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Environmental gene regulation by DNA supercoiling in Borrelia burgdorferi

Janet Alverson
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Environmental Gene Regulation by DNA Supercoiling in *Borrelia burgdorferi*

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The synthesis of the outer surface proteins OspC and OspA increased and decreased, respectively, when cultures of *Borrelia burgdorferi* were shifted from 23°C, which models the temperature of the tick vector, to 35°C, which approximates the mammalian host body temperature. The changes in protein synthesis correlated with changes in mRNA transcript levels showing that this regulation occurs at the gene level. This change in outer surface protein production is due to changes in DNA supercoiling with DNA from *B. burgdorferi* cultures grown at 35°C being less supercoiled than that of cultures grown at 23°C. OspC synthesis increased and OspA synthesis decreased when DNA supercoiling was artificially relaxed by treating cultures with coumermycin A1, a DNA gyrase inhibitor. In addition, OspC synthesis was higher in DNA gyrase mutants than wild type cells and DNA from the gyrase mutants was more relaxed. Promoter studies using a chloramphenicol acetyltransferase (CAT) reporter system in *Escherichia coli* and *B. burgdorferi* cells showed that *ospC* is regulated by DNA supercoiling in both bacteria while *ospA* is responsive to DNA supercoiling in *B. burgdorferi*, but not in *E. coli*. This suggests the presence of an *ospA* repressor in *B. burgdorferi*. These results, taken together, suggest that *B. burgdorferi* senses environmental changes in temperature by altering the level of DNA supercoiling, which then affects the program of gene expression. This implies that DNA supercoiling acts as a signal transducer for environmental regulation of outer surface protein synthesis.

The *gyrB* promoter was also shown to be homeostatically regulated by DNA supercoiling while other *B. burgdorferi* promoters were shown to be non-responsive to DNA supercoiling. The synthesis of heat shock protein, GroEL is up-regulated in *gyrB* mutants. This suggests that expression of *groEL* and *gyrB*, both genes carried on linear DNA molecules, respond to changes in DNA supercoiling *in vivo.*
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Chapter 1

Introduction

1.1 Lyme disease

Lyme disease is the most common arthropod-borne infection of humans in the United States (6, 101). It also affects other animals including dogs, horses, and cattle (1, 78).

_Borrelia burgdorferi_, a bacterium in the spirochete phylum, is the etiological agent of Lyme disease and is transmitted by the bite of an _Ixodes_ tick. The ecology of the tick vectors explains the seasonality and geographic distribution of the illness (83). _Ixodes_ ticks have a three-stage (larva, nymph, and adult), two-year life cycle (Fig. 1), and each stage takes one blood meal. The usual hosts of the larvae and nymphs in the United States are the white-footed mouse, other small mammals, and a variety of passerine birds, while the adult stage feeds primarily on deer and other larger mammals (2, 83). _Ixodes_ ticks hatch from eggs as uninfected larvae. Both larvae and nymphs can acquire infections from reservoir hosts and, after molting can transmit the infection to another host when they feed again. The passage of _B. burgdorferi_ between ticks and mice appears to be the primary means by which a high rate of infection is maintained in tick vectors in endemic areas (15), although experimental findings have shown that dogs can also serve as a reservoir (1). Migrating infected birds may be responsible for introducing the disease to a wider geographic area (2). Humans are most often infected by the bite of nympha-
stage ticks in the late spring or summer months when the nymphs are present in large numbers in endemic areas and people are more active outdoors (83).

Numerous clinical syndromes have been reported in humans and other animals, including a skin rash (erythema migrans), joint disease (arthralgias, arthritis), central nervous system manifestations (meningitis, radiculitis, cranial nerve palsies), cardiac abnormalities (atrioventricular block), and renal disease (1, 101). The tick must remain attached to its mammalian host for at least 36 hours in order to transmit the disease, which is an important point in terms of prevention of the disease. Lyme disease is usually easily treated with antibiotics in the early stages of the infection. If left untreated, however, it can progress to a chronic syndrome. There are also some cases of persistent symptoms of disease despite aggressive and prolonged antibiotic therapy against *B. burgdorferi* (103).

*B. burgdorferi* structurally resemble Gram-negative bacteria (54), with a trilaminar cytoplasmic membrane closely approximated to the cell wall. They can synthesize several different outer surface proteins (Osps), including OspA (31 kDa), OspB (34 kDa), OspC (23 kDa) (39, 115), and others. These are lipoproteins in which the lipid moiety is involved in their immunogenicity (13, 34). The Osps have been targeted as diagnostic and vaccine candidates and have been implicated in the pathogenesis of Lyme disease (42, 88, 89). Variation in expression of Osps may be an adaptation strategy by the spirochete to evade different immune mechanisms or to survive the different physiological changes between a tick and mammal.
1.2 Environmental adaptation of bacteria

Bacteria are able to sense changes in the environment and respond by changing the transcriptional program of the cell, with some genes being upregulated and other genes being downregulated (28). There are promoters that are stimulated by increased DNA supercoiling and others that are inhibited (27, 69). In vivo studies have shown that growth of cells in anaerobic conditions, at high osmolarity, different temperatures, in acidic pH, or in nutrient-poor medium can alter DNA supercoiling (4, 29, 43, 49, 52, 53, 56, 116).

Changes in osmolarity of the growth medium alter the linking number of intracellular DNA and induce the expression of at least one genetic locus, proU, in *Esherichia coli* and *Salmonella typhimurium*. DNA gyrase inhibitors and gyrA or gyrB mutations, which reduce negative supercoiling of DNA, nullify the osmotic induction of proU (49). Furthermore, mutations in topA, which encodes topoisomerase I, can mimic an increase in osmolarity, facilitating proU expression even in media of low osmolarity in which it is not normally expressed (49).

DNA topoisomerase I activity is reduced or absent in strict anaerobic *S. typhimurium* mutants, and DNA gyrase is reduced or absent in strict aerobic mutants (116). In these studies the chromosomal DNA of anaerobically grown wild type cells was supercoiled and that of aerobically grown wild type cells was more relaxed. The authors concluded that topoisomerase I activity, associated with the relaxation of DNA, is necessary for expression of genes required for aerobic growth, and gyrase activity, which introduces negative supercoiling in DNA, is necessary for expression of genes required for anaerobic growth (116). Another group found that tonB gene expression in *E. coli*
and *S. typhimurium* is repressed in anaerobic conditions when DNA is supercoiled, but the expression is increased by novobiocin, a DNA gyrase inhibitor (29).

Transcription of the *gyrA* and *gyrB* genes in *E. coli* is induced by coumermycin, an inhibitor of DNA gyrase, demonstrating that the homeostatic level of supercoiling is maintained by the level of DNA gyrase (20, 33, 37, 67-69). When the DNA encoding the *gyrA* and *gyrB* genes is relaxed, both genes are expressed at a high level; in negatively supercoiled DNA they are expressed at a low level.

