH5α	F$^-$(ϕ80d *lac*ZΔM15) Δ(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17 ($r_k^-m_k^+$) *pho*A *sup*E44 λ^- *thi*-1 *gyr*A96 *rel*A1	Gibco
JM105	*supE endA sbcB15 hsdR4 rpsL thi Δ (lac proAB) /*F$^-$ (*traD36 pro*$^+$ *lacI*^{q}*ZΔM15)*	Stratagene
JM109	e14$^-$ (McrA) *recA1 endA1 gyrA96 thi1 hsdR17(*$r_K^-m_K^+$*) supE44 relA1 Δ (lac proAB) [*F$^-$ *traD36 proAB lacI*^{q}*ZΔM15]*	Stratagene
JM110	*rpsL* (Str^r) *thr leu thi1 lacY galK galT ara tonA tsx dam dcm supE44 Δ (lac proAB) [*F$^-$ *traD36 proAB lacI*^{q}*ZΔM15]*	Stratagene
Rosetta (DE3)	Cm^r; F$^-$ *ompT hsdS*$_B$ ($r_B^-m_B^-$) *recA endA, lacI*^{q}*, gal dcm* (DE3) pLysS/*RARE* (*argU, argW, ileX, glyT, leuW, proL*)	Novagen
Topp1, 2, 4, 5, 6, 10	Tet^R Rif^R [F' *proAB lacI*^{q} ZΔM15 Tn10]	Novagen
YMC11	*gln- ntrB- ntrC-*	(Backman *et al.*, 1981)
Plasmids:		
pT7-7	Ap^r; T7 expression vector	(Tabor, 1990)
pET21b+	Ap^r; T7 expression vector with amino terminal 6XHIS	Novagen
pET28a	Kan^r; T7 expression vector with amino terminal 6XHIS	Novagen
pLysS	Cm^r , T7 lysozyme	Stratagene
pGP1-2	Kan^r; T7 polymerase under control of the λp_L promoter	(Tabor, 1990)
pMAL-C	Ap^r; expression vector with maltose binding protein	New England Biolabs,
pCR2.1-TOPO	Ap^r; PCR product cloning vector	Invitrogen
pAPO1	Ap^r; PCR amplified fragment containing *sisR* inserted into *Nde*I and *Bam*HI of pT7-7	This study
pAPO3	Kan^r; 1.6 kbp *Nde*I and *Sal*I fragment containing	This study

Strain or plasmid	Relevant characteristics	Source
	sisR from pAPO1 in pET28a	
pAPOk	Ap^r; PCR amplified fragment containing *sisK* inserted into *Nde*I and *Sal*I of pET21b+	This study
pAPOxu	Ap^r; 1.27 kbp *Nde*I and *Sal*I fragment containing *sisK* from pAPOk inserted into *Eco*RI and *SalI* of pMAL-C	This study
pAPO-uy3	Ap^r; Taq PCR amplified uy3 inserted into TA pCR2.1-TOPO	This study
pAPO-uy4	Ap^r; Taq PCR amplified uy4 inserted into TA pCR2.1-TOPO	This study
pAPO-uy3&4 overlap	Ap^r; Taq PCR amplified uy3&4 overlap inserted into TA pCR2.1-TOPO	This study
pAPO-fu1	Ap^r; Taq PCR amplified fu1 inserted into TA pCR2.1-TOPO	This study

Ap^r, ampicillin resistance; Kan^r, kanamycin resistance; Cm^r, chloramphenicol resistance; Tet^r, tetracycline resistance; Rif^r, rifampin resistance

Table 2. Synthetic oligonucleotides used in this study

Oligonucleotides	Sequence
act-1	**5'-** GCTAAATTATGAGGAGATATGAATAATT **-3'**
SisKR	**5'-** CTTCTCCGTCGACAGCAGTGAAAAC **-3'**
act-2	**5'-** GGTCAGGCTCTGGATCCAGCTAAATAT **-3'**
act-exp	**5'-** TATTGCATATGAGCAAAATACTTGTAG **-3'**
SPR1	**5'-** CCGGTTGCGTAAATAAGC **-3'**
SPR2	**5'-** TTGAAGATATGCTTTGCG **-3'**
SPR3	**5'-** TTAGCTTGTGTTCTCTTACTG **-3'**
SPR4	**5'-** AAACTCTAGTCATCAATCCTCC **-3'**
uy1F	**5'-** GTGCAAAGGTCAAGAGGAGGGTAGTA **-3'**
uy 1R	**5'-** AATTTCGATGATGTTGAAATACTTGGA **-3'**
uy 2F	**5'-** AAGTGCTCATTTTGTTCTTTCCATA **-3'**
uy 2R	**5'-** AATTGGTAATAGCAGGAGGTGTTG **-3'**
uy 3F	**5'-** ATATCATCTTTGTTGTTAAGTTTTGAGC **-3'**
uy 3R	**5'-** ACAATATGCATACCAGTAATATCTTCGT **-3'**
uy 4F	**5'-** AAGAAATTTTCAATTCGGACCCC **-3'**
uy 4R	**5'-** TCTAATTGATCCTGTGTTTGCTGAC **-3'**
fu1F	**5'-** AAGCCTCTCCACAAGCATCA **-3'**
fu 1R	**5'-** ATAAAATTAAATCAAACCGAACAC **-3'**
fu 2F	**5'-** AGGCAATCTCAGGCACTATG **-3'**
fu 2R	**5'-** GCAGGAAGCGTACCAGAAAT **-3'**
fu 3F	**5'-** TTTCTTTGCCTATTATTTCTGGTA **-3'**
fu 3R	**5'-** ACGACGTGGGCTATGATGA **-3'**
fu 4F	**5'-** AAATGGAATAATAGCATAAGTTA **-3'**
fu 4R	**5'-** GGAATATCATCAAGAAACTGTAAGACA **-3'**
fu 5F	**5'-** CATGACCACTGACTTACAA **-3'**
fu 5R	**5'-** ATTCAACCTATCTCCTGCTC **-3'**
fu 6F	**5'-** CCAGAGAATATTCTTTTTCAAGATAGGG **-3'**
fu 6R	**5'-** CAATGCAAAAGCAAAAAACAAGAT **-3'**
fu 7F	**5'-** CCCGCATATCTTTTTATTATTTT **-3'**
fu 7R	**5'-** AAATAATTCAAAAAATACTCCCCC **-3'**
fu 8F	**5'-** AGTTATTATATTTTCTCCCCTTTC **-3'**
fu 8R	**5'-** CTTGGAGGAAATTGATGGAAAC **-3'**
fu 9F	**5'-** AAAGGATTTTTTTATTTCTACAGA **-3'**
fu 9R	**5'-** AACAACAAAATTAAATCAGACAGC **-3'**
fu 10F	**5'-** TCTATTAATATCTTGGGGTTGGTT **-3'**
fu 10R	**5'-** TTGCACTAGTAAATGTCAGTCTCC **-3'**
fu 11F	**5'-** TCTTTTGAGTCCAGTATTGATGC **-3'**
fu 11R	**5'-** AATAAATAAGGTAAAGAATGAACAAAC **-3'**
fu 12F	**5'-** CGCTTTCAGATAGGCTATTGTTA **-3'**
fu 12R	**5'-** GAAATCCTTAATGAGTACAATACATAC **-3'**
fu 13F	**5'-** TGTTGCGTATAACTTTGTATTTGCT **-3'**
fu 13R	**5'-** ATTCAAAAATATGTAACAATCAAAGC **-3'**
fu 14F	**5'-** ATTTGATAATTTTTTGTAATGTAAT **-3'**
fu 14R	**5'-** GATACCAGCAACGAACCAATA **-3'**

CHAPTER 3

RESULTS AND DISCUSSION

Overexpression of recombinant SisR and SisR-His in *E. coli* – SisR overexpression was initially attempted using the pAPO1 construct which had *sisR* cloned under the control of the T7 promoter in the expression vector pT7-7. T7 RNA polymerase was expressed from plasmid pGP1-2, which carries the gene encoding T7 RNA polymerase under control of λ P_L along with a temperature-sensitive allele of the λ repressor. Plasmid pGP1-2 allows for heat inducible expression of T7 RNA polymerase in any *E. coli* strain. Alternatively, T7 RNA polymerase was expressed in *E. coli* BL21 (DE), pLysS and CodonPlus (RIL), which each bear a chromosomal copy of the gene encoding T7 RNA polymerase that is under control of the *lac* promoter. Several overexpression conditions including different *E. coli* backgrounds (DH5α, *recA*$^-$ DH5α, YMC11, JM105, TOPP 1, TOPP 2, TOPP 4, TOPP 5, TOPP 6), different cell densities for induction, and various induction times, were tested for the two-plasmid system. Likewise, several overexpression conditions, including various IPTG concentrations, inductions times, cell densities, and induction temperatures, were tested for the IPTG-inducible system. None of those conditions resulted in observable levels of expression of SisR (data not shown).

Most amino acids are encoded by more than one codon, and each organism carries its own bias in the usage of the 61 available amino acid codons. In each cell, the tRNA population closely reflects the codon bias of the mRNA population (Ikemura, 1981). Examination of codon usage in all 4,290 *E. coli* genes reveals a number of codons that are underrepresented, a subset of the codons for Arg, Ile, Gly, Leu, and Pro are very rarely used in highly expressed *E. coli* genes (Table 3)

(Henaut and Danchin, 1996). Insufficient tRNA pools can lead to translational stalling, premature translation termination, translation frameshifting, and amino acid misincorporation (Kurland and Gallant, 1996). The most severe effects on expression have been observed when multiple consecutive rare codons are near the N-terminus of a coding sequence.