Novobiocin and coumermycin have also been shown to decrease the transcription of several virulence factors in *Bordetella pertussis* (44). Inhibition of DNA gyrase led to a large decrease in transcription of the virulence regulatory *bvg* locus and several *bvg*-regulated virulence factors that code for proteins involved in adhesion to epithelial or immune cells and toxins. This led the authors to conclude that changes in DNA topology influenced the regulation of individual factors in the Bvg virulence regulon (44).

In *E. coli*, changes in DNA supercoiling have been seen during several types of nutrient changes, especially in starved cells. These variations in the availability of external nutrients are known to trigger changes in gene expression (4). *E. coli* adjusts its metabolism in response to shifts in the available nutrients. The results of this study implied that the level of superhelical energy in a cell is maintained at a constant level except during periods of certain growth transitions, when changes in metabolism and gene expression are accompanied by changes in DNA supercoiling (4).

Alkaline environments decrease the linking number of reporter plasmids in *S. typhimurium* (56). *B. burgdorferi* membrane protein profiles have been observed to change when cells are grown at different pH (17).
The effects of temperature on DNA supercoiling have been reported in several microorganisms. Increased temperature results in more negatively supercoiled DNA in *E. coli* (43) and *Yersinia enterocolitica* (86). In contrast, less negatively supercoiled, or more relaxed, DNA at higher temperatures occurs in *Haloferax volcanii* (72), *S. typhimurium* (30), *Bacillus subtilis* (45), and *Shigella flexneri* (30).

Temperature also affects DNA topology indirectly by influencing DNA bending (87). The *Y. enterocolitica* virulence plasmid, pYV, has been shown to contain multiple regions of intrinsic curvature that are detectable at 30°C, but melt at 37°C, the temperature at which the cells undergo phenotypic switching in protein secretion systems (87). The indirect effects of temperature are also seen in the access of DNA to small DNA-binding proteins (35). H-NS is one of the main nucleoid-associated proteins and controls the temperature-dependent transcriptional expression of the virulence genes of *Shigella* and *E. coli* by repressing the *in vivo* transcription of *virF* only below a critical temperature (32°C) (35).

Adapting to a changing environment is an obvious advantage for bacteria. The evolutionary success of a bacterium depends on an efficient system of environmental sensing and response, including coordination at the transcriptional level. Pathogenic bacteria, such as *B. burgdorferi*, may evade the host immune system or adapt to various environments, such as that found in a mammalian host or tick vector, by varying the synthesis of surface components (85). Previous studies have shown that the Osps are differentially synthesized in mammals and ticks. OspA is produced by the spirochete in the midgut of *Ixodes* ticks that have not yet fed on blood (8). Increased levels of OspC have been observed during tick feeding (97). In addition, an increase in OspC level
occurs when *B. burgdorferi* cultures are shifted from 23°C to 35°C, mimicking the transition from the tick to the mammal (73, 97, 104). The protein level increase followed an increase in steady-state mRNA level (108). Co-cultivation with tick cells enhances the temperature-induced differential synthesis of OspC by the spirochetes, although the mechanism has not yet been defined (76).

1.3 DNA supercoiling

DNA topology can be described by the equation:

\[ \Delta L_k = \Delta T_w + \Delta W_r \]

where \( T_w \) is the twist, which is equivalent to helical pitch, \( W_r \) is the writhe, which describes how the helix coils around itself, and \( L_k \) is the linking number, which is defined as the number of times two DNA strands in a closed-circular DNA molecule cross each other (10, 12). Linking numbers can be positive or negative depending on whether the DNA helix is overwound or underwound compared to a right-handed relaxed DNA molecule. Breaking and resealing of the phosphodiester backbone is the only way to change the linking number (10).

The genome of *B. burgdorferi* is unusual in that it consists of both linear and circular plasmids as well as a linear chromosome (5, 7, 9, 18, 24, 36, 50, 51, 55, 91, 100). The circular DNA of *B. burgdorferi* and other bacteria exists in a negatively supercoiled form. The DNA helix is underwound and writhes in three-dimensional space to relieve the torsional stress (10). Torsional stress can also be relieved when DNA binds to proteins that constrain the supercoils, or by strand separation within a segment of the DNA duplex (as in a transcription complex). Maintenance of negative supercoiling of
DNA is likely essential for intracellular homeostasis and various aspects of DNA-related processes such as replication, transcription, and recombination (10, 12, 31, 61, 80, 112).

The linear DNA of *B. burgdorferi* may also be under superhelical tension, but cannot be studied following release from the cell; perhaps the ends are tethered to the membrane or to a protein complex in the cell.

### 1.4 DNA gyrase and gyrase inhibitors

Supercoiling is dependent on the activities of DNA topoisomerases, enzymes that change the topology of DNA (31, 40, 63, 106, 112). Topoisomerases alter DNA supercoiling by catalyzing interconversions between different topological forms of DNA via single- or double-stranded breaks (113). All topoisomerases have the ability to relax negatively supercoiled DNA (113), but DNA gyrase, an ATP-dependent type II topoisomerase that is found only in prokaryotes, is the only topoisomerase capable of introducing negative supercoiling into closed-circular DNA (Fig. 2) (23, 41, 84, 113). DNA gyrase is an A$_2$B$_2$ heterotetramer. The A and B subunits are encoded by *gyrA* and *gyrB*, respectively (84). The A subunit binds DNA and the B subunit hydrolyzes ATP to drive the DNA supercoiling reaction (84). Negative supercoiling is relaxed by topoisomerase I. This enzyme relieves the torsional stress in the supercoiled DNA molecule, resulting in DNA relaxation.

Coumarin antibiotics, including coumermycin, bind to the B subunit of DNA gyrase, inhibiting its ATPase activity and thus the catalytic supercoiling activity of the holoenzyme, which leads to a decrease in the cellular level of DNA supercoiling (20, 33, 67, 82, 84). Coumermycin has been used as a model for studying resistance of *B. burgdorferi* to antibiotics and the role of DNA gyrase in cell growth and DNA topology.
However, coumermycin is not clinically useful for treatment of Lyme disease because of its insolubility, poor absorption, and high serum inactivation (67, 82). Our laboratory has isolated several *B. burgdorferi* coumermycin-resistant mutants (94, 96). In *E. coli*, coumermycin-resistant mutants have reduced DNA gyrase activity and relaxed DNA (22).

### 1.5 Heat shock proteins

Heat shock proteins (Hsps) are synthesized when cells are exposed to a variety of stresses including elevated temperatures (60, 74). In *E. coli*, genes encoding the major Hsps are expressed at a basal level at normal growth temperature, but are expressed at greater levels at higher temperatures (60, 74). Some Hsps have been shown to act as chaperones for the assembly of complex and oligomeric proteins (11, 48). *B. burgdorferi* synthesizes heat shock proteins with homology to *E. coli* DnaK, GroEL, and DnaJ (3, 38, 107, 110). Pathology to host tissues may be due in part to autoimmune response to *B. burgdorferi* heat shock proteins (62). The major Hsp of ~72 kDa, the DnaK homolog, is immunoreactive and is commonly seen in sera from Lyme disease patients (3). GroEL is a major Hsp of ~60 kDa, and was the first *B. burgdorferi* Hsp that was characterized at the molecular level (47). A previous study found that after heat treatment, DnaK and GroEL were synthesized continuously in *gyrA* mutants of *E. coli*, but only transiently in wild type cells (71). This was found to be due to relaxation of supercoiled DNA. Therefore, Hsp genes are also regulated by environmentally-induced changes in DNA supercoiling.
1.6 Specific Aims

We characterized the effect of temperature, an environmental factor, on DNA supercoiling and the effect of DNA supercoiling on the expression of several \textit{B. burgdorferi} genes. We accomplished the following specific aims:

1. Compare the effects of a temperature shift from $23^\circ$C, the temperature of ticks, to $35^\circ$C, mammalian body temperature, on DNA supercoiling, OspA and OspC protein synthesis, and \textit{ospA} and \textit{ospC} gene expression.

2. Examine the pharmacological effect of coumermycin, a gyrase inhibitor, on DNA supercoiling, OspA and OspC protein synthesis, and \textit{ospA} and \textit{ospC} gene expression.

3. Perturb DNA supercoiling using a genetic approach. Thirty coumermycin-resistant \textit{gyrB} mutants of \textit{B. burgdorferi} were screened for OspC synthesis and compared to thirty wild type clones to determine if a genetic correlation exists. DNA supercoiling was also examined using a small reporter plasmid.

4. Explore the promoter activity of several \textit{B. burgdorferi} genes in response to supercoiling changes with a chloramphenicol acetyltransferase (CAT) reporter expression system in both \textit{E. coli} and \textit{B. burgdorferi}.
Fig. 1. Life cycle of Ixodid ticks in the United States. The white-footed mouse is the principal host for deer tick larvae, which feed in late summer, and for the nymphs, which feed in late spring and early summer. Most Lyme disease in humans is acquired by the bites of the small nymphal ticks (83).
Fig. 2. **Mechanism of DNA supercoiling by DNA gyrase.** Introduction of supercoiling in a circular DNA molecule by action of DNA gyrase, which makes double strand breaks. First, the circular DNA molecule is twisted, then a break occurs where the two chains come together (arrow), and then the broken double helix is resealed on the opposite side of the intact strand (64).
Chapter 2

Methods

2.1 Chapter 3 methods

Bacteria. B. burgdorferi low-passage strain HB19 is an isolate from the blood of a patient with early Lyme disease in Connecticut (102). Cultures (100 ml) were grown at 23°C in BSK-H complete medium (Sigma) until the culture reached mid-log phase (approximately 5 to 6 weeks). The culture was then passaged 1:100 into 100 ml of fresh medium and grown to mid-log phase at 35°C (approximately 10 to 14 days).

Northern Blot. Total RNA was isolated from 100 ml cultures using 10 ml TRIzol™ reagent as described by the manufacturer (Gibco BRL). All solutions and buffers made for RNA gels and Northern blots were treated with diethyl pyrocarbonate (DEPC) to inhibit RNase activity. Fifteen µg of total RNA in 50% formamide, 1X MOPS (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7.0), and 2.2% formaldehyde was heated at 55°C for 15 min. RNA samples were at a final volume of 28 µl, to which 1 µl of 0.5 mg ml⁻¹ EtBr and 1 µl of loading buffer (50% glycerol, 1 mM EDTA, 0.25% bromphenol blue, 0.25% xylene cyanol) were added. RNA was fractionated on 1.2% formaldehyde-agarose gels (1.2% SeaKem agarose, 1X MOPS, and 1.1% formaldehyde) in 1X MOPS running buffer at 70 V. Following electrophoresis, the gel was rinsed with two changes of H₂O for 5 min each. RNA was transferred to nylon membranes (Hybond N⁺, Amersham) using a vacuum blotter with H₂O for 10 min, denaturation buffer (50 mM NaOH, 10 mM NaCl) for 10 min, neutralization buffer (0.5
M Tris-HCl, pH 7.5, 1.5 M NaCl, 1 mM EDTA) for 10 min, and 20X SSC (1X SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) for 3 h. Membranes were UV crosslinked using a Stratalinker (Stratagene). Membranes were dried and rehydrated in 6X SSC. Hybridization with a PCR probe was performed as previously described (14, 65). The probe for the ospC locus was generated by two rounds of PCR amplification from total HB19 DNA with oligonucleotides ospC 1F and ospC 300R (Table 1) (GenBank accession number X69596) as described previously (95). The probe represents the first 300 base pairs of the ospC open reading frame and was labeled with [α-32P]-dATP using a random priming labeling kit (Prime-It II, Stratagene). The probes for the ospA and flaA loci were made the same way as the probe for the ospC locus except using oligonucleotides ospA 86F and ospA 437R (Genbank accession number AE000790), or flaA 67F and flaA 469R (Genbank accession number AE001168) respectively (Table 1).

Membranes were incubated for one hour in prehybridization solution (5X SSC, 1% SDS, 5X Denhardt’s, 50% formamide, and 100 µl salmon sperm DNA). Probes were incubated with membranes in hybridization buffer (fresh prehybridization buffer without the salmon sperm) overnight at 42°C. Membranes were washed twice with 2X SSC-0.1% SDS for 10 min each at 24°C, washed once with 0.2X SSC-0.1% SDS for 1 h at 42°C, wrapped in Saran wrap, and exposed to radiographic film overnight. Band intensity was determined using an Image Acquisition and Analysis system (Ambis), kindly provided by Dr. Bill Holben (The University of Montana). Blots were stripped in preparation for reprobing as described (14); blots were washed in 1 % SDS, 0.1X SSC, 40 mM Tris-HCl pH 7.5 four times for 5 min each at 80°C.
**SDS-PAGE and immunoblotting.** *B. burgdorferi* protein extracts were prepared by pelleting 10 ml cultures, washing twice with PBS (138 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4), obtaining the OD_600 in 1 ml PBS, resuspending in 200 μl H_2O per OD_600, adding an equal volume of 2x Laemmli buffer, and boiling for 5 min. Protein extracts from 3.5 × 10^7 cells were separated by electrophoresis through 12.5% SDS-polyacrylamide gels and visualized by staining with Coomassie brilliant blue. Resolved proteins were also transferred to a PVDF membrane (Immobilon P, Millipore) using a Trans-Blot cell (Bio-Rad) following the instructions of the manufacturer. The OspA antigenic protein was detected using The Immun-Star chemiluminescent detection system and following the instructions of the manufacturer (Bio-Rad). Briefly, membranes were blocked for 30 min at room temperature with 0.2% non-fat dry milk / 20 mM Tris, 500 mM NaCl, pH 7.5 (TBS) and then washed for 10 min with TBS / 0.1% Tween-20 (TTBS). The membranes were then incubated overnight at 4°C with monoclonal mouse anti-OspA antibody 1:100 in 0.2% non-fat dry milk / TTBS. The membrane was rinsed twice for 10 min each with TTBS. Alkaline phosphatase-linked goat anti-mouse IgG (Bio-Rad) was diluted 1:3000 in 0.2% non-fat dry milk / TTBS and incubated for 1.5 h at room temperature. The membrane was washed three times for 10 min each with TTBS and then developed in 5 ml of the substrate solution and exposed to autoradiographic film at room temperature for 3 min. The OspC antigenic protein was detected as follows. The membranes were blocked for 1 h at room temperature with 2% fetal calf serum / PBS / 0.05% Tween 20 and subsequently incubated overnight at 4°C with polyclonal rabbit anti-OspC antibody (98) 1:500 in the blocking solution. The membrane was rinsed five times for 5 min each with PBS / 0.05% Tween 20. Horseradish
peroxidase-linked goat anti-rabbit IgG (Bio-Rad) was diluted 1:50 in PBS / 0.05% Tween 20 and incubated for 1.5 h at room temperature. The membrane was washed three times for 5 min each with PBS / 0.05% Tween 20, ten times for 3 min each in deionized water, and finally once for 5 min in PBS. The membrane was then developed in 50 ml 0.6 mg ml$^{-1}$ 4-chloro-1-napthol (in 20% methanol / PBS) with 20 μl 30% H$_2$O$_2$. The rabbit anti-OspC antiserum and mouse monoclonal OspA antibody were generously provided by Dr. Tom Schwan (Rocky Mountain Laboratories, Hamilton, MT).