Analysis of codon usage in *B. burgdorferi* reveals that all 61 triplet codons are used (Fraser *et al.*, 1997). When both AU- and GC-containing codons specify a single amino acid, there is a marked bias (from 2-fold to more than 20-fold, depending on the amino acid) in the use of AU-rich codons. The most frequently used codons are AAA (Lys, 8.1%), AAU (Asn, 5.9%), AUU (Ile, 5.9%), UUU (Phe, 5.7%), GAA (Glu, 5.0%), GAU (Asp, 4.2%) and UUA (Leu, 4.2%). The most common amino acids are Ile (10.6%), Leu (10.3%), Lys (10.2%), Ser (7.8%) and Asn (7.2%) (Fraser *et al.*, 1997).

Analysis of the codons in SisR reveals not only several of the rare codons (28%), but also multiple consecutive rare codons near the N-terminus of the protein (33.3%) (Table 3). *E. coli* strain Rosetta (DE3) pLysS contains plasmid pLysS/RARE that carries *argU, argW, ileX, glyT, leuW, proL*, genes that encode tRNA for rare codons in *E.coli* (Table 3). SisR-His was expressed to high levels in the *E. coli* Rosetta (DE3) pLysS strain, indicating that the previous problems associated with expression were the result of rare codons within SisR. Indeed, 51% of SisR rare codons had the corresponding tRNAs encoded in the pLysS/RARE plasmid. Optimum conditions for the overexpression of SisR-His were achieved when the Rosetta strain was freshly transformed with pAPO3, grown to OD_{600} 0.8 and induced with 1 mM IPTG at 37 °C for 3 hours (Figure 10). The same conditions were used with success for the overexpression of native SisR (data not shown).

Purification of SisR-His. SisR-His bound efficiently to the Ni-NTA affinity column and was eluted with 250 mM imidazole (Figure 11). SisR-His was purified further by heparin-agarose chromatography which is used frequently for the purification of DNA-binding proteins. SisR-His bound tightly to the heparin-agarose column and eluted from the column at about 250-350 mM KCl (Figure 12A and 12B). Purification was efficient, yielding a final concentration of 0.5-1.0 mg per ml. However 75-100 mM KSCN had to be included in both the elution buffer and the dialysis buffer to keep SisR-His in solution at these concentrations. KSCN is a chaotropic salt that has been used to keep other activators of σ^{54}-holoenzyme in solution (Lee *et al.*, 1994). Future gel filtration experiments using purified SisR-His would determine its ability to form homo-multimeric interactions.

Overexpression and solubility of recombinant SisK-His. Unlike SisR-His overexpression attempts, SisK-His was successfully expressed in *E.coli* strain DH5α (Figures 10 &13). This could be attributed to differences in codon usage between the two proteins since analysis of the codons in *sisK* reveals that 25% of its codons are rare compared to 28% in *sisR*. Furthermore, 26% of *sisK* rare codons compared to 33.3% in *sisR* are located near the N-terminus end of the protein. Moreover, unlike in *sisR*, the rare codon CCC and TCG are absent in *sisK*. Perhaps the most important distinction that could account for the differences in expression of SisR-His and SisK-His in *E. coli*, however, is that unlike SisR, SisK does not have stretches of consecutive rare codons (Table 3).

Under conditions described as optimum for SisR-His expression the level of expression of SisK-His that was achieved in the *E. coli* Rosetta (DE3) strain was

higher than that with *E.coli* strain DH5α (data not shown). This difference in expression was the result of rare codons within SisK, indeed 60% of SisK rare codons had the corresponding tRNAs encoded in the pLysS/RARE plasmid (Table 3).

Following breakage of cells that overexpressed SisK-His and subsequent clarification of the crude cell extract by centrifugation, the bulk of SisK-His was present in the cell pellet (Figure 13). Attempts to purify any SisK-His that remained soluble by Ni-NTA affinity chromatography were unsuccessful (data not shown), as were attempts to solubilize SisK-His with sarkosyl and then purify it by Ni-NTA affinity chromatography (data not shown).

Overexpression and purification of recombinant MBP-SisK in *E. coli.* Kapust and co-workers suggest that the MBP functions as a general molecular chaperone in that it plays a passive role in protein folding serving to prevent the off-pathway aggregation of intermediates in the folding process. Further, they have determined that MBP is a far more effective solubilizing agent than two other fusion proteins that have been claimed as solubilizing agents, thioredoxin and glutathione S-transferase (Kapust and Waugh, 1999).

The use of MBP fused with SisK had a great potential for keeping SisK in solution. MBP-SisK protein fusion was efficiently expressed in both *E. coli* Rosetta (DE3) and DH5α (Figures 14) under the same conditions described for SisR-His. The level of expression that was achieved in the *E. coli* Rosetta (DE3) strain was much higher than that with *E.coli* strain DH5α (data not shown), which may have been attributed to codon usage in *sisK* (Table 3). SisK-MBP was purified from these strains in a single step by affinity chromatography with an amylose resin column (Figure 14)

MBP-SisK has an associated flavin that may be involved in signal perception. Purified MBP-SisK had a faint yellow color, and an absorption spectrum of the purified protein revealed a peak at about 410 nm, suggesting the presence of a flavin cofactor (Figure 15A and 15B). Attempts to determine whether of the flavin cofactor was FMN or FAD were not successful due to the low amount of flavin cofactor in the protein preparation. It is possible that the flavin was lost during purification of SisK-MBP. Alternatively, the MBP-SisK may not have been saturated with flavin *in vivo.*

Inspection of the predicted amino acid sequence of SisK suggests that it contains a PAS domain (Figure 8A and 8B). PAS-domain proteins are associated with cofactors such as heme, flavin or 4-hydroxycinnamoyl (Taylor and Zhulin 1999) which often sense redox potential by recognizing signals such as oxygen, reduction state of redox sensors or light (Gong, et al. 1998). PAS domains have been found in many other proteins, including other histidine protein kinases, light receptor and regulator proteins, clock proteins, oxygen/redox sensors, ion channels, and a Ser/Thr kinase with a putative redox-sensing or flavin-binding domain (Pellequer, Wager-Smith et al. 1998).

Low-passage SisK and σ^{54} mutants isolated under low O_2 conditions (<5% O_2) and quantitative RT-PCR and immunoblots of wild-type B31A show an increase in *rpoS* mRNA and σ^s levels as cells entered stationary phase. However, these increases are not observed in the σ^{54} mutant (Treglown *et al.*, 2003). Furthermore, the low-passage σ^{54}, σ^s and SisK mutants are significantly more sensitive to tertiary-butyl peroxide (reactive oxygen species) than the wild-type strain (Treglown *et al.*, 2003). These mutants would probably be much more sensitive than the wild type strain in vivo as well, as they would be encountering a series of reactive oxygen species in the

different host environments encountered during the tick life cycle. Our lab postulates that the flavin in SisK serves as a redox sensor that detects oxygen, reactive oxygen species or other redox signals to control either the autophosphatase or kinase activities of the protein and thereby regulate SisR-mediated transcriptional activation of *rpoS*.

***In Vitro* Phosphorylation Assays.** Protein histidine kinases of two component system are bifunctional having both kinase activity (acting on histidine) and phosphatase activity (acting on phophoaspartate) (Klumpp S. and Krieglstein, 2002) upon sensing of an environmental signal. In order to characterize *B. burgdorferi* SisK/SisR two-component system, purified MBP-SisK and the SisR proteins (both native and histidine-tagged) were used for *in vitro* phosphorylation assays. Purified MBP-SisK phosphorylated itself in the presence of ^{32}P-(γ)-ATP demonstrating that it has autokinase activity (Figures 16 lanes 1). The transfer of the phosphate to SisR required freshly purified MBP-SisK since older preparations appeared to lose activity with time (data not shown). A 1:1 ratio of MBP-SisK to SisR proteins (5μg) proved to be effective. MBP-SisK-phosphate was a phospho-donor to both native SisR and SisR-His, however phosphate transfer was much more efficient with SisR-His (Figure. 16 lanes 3-6 compared to lanes 8-11). We do not understand the reason for this, but there may be another factor that is required for efficient transfer of phosphate from SisK-phosphate to SisR. The histidine-tag may alleviate the need for this other factor by altering the structure of the receiver domain of SisR or by promoting productive interactions between SisK and SisR. Mutational studies of SisK H-box and SisR Asp-49, Asp-54 residues, pulse-chase experiments and measurements of phosphate released would better elucidate the phosphorelay system.

Gel mobility shift assays. Identification of the *rpoS* enhancer would provide important information about the regulation of *rpoS* and could lead to the identification of other σ^{54}- regulated genes in *B. burgdorferi*. The purified recombinant activator SisR (data not shown) and SisR-His (Figures 17, 18, and 19), as well as their phosphorylated forms by their cognate recombinant histidine kinase MBP-SisK (data not shown), were assayed for their ability to bind DNA. Affinity for heparin agarose during the protein purification gave me confidence that the DNA binding activity of the purified activator was not impaired.