**DNA isolation and hybridization probe.** DNA was extracted from 10 ml of an HB19 culture grown at 23°C and 35°C as described previously (93). Bacteria from 10 ml cultures were washed with 1 ml of PBS and resuspended in 0.2 ml TES (50 mM Tris-HCl, pH 8.0, 50 mM EDTA, 15% sucrose). The cells were lysed by adding 20 μl of 10% SDS and incubated at 37°C for 1 h after 3 μl of 20 mg ml$^{-1}$ proteinase K solution was added. The lysate was extracted once with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1). It was then extracted once with an equal volume of chloroform-isoamyl alcohol (24:1). Nucleic acid was precipitated by adding 9 μl of 5 M NaCl and 0.4 ml of ethanol and incubating at −20°C for at least 30 min. The PCR probe for the *ospC* locus was made as described above for the Northern blot.

**Two-dimensional gel electrophoresis and Southern blot.** Two-dimensional gel electrophoresis to resolve circular topoisomers (79) was performed by a modification of a previously published protocol (93). Total chromosomal HB19 DNA was fractionated on a 0.35% agarose gel in TAE (40 mM Tris, 20 mM acetate, 1 mM EDTA) with 1.5 μM chloroquine at 20 V for 36 h. The gel was then rotated 90° and equilibrated with an additional 3.5 μM chloroquine (5 μM final concentration) for 5 h. The gel was then run
in the second dimension for 36 h at 20 V. The gel was rinsed in three changes of water (1 h each) to remove the chloroquine before being stained with ethidium bromide for 45 min. The DNA was transferred to a nylon membrane by vacuum blotting for 1 h, cross-linked by ultraviolet light, and air-dried. The membrane was rehydrated in 6X SSC and incubated in prehybridization solution (6X SSC, 0.5 % SDS, 5X Denhardt’s) for 1 h. The membrane was then hybridized overnight at 68°C with the 300 bp ospC probe as described previously (95). The blots were washed twice for 15 min at 24°C in 2X SSC-0.1% SDS, once for 30 min at 65°C in 0.2X SSC-0.1% SDS, and exposed to Hyperfilm (Amersham).

**Transformation of B. burgdorferi.** Competent cells were prepared by a previously published protocol (92). One-hundred ml cultures of each strain were grown to a density of about $5 \times 10^7$ cells ml$^{-1}$. The cells were harvested by centrifugation at 4000 $\times$ g for 20 min at 4°C. Pellets were washed twice in 30 ml cold dPBS and centrifuged at 3000 $\times$ g for 10 min at 4°C. Pellets were then washed three times in 10 ml cold electroporation solution (EPS: 93 g L$^{-1}$ sucrose, 15% [v/v] glycerol) and centrifuged at 2000 $\times$ g for 10 min at 4°C. The cell pellets were then resuspended in 0.15 ml of cold EPS and distributed in 50 µl aliquots in sterile 1.7 ml tubes for use. Plasmids were transformed into B. burgdorferi cells by electroporation using a previously published protocol (92). Three µl of plasmid DNA was transferred to a tube of fresh competent cells, mixed gently, and incubated on ice for 1 min. The cell/DNA mixture was then transferred to a chilled electroporation cuvette (0.2 cm) and pulsed with a single exponential decay pulse of 2.5 kV. Cultures were allowed to recover for 20 h in 10 ml BSK at 34°C.
**One-dimensional gel electrophoresis.** Plasmid DNA was purified using an alkaline lysis plasmid miniprep kit following the directions of the manufacturer (Promega Wizard®). The DNA was resuspended in 30 μl H₂O and stored at -20°C.

Plasmid DNA was fractionated on a 0.8% agarose gel in TAE for 4 h at 50 V with 0 or 15 μM chloroquine added to the gel and to the running buffer. The gel was rinsed in three changes of water (1 h each) to remove the chloroquine before being stained with ethidium bromide for 45 min.

### 2.1 Chapter 4 methods

**Antibiotics.** Coumermycin A₁ (referred to as coumermycin; Sigma) at 50 mg ml⁻¹ in dimethyl sulfoxide (DMSO), ciprofloxacin (Sigma) at 25 mg ml⁻¹ in H₂O, and kanamycin (Sigma) at 25 mg ml⁻¹ in H₂O were stored at -20°C. The effect of coumermycin on bacterial growth was assayed as previously described (93).

**Bacteria.** The gyrB mutants are B31 clones selected in 0.1 μg ml⁻¹ coumermycin; they contain various substitutions of conserved residue Arg-133 (67, 94, 95). Each mutant is an independent clone based on their gyrB DNA sequence. CR9C is a gyrB mutant described previously (95) with an Arg 133 to Ile mutation.

**SDS-PAGE and immunoblotting.** SDS-PAGE and immunoblotting were performed as described above for the OspC protein except the second incubation was done with peroxidase-linked *Staphylococcus aureus* Protein A (ICN Biochemicals) diluted 1:20,000 in PBS/0.05% Tween 20 and incubated for 1.5 h at room temperature.
2.2 Chapter 5 methods

**Bacteria.** *E. coli* strains N99 and N4177 were kindly provided by Drs. Marty Gellert and Mary O' DEA (National Institutes of Health, Bethesda, MD). N99 and N4177 were first described by Menzel and Gellert (70). N99 carries a wild type gyrB, and strain N4177 is isogenic except for two gyrB mutations, gyrB221 (Cou') and gyrB203 (TS), which together confer a coumermycin-resistant and temperature-sensitive phenotype (22, 70).