Activators of σ^{54}-holoenzyme generally bind to enhancers located 100-200 bp upstream of the target promoter and contact polymerase bound at the promoter to activate transcription (Weiss *et al.*, 1992a). Transcriptional enhancers are relatively short (30-200 bp) DNA sequences usually composed of several binding sites for activator protein(s). Enhancers are usually able to activate genes over a considerable distance, up to 60 kb in Eukaryota and up to 15 kb in Prokaryota using a mechanism yet unknown (Bondarenko *et al.*, 2002). A series of 16 overlapping PCR products labeled at their 5'-ends with ^{32}P-(γ)-ATP, spanning 2 kb on either side of the *rpoS* σ^{54}-dependent promoter was examined for the presence of an enhancer in a gel mobility shift assay. SisR bound to regions uy3 and uy4 (Figure 17, lanes 6 and 8, respectively), which corresponded to a region within *pdxK* (Figure 9), however binding to these regions were not reproducible (data not shown). Reproducible binding of SisR-His was observed to a DNA fragment designated fu1, located about 1.6 kb downstream of the promoter (Figures 17, 1ane 10 and Figures 18, 19), which corresponded to a region within *gcp* (Figure 9). And at least two shifted species were observed at 250 nM SisR-His or higher (Figures 18 and 19), suggesting that the fu1 probe contains more than one binding site for SisR. Binding to fu1 region appeared

to be specific due to the concentration dependent increase in shifts 1 and 2 and the observed shift at the low 50 nM SisR-His concentration (Figures 17 and 18). Cooperative binding of a σ^{54}- dependent transcriptional activator to an upstream activation sequence has been shown for NtrC in both *E. coli* (Weiss *et al.*, 1992b) and *S. typhimurium* (Porter et al., 1993), and for DctD in *Rhizobium meliloti* (Scholl and Nixon, 1996). Moreover, binding of FleQ, the major flagellar gene regulator in *Pseudomonas aeruginosa,* to a downstream vicinity of the promoters, *flhA, fliE*, and *fliL* has also been shown and this activator then activates transcription without looping. Furthermore, FleQ also binds upstream the *fleSR* promoter and activates transcription from a distance via looping (Jyot *et al.*, 2002).

The mechanism of action of the SisR homolog NtrC has been intensely studied using the *glnAp2* promoter as a model. The NtrC dependent transcriptional enhancer participates in the regulation of σ^{54} dependent genes involved in metabolism of nitrogen in *E. coli*. The enhancer is localized ~110 bp upstream of the *glnAp2* promoter but strongly activates transcription when positioned up to 15 kb away *in vivo* (Reitzer and Magasanik, 1986) and up to 0.9 kb *in vitro* (Ninfa *et al.*, 1987); it functions both upstream and downstream from the promoter (Bondarenko *et al.*, 2002). NtrC dimers form tetramers or higher-order oligomers, and phosphorylation increases the ability of NtrC to oligomerize and more importantly to hydrolyse ATP. Thus, phosphorylation is required for transcription activation by NtrC (Parkinson and Kofoid, 1992; Weiss *et al.*, 1992b). The phosphorylation-dependent activation of NtrC inspired the use of phosphorylated SisR-His and SisR in DNA binding assays; however binding of SisR-His nor SisR to fu1 probe did not improve (data not shown). Moreover, the other 15 generated DNA fragments were also tested in DNA binding assays and phosphorylated SisR-His and SisR did not bind to any of the 15 fragments

(data not shown). But a caveat is I don't know to what extent the protein was phosphorylated. The percentage phosphorylated protein appeared to be low (Figure 16).

E. coli cell extract containing overexpressed pAPO1 was used to assess native SisR binding to ^{32}P-labelled fu1 probe at various cell extract volumes. Binding reactions were carried out in the presence of sonicated competitor salmon sperm DNA (41% G+C)An increase in shift was observed with increasing volumes of cell extract to fu1 probe (Figure 20), however similar patterns were observed in the absence of pAPO1 (data not shown). Moreover, similar patterns were also observed to the addition of cell extract to probes uy1-uy4 in the presence and absence of pAPO1 (data not shown). Thus, shifts were concluded to be independent of SisR binding. Perhaps the use of a more AT rich competitor such as a heteropolymer poly (dAdT).poly(dAdT) would have made this assay more specific to SisR binding.

Target regions fu1, uy3 and uy 4 were successfully cloned (Table 1), however SisR binding to target regions was very poor and therefore not worth footprinting. Unfortunately, the poor binding of SisR to fu1 region prevented the identification of the enhancer sequence.

We do not know if the sites on the ful probe serve as the enhancer for *rpoS* or if there are SisR-binding sites located >2 kb from the *rpoS* promoter. This distance may be needed to accommodate interactions between enhancer-bound SisR and σ^{54}-RNA polymerase bound at the promoter since *rpoS* is on the linear chromosome of *B. burgdorferi.* The linear chromosome of *B. burgdorferi* has telomeres, or termini of the chromosome, that are covalently closed hairpin loops, in which a DNA strand loops around to become the complementary strand (Casjens *et al.*, 1997), thus allowing DNA supercoiling to take place. DNA supercoiling is believed to greatly

facilitate prokaryotic enhancer action over a large distance (2.5 kb), however unlike DNA bending, DNA supercoiling is not essential for action over a short distance (0.11 kb) (Liu *et al.*, 2001).

Integration host factor F (IHF), a histone-like protein is known to bind and bend DNA. OmpR acts as an activator and a repressor of *ompF*. IHF is essential for normal *ompF* regulation as it is required to bend DNA to form the repressive loop. In an IHF mutant, OmpR is unable to repress *ompF* transcripts (Tsui *et al.*, 1988). *B. burgdorferi* lacks IHF (Fraser *et al.*, 1997), thus making it difficult for DNA bending. Supercoiling would be a way for the activator protein to be able to touch the σ^{54}-dependent RNA-polymerase holoenzyme.

Conceivably, the *rpoS* enhancer could be found by PCR-assisted binding–site selection, if *rpoS* indeed has an enhancer. Perhaps *in vivo*, *rpoS* does not have only one strong enhancer, it could have several weak ones including the one found in these studies. Not surprisingly, the weak enhancers could be located very far downstream or upstream of the promoter region and the contact between SisR and σ^{54} could be facilitated by DNA supercoiling. Furthermore, it would also be possible that SisR activates transcription of *rpoS* by a novel mechanism without binding to an enhancer sequence as σ^{54}-dependent expression of *B. burgdorferi rpoS* is responsive to a signal that accumulates as cultures approach stationary phase. NtrC mutant forms that have no detectable DNA binding ability still retain their ability to activate basal levels of transcription when present at high concentrations (Porter *et al.*, 1993). An *rpoS* reporter system should be built in the future with the downstream 1.6 kb enhancer region determined in these studies to test if SisR and SisK activate transcription of *rpoS* from the σ^{54}-dependent promoter. Furthermore, future studies should investigate if σ^{54} is also regulating expression of repressors in *B. burgdorferi*, as it was suggested

by two-dimensional gel electrophoresis (Dr. Jonathan Frye, personal communication) and by DNA microarray (Dr. Frank Gherardini, personal communication) when the *rpoN* mutant expressed less genes than wild type. In *Alcaligenes eutrophus*, the phosphorylated transcriptional activator of H16 hydrogenase system HoxA has a negative effect on transcriptional activation (Lenz and Friedrich, 1998).

Table 3. Position and % of *B. burgdorferi* rare codons in SisR and SisK generated by *E. coli* Codon Usage Analyzer 2.0
(http://128.111.208.226/codonUsage/usage2.0c.html) Codons shown here represent less than 10% of codons for the same amino acid for *E. coli* Class II gene data (Henaut and Danchin, 1996) when synthesizing SisR.

Rare Codon	Amino Acid	Position in SisR*	Position in SisK*
ATA	ILE	51, 52, 60, 67, 68, 76, 114, 115, 135, 137, 162, 164, 173, 183, 184, 200, 248, 313, 315, 325, 331, 333, 376, 399, 402, 445, 450, 451	31, 33, 37, 54, 73, 85, 88, 98, 99, 109, 173, 199, 203, 209, 220, 233, 249, 258, 273, 290, 293, 304, 308, 315, 323, 337, 345, 346
AGA	Arg	16, 56,95, 110, 117, 138, 194, 234, 261, 301, 307, 319, 321, 342, 365	93, 145, 146, 158, 205, 218,
TTG	Leu	1, 65, 74, 112, 388, 452	150, 239, 252, 314
GGA	Gly	18, 27, 36, 62, 75, 85, 149, 222, 226, 273, 362,	36, 44, 100, 169, 243, 312, 334, 336, 338, 350
ACA	Thr	42, 82, 86, 103, 242, 276, 287, 329, 403, 427, 428, 444	10, 68, 94, 154, 211, 286, 330, 366
CTT	Leu	23, 40, 48, 108, 111, 160, 172, 191, 207, 212, 216, 237, 245, 259, 283, 347, 371, 398, 442	48, 76, 124, 168, 253, 284, 339, 349,
CCC	Pro	58, 105	Not present
AGT	Ser	34, 214, 373	335
TTA	Leu	102, 106, 132, 153, 260, 304, 308, 318, 328, 367, 375, 377, 408	9, 15, 61, 65, 84, 87, 101, 130, 175, 214, 224, 240, 244, 268, 378
TCA	Ser	167, 192, 118, 252, 378	40, 71, 106, 114
CTA	Leu	81, 113, 119 , 136, 141, 152, 332, 404	12, 91, 143, 178, 215, 267, 372
CTC	Leu	66, 355	262, 297,
TCG	Ser	448	Not present
GGG	Gly	272	318, 381
AGG	Arg	282	155

* 28% of SisR codons and 25% of SisK codons are rare to *E. coli* , of which 33.3% and 26%, respectively, are located in the N-terminal end of the proteins
blue= rare codons that have tRNA genes encoded by the plasmid pLysS/RARE present in the Rosetta (DE3) strain
red= long stretch of rare codons present in the N-terminal region

Figure 10. Overexpression of native SisR, SisR-His and SisK-His and σ^{54}-His. Rosetta (DE3) only (lane 2 and 3) or carrying plasmids pAPO1 (~52 kDa native SisR, lanes 4 and 5) or pAPO3 (~54 kDa SisR-His, lanes 6 and 7), or pAPOk (~44 kDa SisK-His, lanes 8 and 9), or pAPOσ^{54} (~51 kDa σ^{54}-His, lanes 10 and 11) was grown at 37^{o}C to OD_{600} 0.8 at which point 1 mM IPTG was added to induce expression of encoded proteins for 3 hours. Lane 1, protein standards; Lanes 2, 4, 6, 8, and 10 cell extracts from uninduced cultures and lanes 3, 5, 7, 9, and 11 extracts of cells following induction

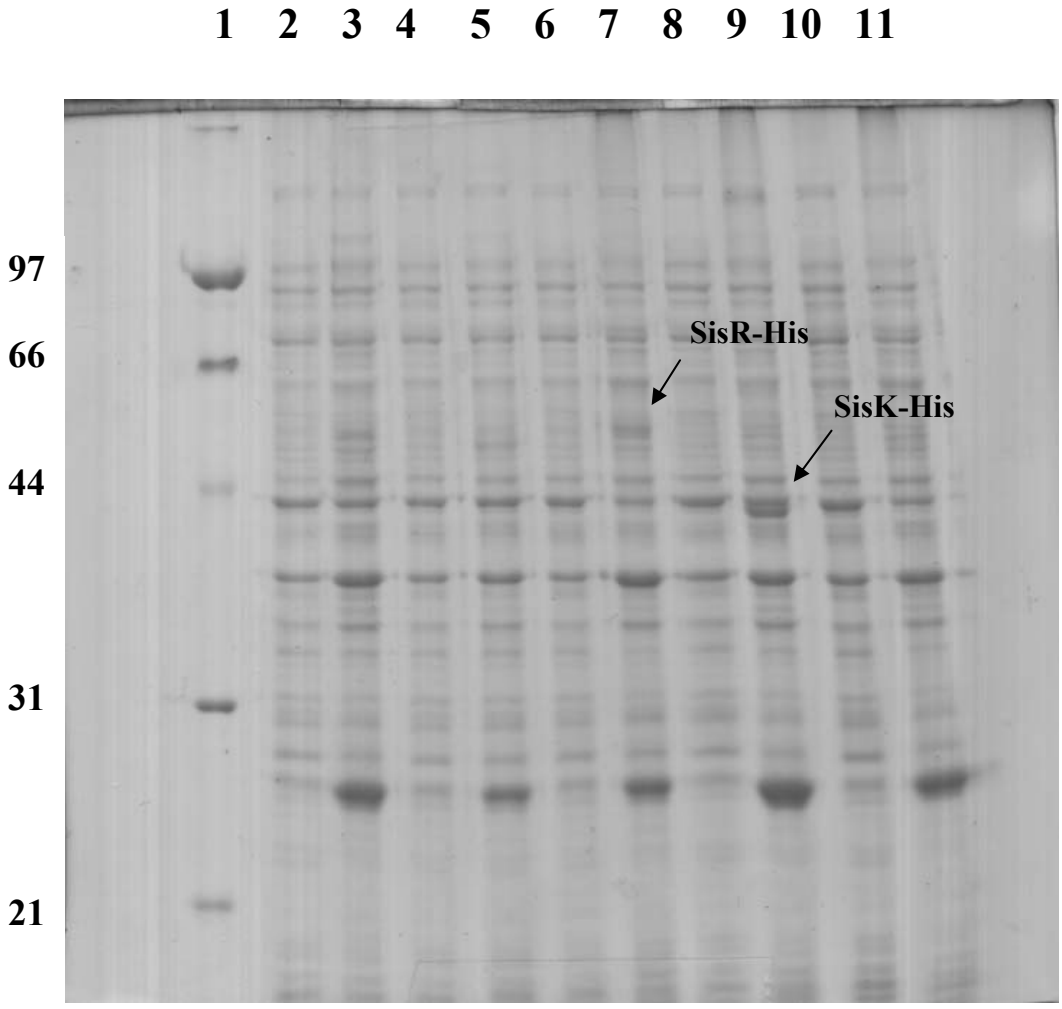

1 2 3 4 5 6 7 8 9 10 11
97
66
44
31
21
SisR-His
SisK-His

Figure 11. Purification of SisR-His by affinity chromatography. The ~ 54 KDa recombinant SisR-His eluted from the Ni-NTA column with ~250 mM imidazole (lanes 2-9). Protein standards are shown in lane 1.

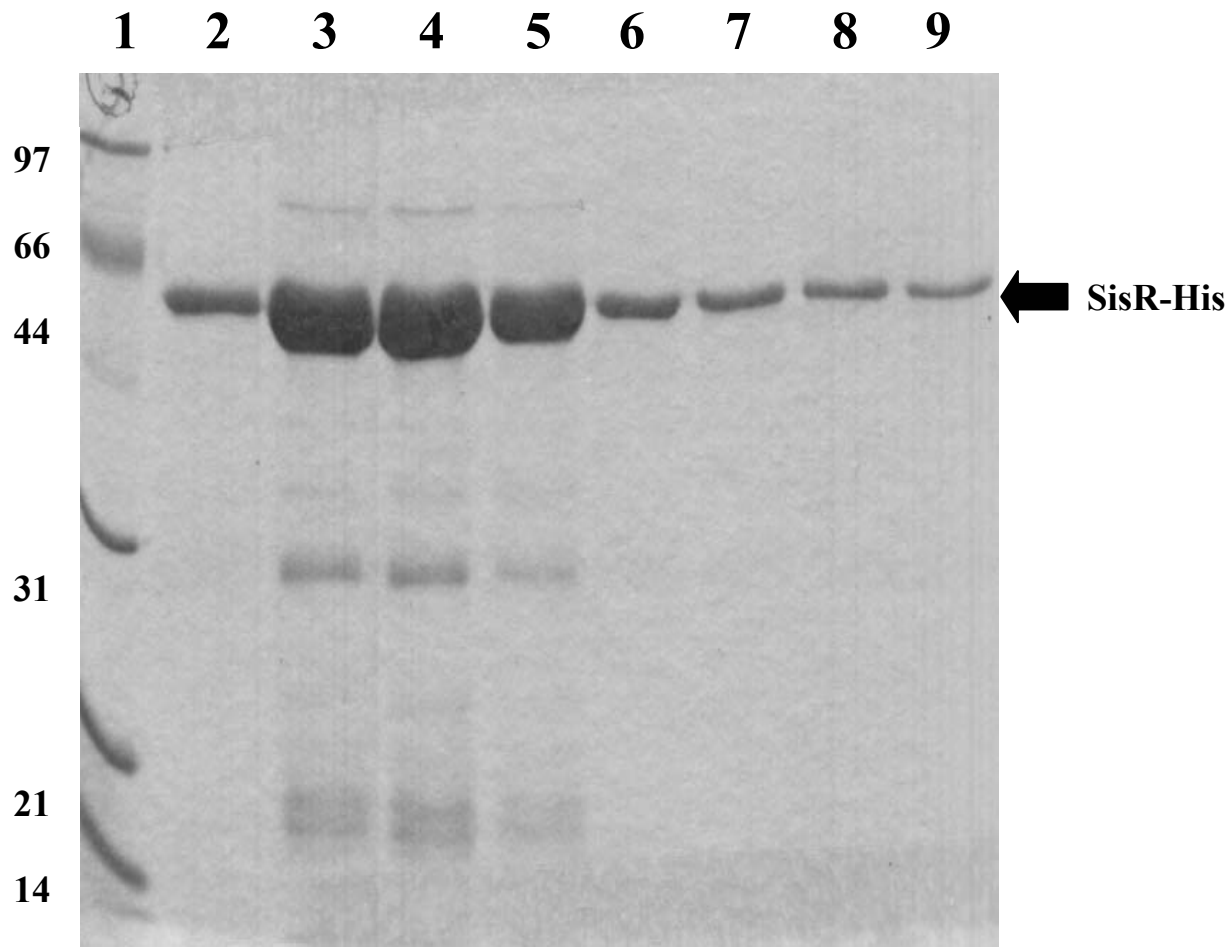
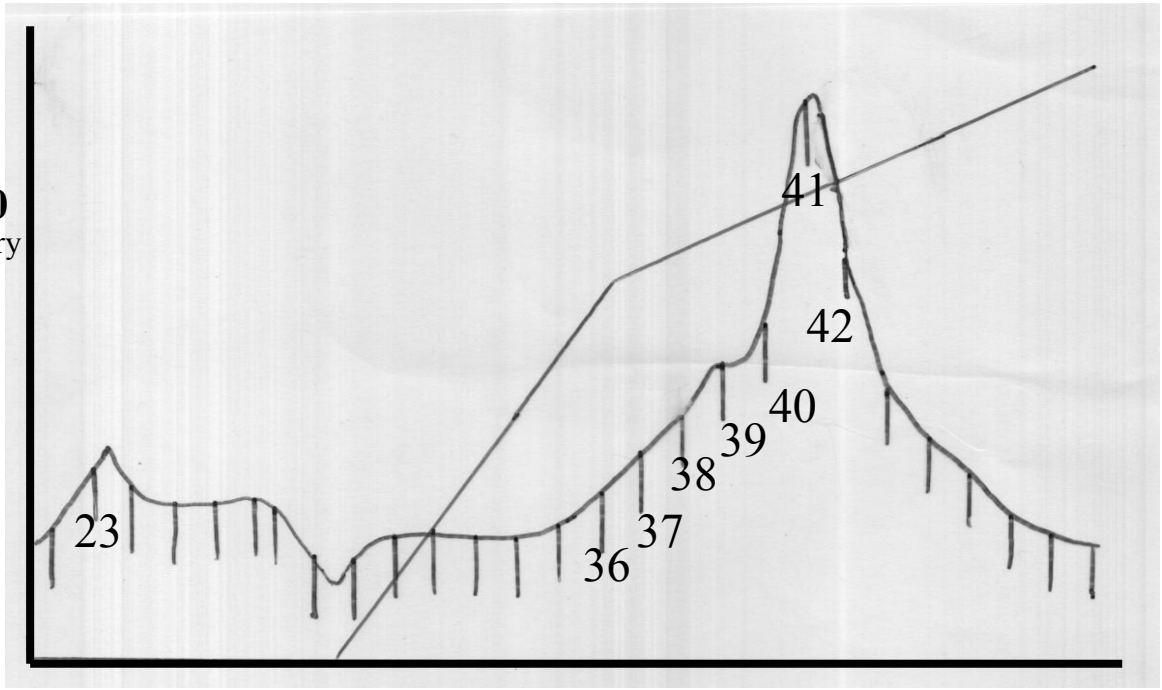