**Plasmids.** Plasmids containing *B. burgdorferi* promoters for *ospA* (pGOΔ5) and *ospC* (pGOΔ15) cloned upstream of a promoterless *Streptococcus agalactiae cat* gene were kindly provided by Chuck Sohaskey (Veterans Administration Medical Center, Long Beach, CA) (99) (Table 2). Promoter fusions of gac, gac.2, and gyrB cloned into a promoterless pGOΔ1 were constructed by Scott Knight and Corbin Schwanke (58) (Table 2). We transformed these plasmids along with a control promoterless plasmid (pGOΔ1) into *E. coli* DH5α cells using a calcium chloride transformation protocol (46). Selection and maintenance of transformants with these plasmids were carried out on Luria-Bertani (LB) agar with 10 μg ml⁻¹ chloramphenicol.

**MIC.** For an *in vivo* analysis of promoter activity, the minimum inhibitory concentrations (MICs) of chloramphenicol for *E. coli* with and without the plasmids were determined. LB agar with chloramphenicol at 0, 5, 10, 20, 40, 80, 160, 320, and 640 μg ml⁻¹ was used for agar plate dilutions. Cell densities of overnight *E. coli* transformant cultures were determined by spectrophotometry. The cultures were diluted with LB broth, and an equal amount (A₆₆₀ = 0.071) of each culture was placed onto the plates in 10 μl aliquots. The plates were incubated at 37°C for 16 hours. The MIC was defined as...
the lowest concentration of chloramphenicol that prevented visible growth of the bacteria on the plate.

**CAT ELISA.** *E. coli* DH5α cells were transformed as described above. *B. burgdorferi* HB19 cells were transformed by electroporation as described in section 2.1 above (92). Plasmids were introduced into *E. coli* strains N99 and N4177 by modifying a previously published transformation protocol (21) such that the culture temperature of N4177 was held below 30°C throughout the transformation procedure. N99 was transformed at 37°C. An ELISA assay to measure the level of CAT in cell extracts from *E. coli* carrying each plasmid has been developed using a kit from Boehringer Mannheim. Modifications in the kit protocol were made to adapt the assay for a prokaryotic system: *E. coli* cells from 5 ml cultures were grown to an *A*₂₆₀₀ of 0.6 and pelleted by centrifugation. *B. burgdorferi* cells were pelleted when they reached mid-log phase for the 23°C and 35°C cultures, and after 72 hours for the cultures with coumermycin added. The cell pellet was washed with 1 ml of PBS (138 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄) and resuspended in TES (50 mM Tris-HCl pH 8, 50 mM EDTA, 15% sucrose). The suspension was then sonicated and Cell Lysis solution from the ELISA kit added and incubated at 23°C for 30 min. The CAT ELISAs were read on a microtiter plate reader (Molecular Devices), kindly provided by Dr. Jack Nunberg (The University of Montana). The results from the CAT activity assay were normalized with respect to protein concentration (Pierce BCA Protein Assay) by dividing the CAT activity in ng ml⁻¹ by the total protein concentration in µg ml⁻¹.

**Protein Assay.** Pierce bicinchoninic (BCA) protein assay was used to measure the protein concentration of the cell lysates following the instructions provided by the
manufacturer. Protein standards were made using the Albumin Standard supplied with the kit, diluted in the Cell Lysis solution from the CAT ELISA kit for final albumin concentrations of 200 to 1200 μg ml⁻¹. One hundred μl of each sample or protein standard was placed in a test tube and 2 ml of Working Reagent from the kit was added. One hundred μl of the Cell Lysis solution with 2 ml of Working Reagent added was used as a blank. The tubes were incubated for 2 h at 23°C and then the absorbance was read on a spectrophotometer at 562 nm. The absorbance of the blank was automatically subtracted from the values of the standards and the unknowns. A standard curve was prepared by plotting the net (blank corrected) absorbance at 562 nm vs. protein concentration. The protein concentration was determined for the unknown samples using this standard curve.

2.3 Chapter 6 methods

**GroEL purification.** A crude lysate of a low-passage coumermycin-resistant gyrB mutant of *B. burgdorferi* strain B31 was prepared from a 1.5 L culture grown at 32°C in BSK-H medium (Sigma) as previously described (66) with the following modifications. Cells from a 1.5 L culture (in three 500 ml bottles) were collected at 10,500 × g for 20 min in a GSA rotor. The cell pellet was washed twice in 30 ml of Dulbecco’s phosphate-buffered saline (dPBS). Cells were collected in a SS-34 rotor at 7500 × g for 10 min after the first wash and at 6000 × g after the second wash. Cells were resuspended in 1.5 ml of TS (50 mM Tris-HCl, pH 8.0, 15% sucrose) and stored at −80°C. Four 1.5 ml aliquots were thawed at 37°C and DTT (final concentration = 2 mM), EDTA (final concentration = 1 mM), and PMSF (final concentration = 0.5 mM) were added to each aliquot. The cells were then lysed by sonication (eight 15 s pulses for
each of the four aliquots. Nucleic acid was precipitated by slowly adding 1/5 the volume of 1 M KCl and 2/5 the volume of 5% streptomycin sulfate (pH 7.2 with NH₄HCO₃) followed by rotation at 4°C for 10 min. The lysate was clarified first by centrifugation at 7,500 x g for 10 min in an SS34 rotor and then by ultracentrifugation at 435,000 x g for 30 min in a TLA-100.2. The clarified lysate was dialysed overnight at 4°C against 50 mM Tris-HCl, pH 7.5 (the pH of Tris solutions was measured at 25°C), 10% glycerol, 1 mM EDTA, 5 mM DTT (A buffer), and loaded onto a 5 ml Econo-Pac Heparin Cartridge (Bio-Rad). The column was eluted with a 60 ml linear gradient from 0-0.6 M NaCl in A buffer at 2 ml min⁻¹ and thirty 2-ml fractions were collected. The 70 kDa protein of interest was in the flow-through from the column, which was combined with an equal volume of 3.4 M (NH₄)₂SO₄, 50 mM Tris pH 7.5, 1 mM EDTA, 5 mM DTT and loaded onto a 1 ml Phenyl Superose column (Pharmacia). The column was eluted with a 20 ml linear gradient from 1.7-0 M (NH₄)₂SO₄ in P buffer (50 mM Tris pH 7.5, 1 mM EDTA, 5 mM DTT) at 0.3 ml min⁻¹ and twenty 1-ml fractions were collected. Fractions containing the 70 kDa protein (which eluted at ~0.85 M (NH₄)₂SO₄) were dialysed against A buffer overnight at 4°C and loaded onto a 1 ml Mono-Q column (Pharmacia). The column was eluted with a 20 ml linear gradient from 0-1 M NaCl in A buffer and twenty 1-ml fractions were collected. The fractions containing the 70 kDa protein (which eluted at ~0.5 M NaCl) were concentrated using a Centricon 10 concentrator (Amicon) in a SS-34 rotor for 60 min. An equal volume of SDS-PAGE loading buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 1.4 M 2-mercaptoethanol, 0.2% bromphenol blue) was added to the concentrated fractions which were then boiled for 5 min. SDS-PAGE gels were run with the sample and transblotted to PVDF membranes as previously described.
in Chapter 2.1. The 70 kDa bands were excised, wet with methanol, stored in 1 ml dH2O, 2 mM DTT at 4°C, and given to Joan Strange (Murdock Molecular Biology Facility, The University of Montana) for N-terminal sequencing (Applied Biosystems 373A DNA Sequencer).