1 2 3 4 5 6 7 8 9
97
66
44
31
21
14
SisR-His

Figure 12. Further purification of SisR-His by heparin-agarose chromatography. SisR-His eluted from the heparin-agarose column with about 250-300 mM KCl. A) Fractions 39-42 on the chromatograph. B) SDS-PAGE showing proteins in selected fractions from the heparin-agarose chromatography. Proteins standards were included in lane indicated as "std".

A


A280
Arbitrary
units
41
42
40
39
38
37
36
23
Fraction number

B

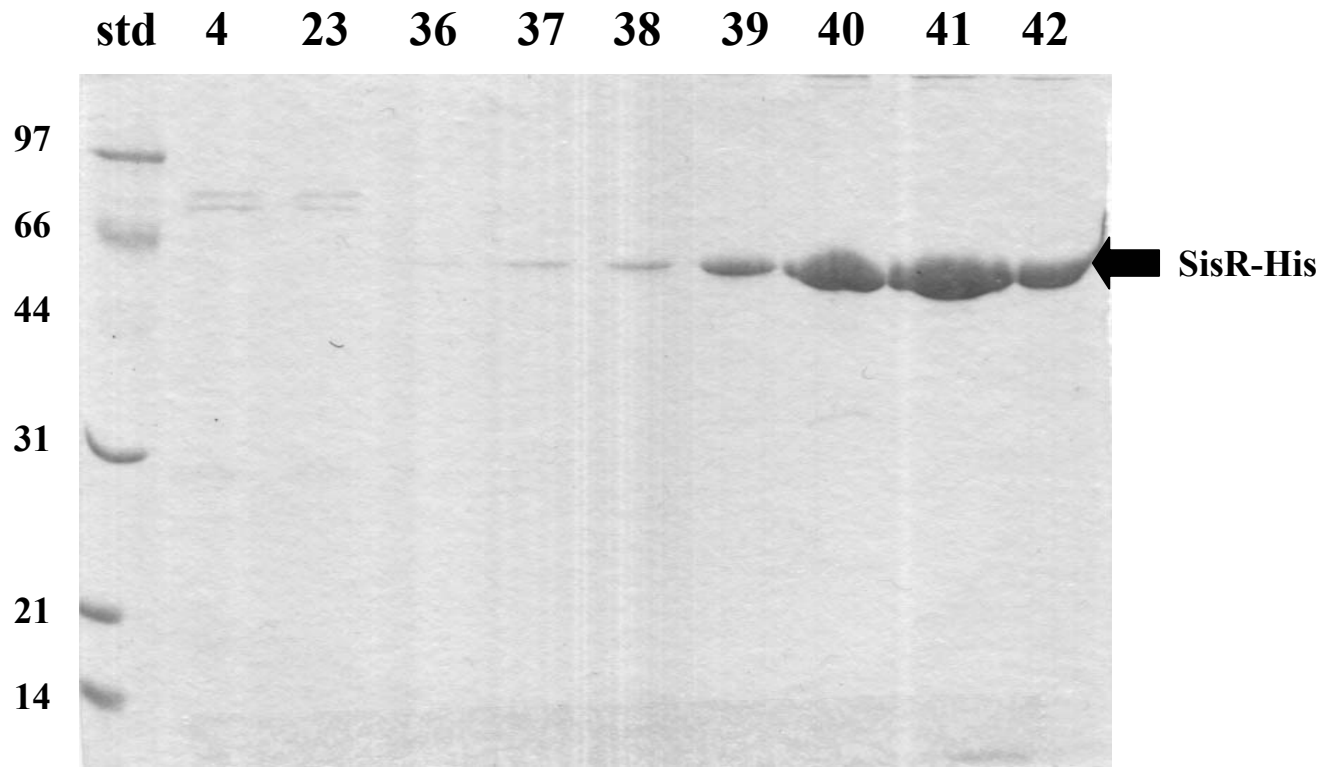

std
4
23
36
37
38
39
40
41
42
97
66
44
31
21
14
SisR-His

Figure 13. Overexpression of SisK-His and SisR-His, and SisK-His localization. *E. coli* strain DH5α carrying either plasmid pAPOk (lanes 2-5) or plasmid pAPO3 (lanes 6-10) was grown at 37^{o}C to OD_{600} 0.8 at which point 1 mM IPTG was added to induce expression of recombinant proteins. After 3 hours, cells were harvested and lysed. Lane 1, protein standards; lane 2 and 6, cell extracts from an uninduced culture; lane 3, 4, and 7-10 extracts of cells following induction, lane 5, cell pellet from induced and lysed culture. Asterisks are next to the bands for SisK-His while the arrow indicates the band for SisR-His