**Heat-shock of cells.** B31 wild type cultures were heat-shocked by incubating cells at 42°C for 1 h before harvest for protein analysis or RNA isolation.

**Hybridization probe.** PCR probes for the *groEL* locus were made as previously described for the *ospC*, *ospA*, and *flaA* loci except oligonucleotides *groEL* 750F and *groEL* 1122R (Table 1; GenBank accession number AE001166) were used.

**SDS-PAGE and immunoblotting.** SDS-PAGE and immunoblotting were performed as previously described for the OspA antigen except monoclonal mouse anti-GroEL antibody (62), kindly provided by Drs. Barbara Johnson and Christian Rittner (Center for Disease Control, Fort Collins, CO) was used for antigenic detection.

**One-dimensional gel electrophoresis.** Competent cells of the coumermycin-resistant *gyrB* mutant used in the above GroEL purification and wild type B31 were made and transformed using the same protocols cited in section 2.1 above. One-dimensional agarose gels in the presence or absence of chloroquine were also run using the same protocols as in section 2.1 above.
Table 1. Oligonucleotides

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<tr>
<td>ospC 300F</td>
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</tr>
<tr>
<td>ospA 86F</td>
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</tr>
<tr>
<td>ospA 437R</td>
<td>TCAAGTCTGGTCCGTCTGCTC</td>
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<tr>
<td>flaA 67F</td>
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</tr>
<tr>
<td>flaA 469R</td>
<td>ATTAGATGGCCACCTTCGCCCTG</td>
</tr>
<tr>
<td>groEL J750F</td>
<td>TGAGGATATTGAGGGGATGCG</td>
</tr>
<tr>
<td>groEL 1122R</td>
<td>AACTCCGCAACAAGTTTTGC</td>
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Table 2. Plasmids

<table>
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<th>Function</th>
<th>Reference</th>
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<td>(93)</td>
</tr>
<tr>
<td></td>
<td>gene</td>
<td></td>
</tr>
<tr>
<td>pGOΔ5</td>
<td>ospA promoter</td>
<td>(93)</td>
</tr>
<tr>
<td>pGOΔ15</td>
<td>ospC promoter</td>
<td>(93)</td>
</tr>
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<td>pGOΔGac</td>
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Chapter 3

Temperature shift affects gene expression and relaxes DNA supercoiling

Ticks become infected with spirochetes by feeding on infected animals. Spirochetes enter and multiply in the tick midgut until the tick takes its next blood meal, usually during the tick's next life stage. The spirochetes then move into the tick salivary glands and are injected into the mammalian host (15). The spirochete rapidly changes production of its outer surface proteins during this process. In the time between the blood meal and the entry of the spirochete into its new host, OspA production is down-regulated while production of OspC is up-regulated (25, 73, 97). *B. burgdorferi* OspC synthesis has been shown to increase in infected ticks after they have ingested a blood meal (97). One of the major environmental signals for this physiological change is temperature, since OspC levels also increase when *B. burgdorferi* cultures are shifted to an increased growth temperature, similar to that of a mammalian host (73, 97, 104). We confirmed this finding in strain HB19. SDS-PAGE gels and immunoblots using anti-OspC antiserum were performed on protein extracts from cultures grown at 23°C and then shifted to 35°C (Fig. 3). We also performed SDS-PAGE gels and immunoblots using anti-OspA antibody and showed that OspA levels decrease when cultures are shifted from 23°C to 35°C (data not shown).

We performed Northern blots to determine if this increase in protein synthesis was due to an increase in transcription of the gene. Previous studies have shown that
ospC mRNA levels increase when *B. burgdorferi* cultures are shifted to a higher growth temperature (108). A 16-fold (± 1 standard error of the mean, SEM) increase in *ospC* steady-state mRNA levels occurs in strain HB19 upon temperature shift from 23°C to 35°C (Fig. 4), confirming that regulation is at the level of gene expression. This study is the first quantification of the increase in *ospC* mRNA levels. The mRNA levels for *ospA* decreased by 1.6-fold (± 0.2 SEM) (Fig. 5).

Temperature regulation of virulence gene expression in other systems has been correlated with temperature-induced changes in supercoiling of circular DNA molecules (27, 28, 44, 45, 86). *ospC* was the first gene mapped to a circular DNA molecule, cp26, in *B. burgdorferi* (65, 90). Circular DNA is topologically constrained and exists in different forms, called topoisomers. More highly supercoiled topoisomers migrate at faster rates during agarose gel electrophoresis than nicked circular DNA or relaxed DNA due to the compact nature of supercoiled molecules (10, 12, 57, 79). To determine if DNA supercoiling transitions are associated with temperature changes in *B. burgdorferi*, we used two-dimensional agarose gel electrophoresis and Southern blotting to measure differences in the topoisomer distribution of cp26 (Fig. 6). Different concentrations of chloroquine, which intercalates into DNA and decreases negative DNA supercoiling, are utilized to separate topoisomers. At non-saturating amounts of chloroquine, positively supercoiled DNA is accelerated and negatively supercoiled DNA is retarded.

Resolving individual topoisomers of large DNA molecules such as cp26 is difficult due to the relatively small differences in migration rate of the topoisomers. Furthermore, one-dimensional gels, which are usually used to assay the DNA supercoiling of small reporter plasmids, are not sensitive enough to discern differences in
the DNA supercoiling of large plasmids. During two-dimensional gel electrophoresis, a small concentration of chloroquine in the first dimension retards migration of circular plasmids and the increased concentration of chloroquine in the second dimension further retards their migration. Circular plasmid topoisomers that are more negatively supercoiled require larger concentrations of chloroquine to effect a similar reduction in migration during electrophoresis (79). Therefore, in a two-dimensional gel, the entire population of topoisomers produces an arc with the most supercoiled molecules migrating the fastest in both dimensions. DNA isolated from an HB19 culture grown at 23°C was more negatively supercoiled than that grown at 35°C, as evidenced by the differential distribution of topoisomers along the arc (the topoisomers from the 23°C culture are migrating faster toward the bottom right) (Fig. 6).

Due to the difficulty in resolving topoisomers of the large cp26 plasmid, a small (4 kb) plasmid, pGOΔ15 (also used for the promoter analysis in chapter 5), which is transiently maintained in *B. burgdorferi*, was assayed on a one-dimensional agarose gel. Plasmids isolated from HB19 cultures grown at 23°C and 35°C were resolved by gel electrophoresis in the presence and absence of chloroquine. More negatively supercoiled molecules migrate faster through the gel. The plasmids from cultures grown at the higher temperature were relaxed (Fig. 7). Chloroquine intercalates into the plasmid DNA introducing positive supercoiling that retards migration of negatively supercoiled DNA and expedites migration of relaxed DNA. Chloroquine retarded the migration of the supercoiled topoisomers from the cells grown at 23°C, and expedited the migration of the relaxed DNA from the cells grown at 35°C (Fig. 7).
Fig. 3. OspC immunoblot of HB19 after a temperature shift. Effect of temperature shift on OspC synthesis. A culture of *B. burgdorferi* HB19 was grown at 23°C and then passaged at 35°C. Cell lysates were resolved by 12.5% SDS-PAGE, blotted to a PVDF membrane, and probed with an anti-OspC antiserum.
Fig. 4. Northern blot analysis of *ospC* after a temperature shift. Effect of temperature shift on *ospC* expression. A culture of *B. burgdorferi* HB19 was grown at 23°C and then passaged at 35°C. Equal amounts of RNA (15 μg) were separated on a formaldehyde-agarose gel, blotted to a nylon membrane, and hybridized with an *ospC* probe. Although the hybridization signal from the 23°C culture is not detected in this exposure, *ospC* RNA levels in the 35°C culture are 16-fold greater (± 1 SEM from three independent experiments) as directly quantified by an Image Acquisition and Analysis System.
Fig. 5. Northern blot analysis of ospA after a temperature shift. Effect of temperature shift on ospA expression. A culture of *B. burgdorferi* HB19 was grown at 23°C and then passaged at 35°C. Equal amounts of RNA (15 μg) were separated on a formaldehyde-agarose gel, blotted to a nylon membrane, and hybridized with an ospA probe. ospA RNA levels in the 23°C culture are 1.6-fold greater (± 0.2 SEM from three independent experiments) as directly quantified by an Image Acquisition and Analysis System.
Fig. 6.

A

23°C  35°C

First dimension
(+ 1.5 μM chloroquine)

Second dimension
(+ 5 μM chloroquine)

B

Fig. 6.
Fig. 6. Effect of a temperature shift on the topology of circular plasmid cp26.

(A) The topology of DNA from *B. burgdorferi* HB19 cultures grown at 23°C and after a shift from 23°C to 35°C was determined by two-dimensional 0.35% agarose gel electrophoresis and Southern blotting with an *ospC* probe. (B) Schematic representation of the Southern blot. The covalently closed supercoiled topoisomers (sc) of cp26 form an arc, with more negatively supercoiled molecules migrating faster in both the first and second dimensions. Also visible are nicked open circular (n) and linearized (l) forms of cp26 that migrate along the diagonal (dotted line).
Fig. 7. Effect of a temperature shift on DNA topology. Plasmid pGOΔ15 was transiently introduced into *B. burgdorferi* HB19 cultures and grown at either 23°C or 35°C. The topology of the plasmid was determined by one-dimensional 0.8% agarose gel electrophoresis in the absence (native) or presence of 15 μM chloroquine (CQ). More negatively supercoiled molecules migrate faster through the gel; chloroquine intercalates into the plasmid DNA introducing positive supercoiling that retards migration of negatively supercoiled DNA (sc) and expedites migration of relaxed DNA (r). The migration of the linearized (l) form of the plasmid is not affected by chloroquine. Molecular size standards are indicated in kilobase pairs (kb).
Chapter 4

The effect of pharmacological and genetic manipulation of DNA supercoiling on gene expression and protein synthesis

Effect of coumermycin-induced relaxation of DNA supercoiling on OspC synthesis. The coumarin group of antibiotics binds to the B subunit of DNA gyrase and inhibits DNA supercoiling by blocking its ATPase activity (33, 67). This group of antibiotics includes novobiocin, coumermycin, and chlorbiocin. They are structurally related and are produced by *Streptomyces* species. Coumermycin inhibits DNA gyrase and induces relaxation of negatively supercoiled circular plasmids in *B. burgdorferi* (86) and other bacteria (22, 33, 67, 84, 93).

The IC$_{50}$ of coumermycin in HB19 is 14 ng ml$^{-1}$ (Fig. 8). We performed dose-response experiments by treating *B. burgdorferi* strain HB19 with increasing concentrations of coumermycin (0.2 ng ml$^{-1}$ to 20 ng ml$^{-1}$), which allow for at least some bacterial growth. In fact, low concentrations of coumermycin (0.2 ng ml$^{-1}$ to 0.5 ng ml$^{-1}$) slightly stimulate growth (Fig. 8). Coumermycin treatment induced OspC synthesis in the absence of a temperature shift (Fig. 9). The synthesis of other proteins also changed in response to coumermycin treatment (Fig. 9). Most notable of the change in protein synthesis is the decrease in a 31 kDa protein. An immunoblot with an anti-OspA antibody showed that the 31 kDa protein, whose synthesis decreased with increasing concentrations of coumermycin (Fig. 10), was the outer surface protein OspA. This is consistent with the coordinate regulation of OspA and OspC synthesis observed by others.
Control experiments were carried out using analogous subinhibitory concentrations of two different antibiotics that do not affect DNA supercoiling, kanamycin and ciprofloxacin. These antibiotics had no effect on OspC synthesis (data not shown). Therefore, OspC and OspA synthesis is altered by decreased DNA supercoiling and not merely in response to antibiotic treatment.

To investigate the kinetics of supercoiling-induced gene expression, a time course was performed by treating HB19 cells with a high dose of coumermycin (1 μg ml⁻¹) and harvesting cells for protein analysis 0, 8, 16, and 32 h after antibiotic addition. The immunoblot for OspC shows the increase in protein synthesis after just 8 h (Fig. 11). A time course was also performed to determine when the ospC (Fig. 12) and ospA (Fig. 13) genes are producing increased and decreased steady-state levels of mRNA transcripts, respectively. The ospC transcript increased 2.5-fold 2 h after the addition of antibiotic, and 16-fold after 32 h. The ospA transcript decreased by 31% within 2 h after the addition of coumermycin, and 77% after 32 h. A previous study found that coumermycin induced the relaxation of negatively supercoiled plasmids within 2 h (93).

Dose-response Northern blot experiments were performed to study the relationship between coumermycin concentration and gene expression. Membranes were probed with ospC and ospA after adding 0, 10, and 20 ng ml⁻¹ coumermycin. The ospC transcript increased 11-fold at the highest antibiotic dose (Fig. 14). The ospA transcript decreased by 62% after adding 20 ng ml⁻¹ coumermycin (Fig. 15).

Controls for the coumermycin time course and temperature shift Northern blots were performed by probing the membranes with a constitutively expressed gene, flaA, a
gene encoding a flagellum subunit (Fig. 16). The steady-state levels of $\text{flaA}$ mRNA did not change appreciably after a temperature shift or after the addition of coumermycin.

One-dimensional agarose gels with and without the addition of chloroquine were run as previously described in chapter 3 with a small reporter plasmid, pGOΔ15, isolated from HB19 cultures after the addition of 0, 10, and 100 ng ml$^{-1}$ coumermycin (data not shown). The topoisomer distribution was similar to that of the temperature shift experiment in chapter 3: cultures with no coumermycin had a supercoiled topoisomer like the cultures grown at 23°C. The cultures grown in the presence of 10 or 100 ng ml$^{-1}$ coumermycin had relaxed DNA like the culture grown at 35°C.