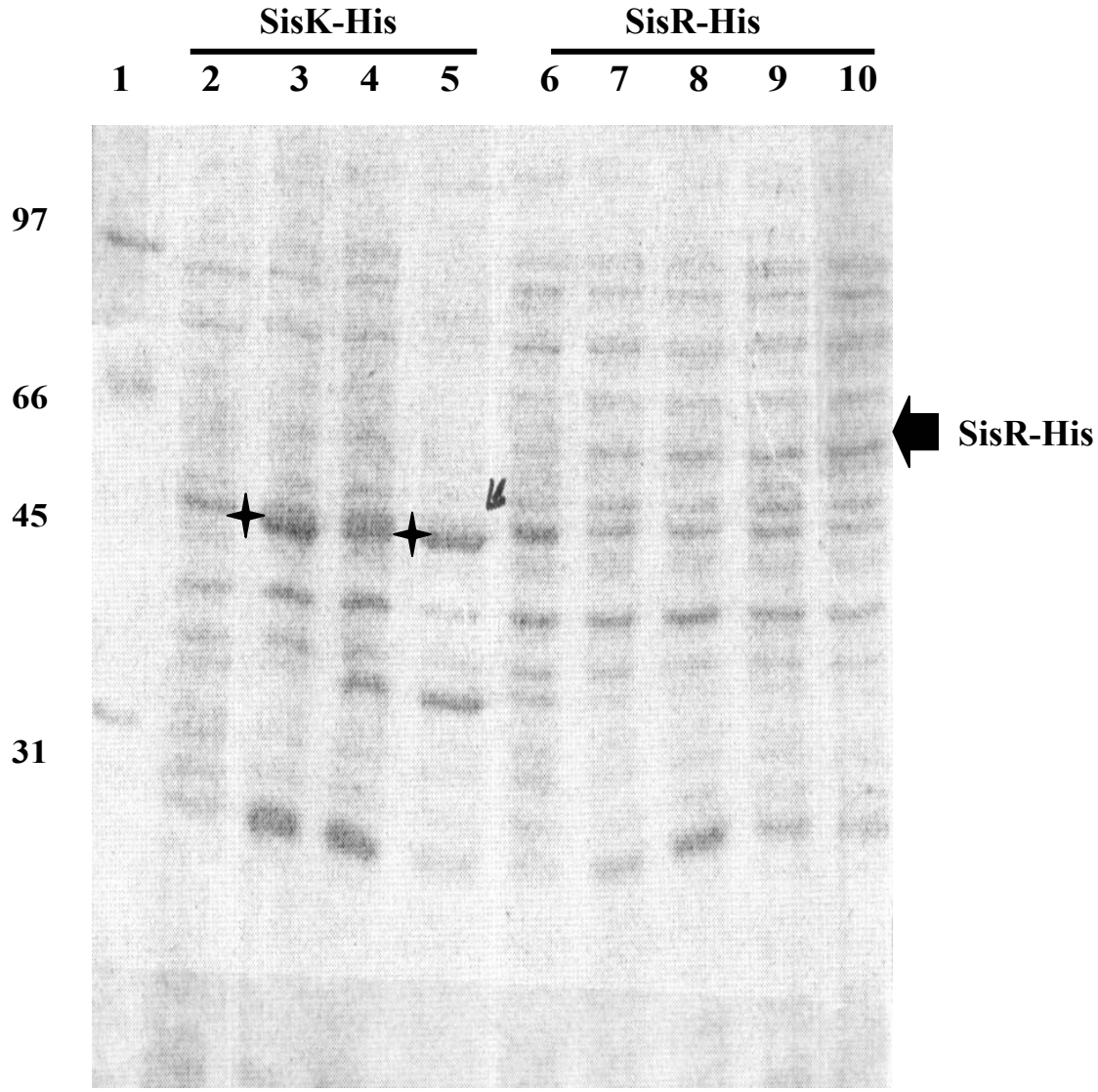

SisK-His
SisR-His
1
2
3
4
5
6
7
8
9
10
97
66
45
31
SisR-His

Figure 14. Overexpression and purification of MBP-SisK. *E. coli* strain DH5α carrying plasmid pAPOxu was grown at 37°C to OD_{600} 0.8 at which point 1 mM IPTG was added to induce expression of MBP-SisK. After 3 hours, cells were harvested and lysed and the MBP-SisK was purified. Lane 1, protein standards; lane 2, extracts of cells following induction of MBP-SisK; lane 3, cell extracts from an uninduced culture. The ~ 92 KDa recombinant MBP-SisK was purified by affinity chromatography using an amylose resin column (New England Biolabs). The MBP-SisK was eluted from the column by including 20 mM maltose in the buffer (lanes 4-9) with the bulk of the protein eluting in the earlier fractions.

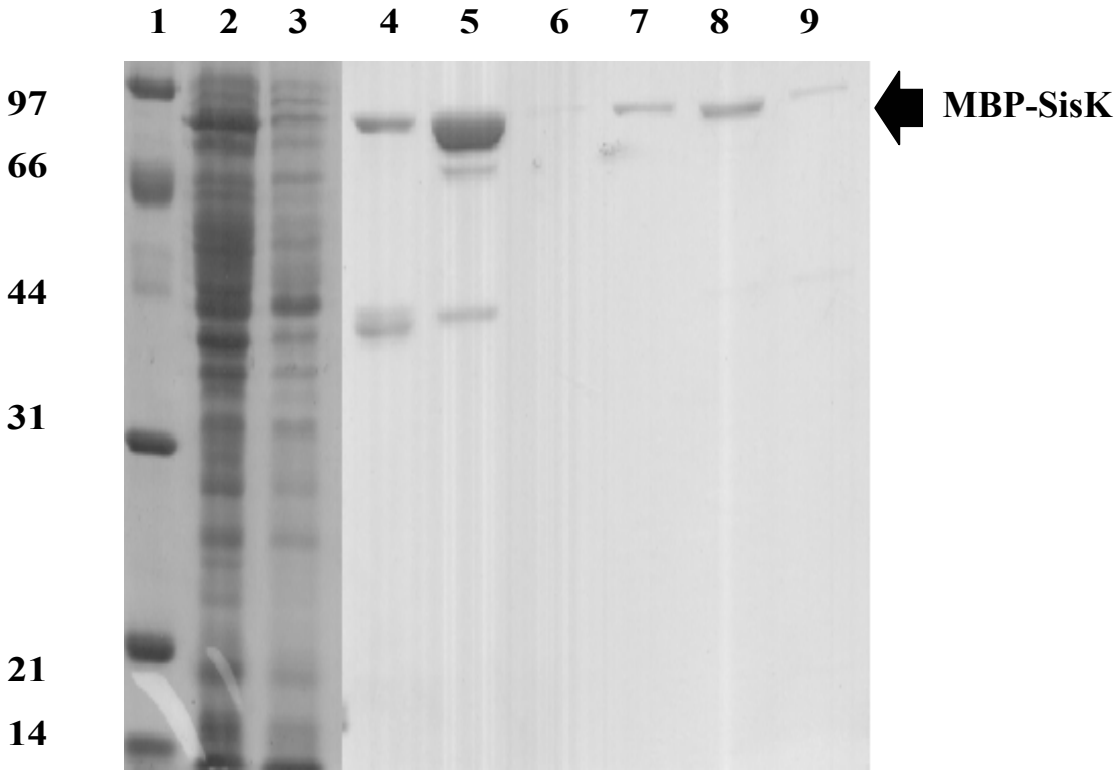

1 2 3 4 5 6 7 8 9
97
66
44
31
21
14
MBP-SisK

Figure 15. Absorption spectrum of purified MBP-SisK. Purified MPB-SisK (750 μg/ml) was analyzed on a Shimadzu UV 160U/UV visible scanning spectrophotometer from 350 to 750 nm wavelength light. (A) Note the small peak near 400 nM in the absorbance spectrum of the protein (B) Derivation of the absorption spectrum from Figure 19A. A first derivative of the absorption spectrum scan was done to determine more precisely the wavelength of light which gave maximal absorbance for the peak shown in Panel A. Maximum absorbance was estimated at 410 nM from this analysis.

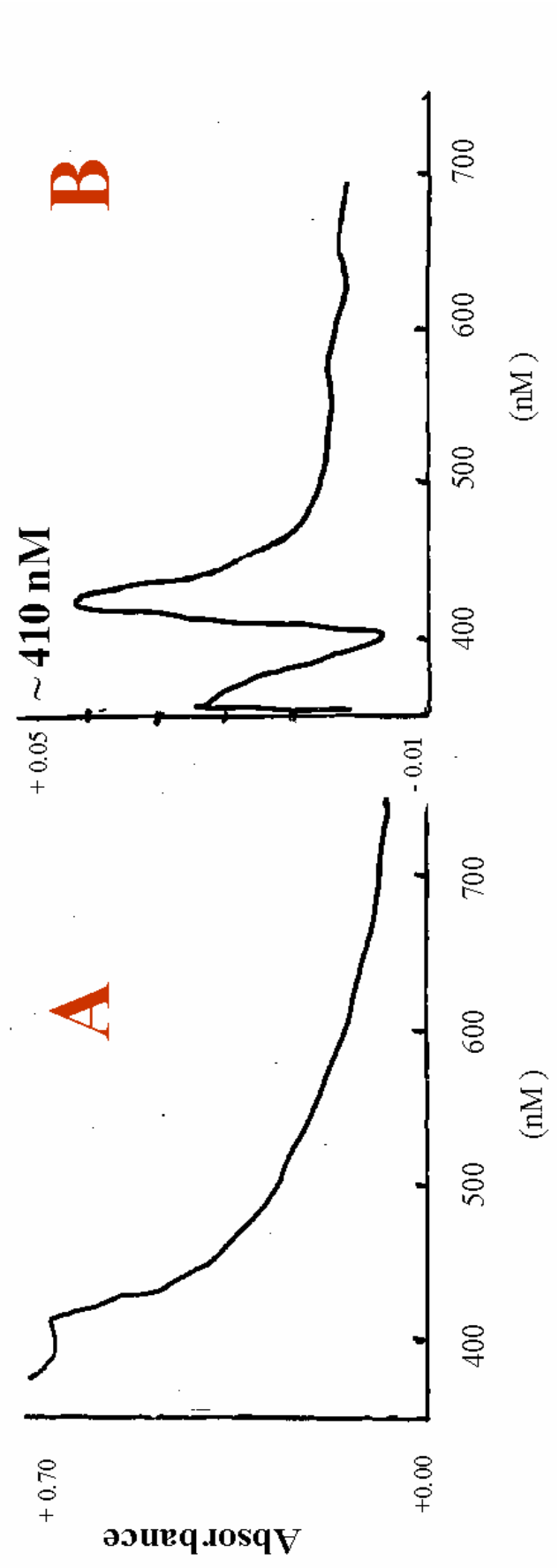

B
~ 410 nM
+ 0.05
- 0.01
400
500
600
700
(nM)
A
Absorbance
+ 0.70
+0.00
400
500
600
700
(nM)

Figure 16. Autophosphorylation of MBP-SisK and subsequent transfer of phosphate to SisR-His or to native SisR. Lane 1, MBP-SisK alone (5 mg); lane 2, native SisR alone (5 mg); lanes 3-6, MBP-SisK plus native SisR (5 mg each); lane 7, SisR-His alone (5 mg); lanes 8-11, MBP-SisK plus SisR-His (5 mg each). For lanes 3-11 MBP-SisK and the SisR proteins were mixed and the labeling reaction was initiated by the addition of ^{32}P-(γ)-ATP. Reactions were stopped following 30^{o}C incubation after either 2.5 minutes (lanes 6 and 11), 5 minutes (lanes 4 and 9), or 15 minutes (lanes 1, 2, 3, 7 and 8)

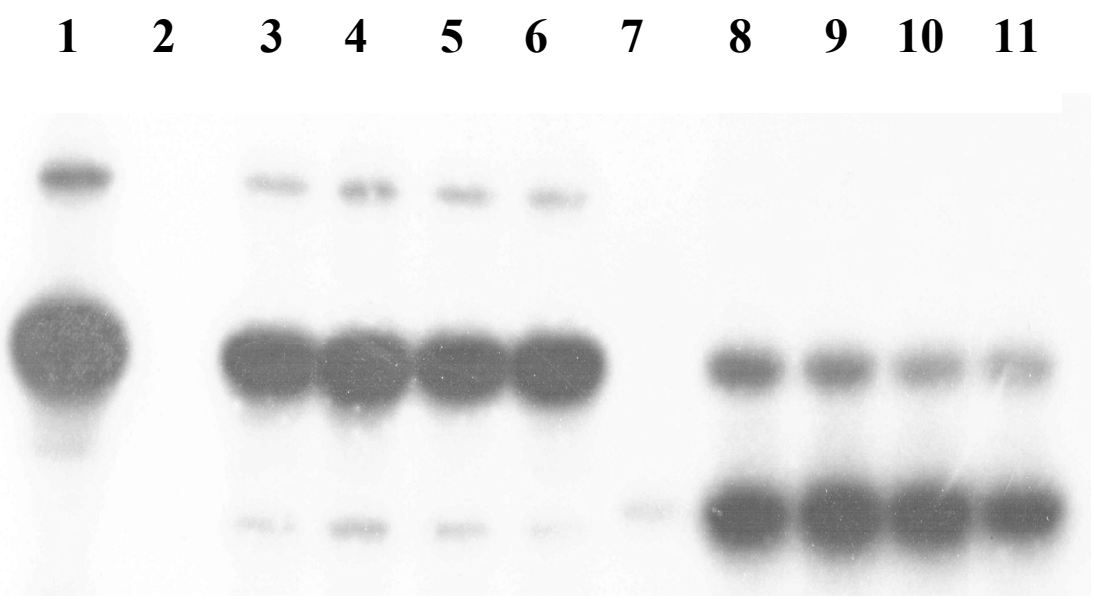

1
2
3
4
5
6
7
8
9
10
11

Figure 17. SisR-His binding to DNA fragments from regions downstream of *rpoS*. Purified SisR-His (1 μM monomer) was incubated with various ^{32}P-labelled DNA probes (even numbered lanes). DNA-protein complexes were analyzed on a 5% non-denaturing polyacrylamide gel as described by Parsek *et al.* (1994). No protein controls are presented in odd numbered lanes. Lanes 1 and 2, uy1 probe; lanes 3 and 4, uy2 probe; lanes 5 and 6, uy3 probe; lanes 7 and 8, uy4 probe; and lanes 9 and 10, ful probe. The relative positions to which these probes correspond to are indicated in Figure 1. The black arrows in lanes 5, 7, and 9 indicate free DNA and shifted species observed in lanes 6, 8, and 10 for probes uy3, uy4, and ful, respectively. Binding reactions were carried out for 15 minutes at 37°C in 50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 1 mM EDTA, 40 mM KCl, 4% glycerol, 1 mM β-mercaptoethanol, 0.05 mg/ml bovine serum albumin, and 0.50 mg/ml sonicated salmon sperm DNA (binding buffer).

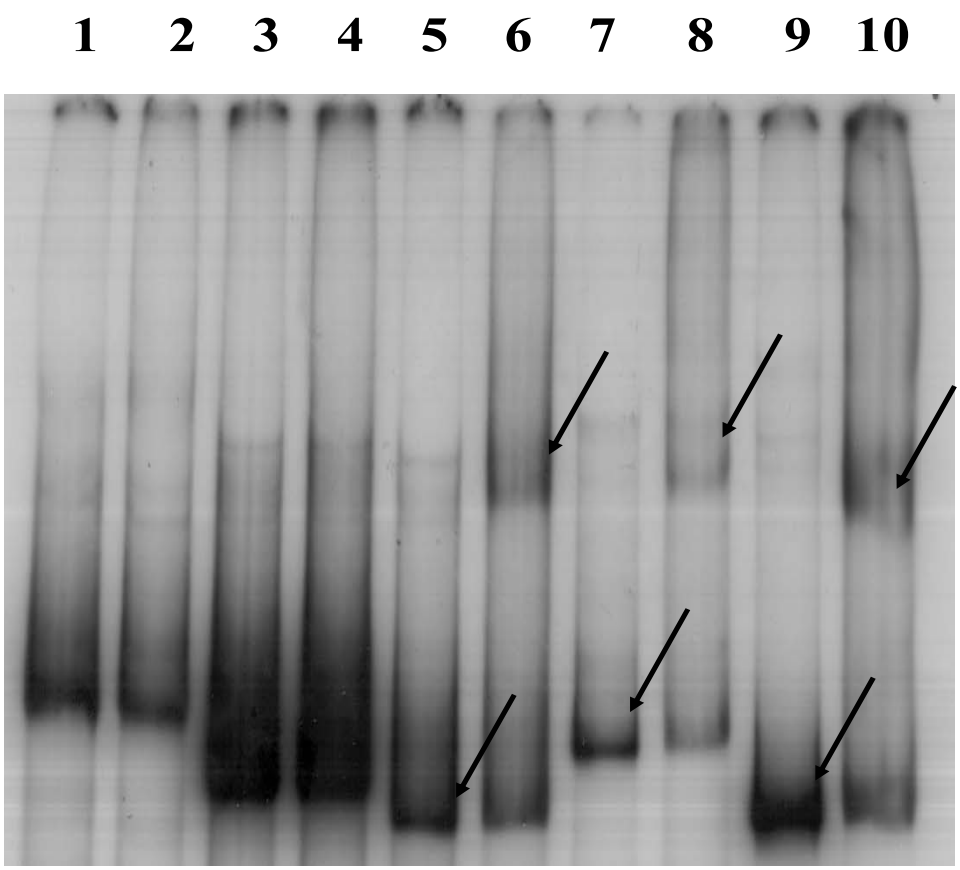

1 2 3 4 5 6 7 8 9 10

Figure 18. Titration of SisR-His in the gel mobility shift assay with the fu1 probe at 30°C and 37°C. SisR-His binding to ^{32}P-labelled fu1 probe was assessed at various SisR-His monomer concentrations ranging from 50 nM to 1000 nM. Binding reactions were done as described in Figure 17 legend at either 30^{o}C or 37^{o}C. Free probe and two shifted species were observed under these assay conditions and are indicated with black arrows (Shift 1 and Shift 2).

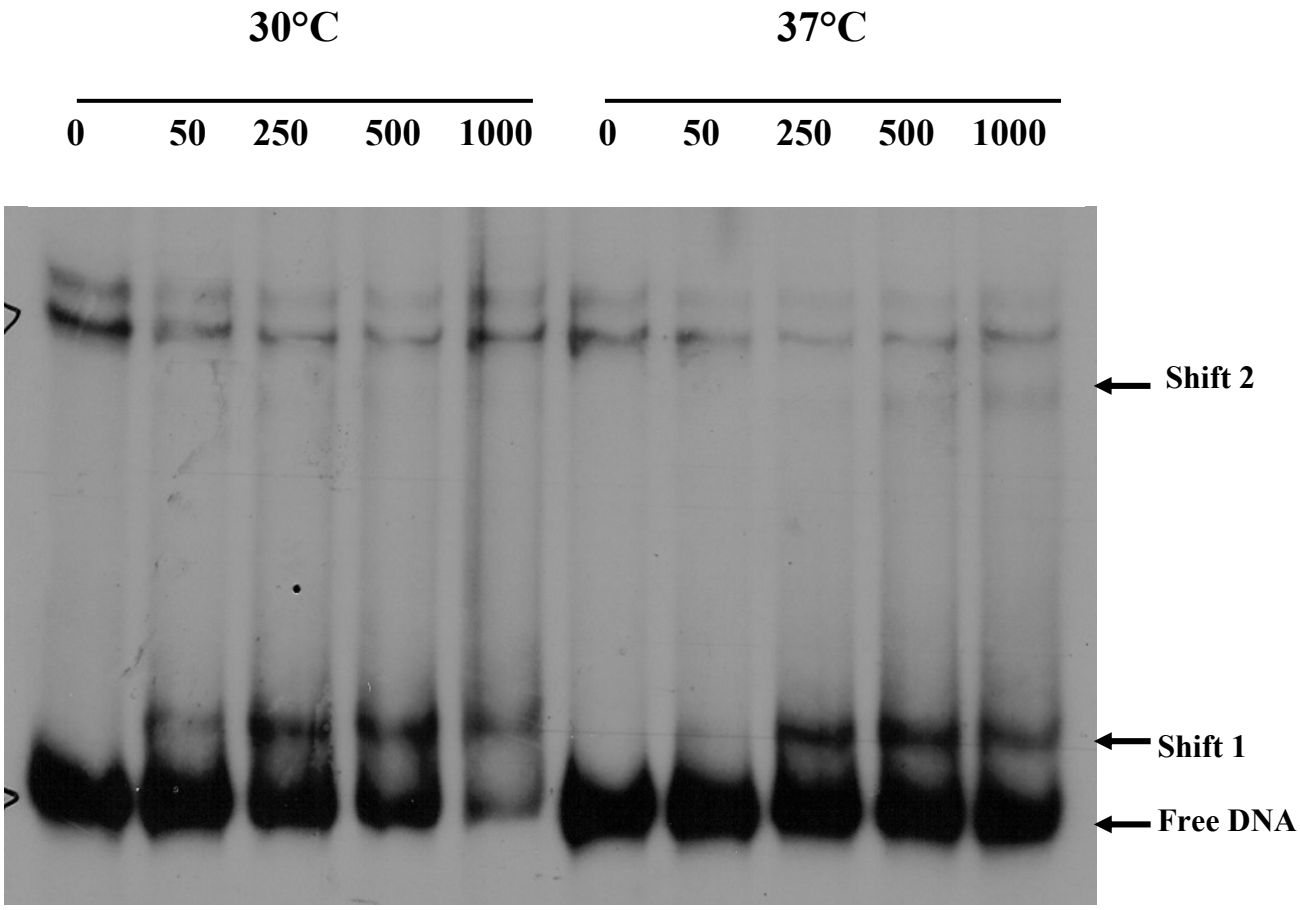

30°C
37°C
0
50
250
500
1000
0
50
250
500
1000
Shift 2
Shift 1
Free DNA

Figure 19. Quantitation of SisR-His binding to the fu1 probe. Bands for free DNA, shift 1 species, and shift 2 species for the DNA binding reactions done at 37^{o}C were quantitated with a PhosphorImager.

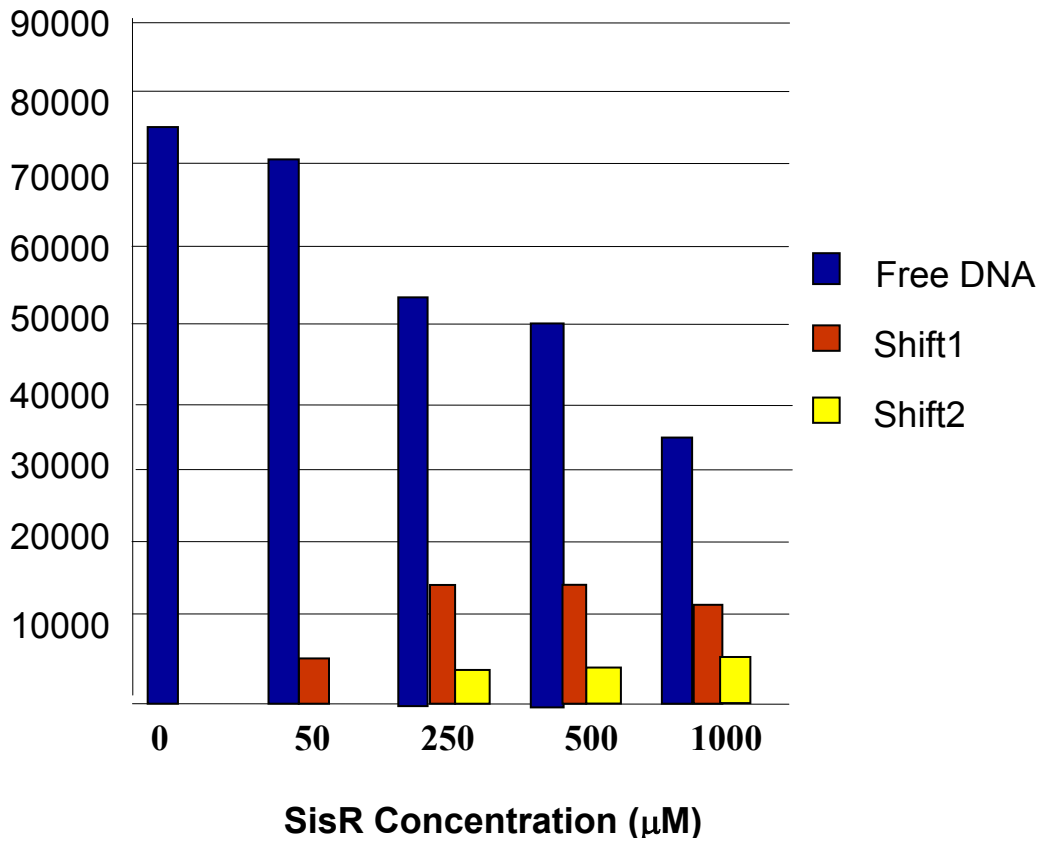
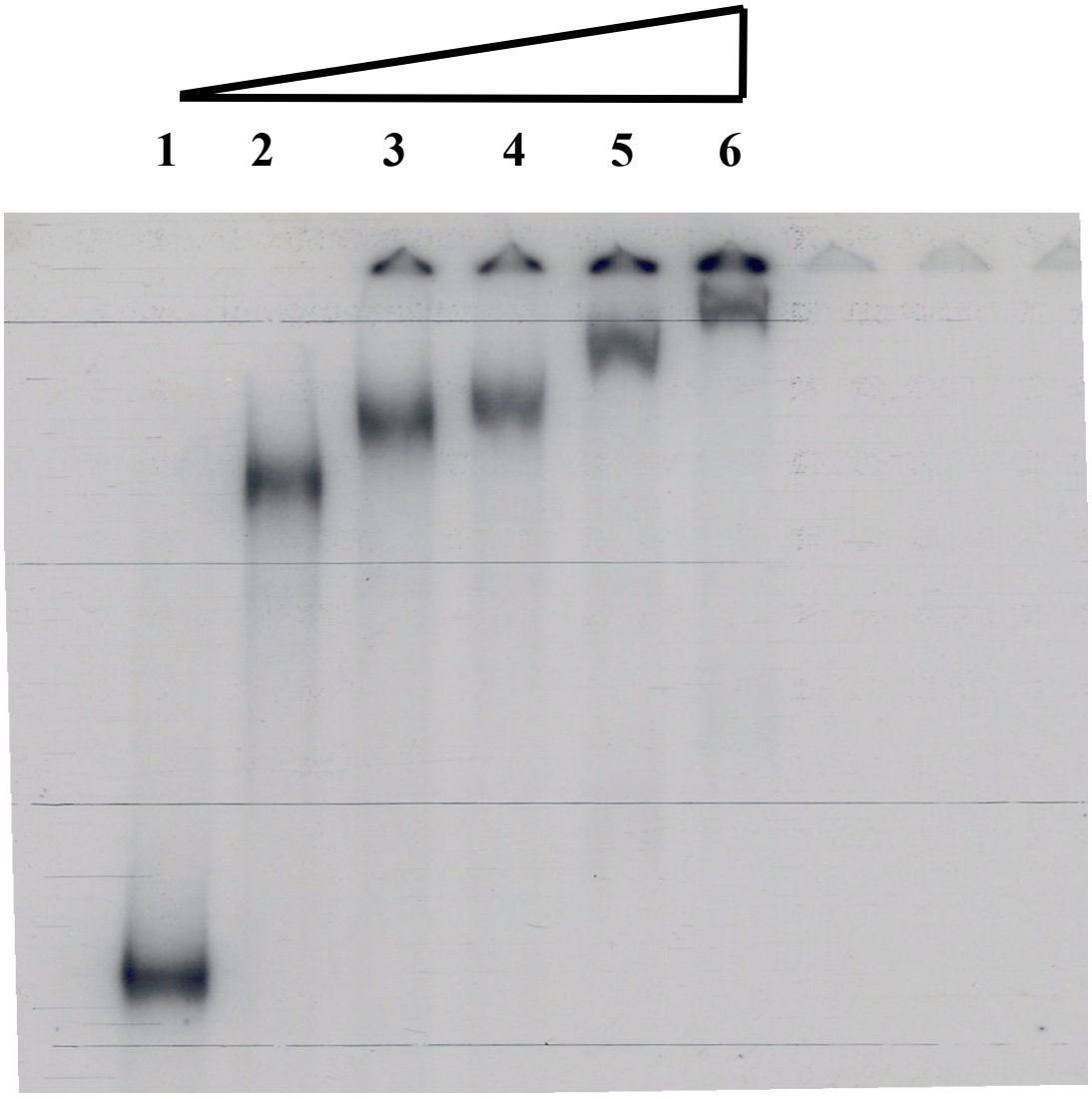

90000
80000
70000
60000
50000
40000
30000
20000
10000
0
50
250
500
1000
SisR Concentration (μM)
Free DNA
Shift1
Shift2

Figure 20. Binding to fu1 region of *E. coli* cell extract carrying pAPO1. Native SisR binding to ^{32}P-labelled fu1 probe was assessed at various cell extract volumes containing overexpressed pAPO1 ranging from 0 to 10 μl. Lane 1-10, 0, 2, 4, 6, 8 and 10 μl, respectively. Binding reactions were carried out for 3 minutes at 20°C in 50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 1 mM EDTA, 40 mM KCl, 4% glycerol, 1 mM β-mercaptoethanol, 0.05 mg/ml bovine serum albumin, and 0.50 mg/ml sonicated salmon sperm DNA (binding buffer).


1 2 3 4 5 6

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