**Genetic manipulation of DNA supercoiling: OspC synthesis increases in $\text{gyrB}$ mutants.** We used a genetic approach to further analyze the effect of DNA supercoiling on $\text{ospC}$ expression. We compared OspC synthesis in a $\text{gyrB}$ mutant and a wild type clone by immunoblot analysis. *E. coli* $\text{gyrB}$ mutants have reduced DNA gyrase activity and a lower level of DNA supercoiling than wild type cells (22).

OspC synthesis, as assayed by immunoblotting, is upregulated in a $\text{gyrB}$ mutant compared to wild type clone of *B. burgdorferi* strain B31 (Fig. 17). B31 was utilized because of our laboratory’s extensive characterization of $\text{gyrB}$ mutants in this strain (94, 95). The small reporter plasmid pGOΔ15 transiently maintained in wild type B31 and CR9C, a $\text{gyrB}$ mutant, was resolved by electrophoresis with and without chloroquine (Fig. 18). The plasmid isolated from the $\text{gyrB}$ mutant is relaxed while the plasmid from the wild type clone is negatively supercoiled (Fig. 18).

Thirty *B. burgdorferi* $\text{gyrB}$ mutant clones and thirty wild type clones were examined by immunoblotting. The large number of clones was used because changes in
DNA supercoiling in the mutants can be compensated either by homeostatic upregulation of the *gyrB* gene (69) (Chapter 5, Figs. 25-28), or possibly by extragenic *topA* suppressors. We found that significantly more *gyrB* mutants (98%) synthesized OspC compared to wild type clones (72%) (*P* ≤ 0.02 as determined by a $\chi^2$ test). In addition, almost double the number of *gyrB* mutants (60%) had a moderate to high level of OspC production compared to the wild type clones (32%). Therefore, DNA relaxation in *gyrB* mutants also results in increased OspC synthesis.
Fig. 8. Effect of coumermycin concentration on growth. Cultures of *B. burgdorferi* HB19 were grown in BSK-H medium containing from 0 to 20 ng ml⁻¹ of antibiotic. Plotted are the means of four independent experiments relative to no treatment for each experiment. Error bars represent standard errors of the means.
Fig. 9. **Effect of coumermycin concentration on OspC synthesis.** Cultures of *B. burgdorferi* HB19 were grown in BSK-H medium containing from 0 to 20 ng ml\(^{-1}\) of antibiotic. Cell lysates were resolved by 12.5% SDS-PAGE and stained with Coomassie brilliant blue (CBB), blotted to a PVDF membrane and probed with an anti-OspC antibody (immunoblot).
Fig. 10. Effect of coumermycin concentration on OspA synthesis. Cultures of *B. burgdorferi* HB19 were grown in BSK-H medium containing from 0 to 20 ng ml\(^{-1}\) of antibiotic. Cell lysates were resolved by 12.5\% SDS-PAGE and stained with Coomassie brilliant blue (see Fig. 9 for CBB), blotted to a PVDF membrane and probed with an anti-OspA antibody (immunoblot).
Fig. 11. Time course of OspC synthesis after coumermycin addition. Cultures of *B. burgdorferi* HB19 were grown in BSK-H medium containing 0 (−) or 1(+) μg ml⁻¹ coumermycin, and then were harvested at 0, 8, 16, and 24 h after antibiotic addition. Cell lysates were resolved by 12.5 % SDS-PAGE and stained with Coomassie brilliant blue (not shown), blotted to a PVDF membrane and probed with an anti-OspC antibody.
Fig. 12. Effect of coumermycin on ospC mRNA transcript. Cultures of B. burgdorferi HB19 were grown in BSK-H medium containing 1 μg ml⁻¹ coumermycin. RNA was isolated from the cultures at 4, 8, 16, and 32 h after the antibiotic addition, and after no (0) antibiotic addition. Equal amounts of RNA (15 μg) were separated on a formaldehyde-agarose gel, blotted to a nylon membrane, and hybridized with an ospC probe.
Fig. 13. Effect of coumermycin on *ospA* mRNA transcript. Cultures of *B. burgdorferi* HB19 were grown in BSK-H medium containing 1 μg ml⁻¹ coumermycin. RNA was isolated from the cultures at 4, 8, 16, and 32 h after the antibiotic addition, and after no (0) antibiotic addition. Equal amounts of RNA (15 μg) were separated on a formaldehyde-agarose gel, blotted to a nylon membrane, and hybridized with an *ospA* probe. The identity of the transcript migrating above *ospA* after 8 h is not known. Speculation as to source is discussed in chapter 7.
Fig. 14. Effect of coumermycin concentration on ospC mRNA transcript. Cultures of B. burgdorferi HB19 were grown in BSK-H medium containing 0, 1, 10, and 20 ng ml\(^{-1}\) coumermycin. Equal amounts of RNA (15 \(\mu\)g) were separated on a formaldehyde-agarose gel, blotted to a nylon membrane, and hybridized with an ospC probe.
Fig. 15. Effect of coumermycin concentration on ospA mRNA transcript. Cultures of *B. burgdorferi* HB19 were grown in BSK-H medium containing 0, 1, 10, and 20 ng ml\(^{-1}\) coumermycin. Equal amounts of RNA (15 μg) were separated on a formaldehyde-agarose gel, blotted to a nylon membrane, and hybridized with an ospA probe.
Fig. 16. Effect of a temperature shift and coumermycin on flaA mRNA transcript.
Cultures of B. burgdorferi HB19 were grown in BSK-H medium at 23°C, 35°C or containing 1 µg ml⁻¹ coumermycin. RNA was isolated from the cultures at mid log phase for the temperature shift cultures and at 0 (the 23°C culture served as a 0 time point with no antibiotic added), 4, 8, 16, and 32 h after the antibiotic addition. Equal amounts of RNA (15 µg) were separated on a formaldehyde-agarose gel, blotted to a nylon membrane, and hybridized with an flaA probe.
Fig. 17. Effect of a *gyrB* mutation on OspC synthesis. OspC immunoblot of *B. burgdorferi* wild type B31 and CR9C, a *gyrB* mutant. Cell lysates were resolved by 12.5% SDS-PAGE, blotted to a PVDF membrane, and probed with an anti-OspC antiserum.
Fig. 18. Effect of a gyrB mutation on DNA topology of a small reporter plasmid.

Plasmid pGOA15 was transiently introduced into wild type B31 and CR9C. The topology of the plasmid was determined by one-dimensional 0.8 % agarose gel electrophoresis in the absence (native) or presence of 15 μM chloroquine (CQ). More negatively supercoiled molecules migrate faster through the gel; chloroquine intercalates into the plasmid DNA introducing positive supercoiling that retards migration of negatively supercoiled DNA (sc) and expedites migration of relaxed DNA (r). The migration of the linearized (l) form of the plasmid is not affected by chloroquine. Molecular size standards are indicated in kilobase pairs (kb).
Chapter 5

Regulation of promoter activity by DNA supercoiling

There are currently no shuttle vectors available for *B. burgdorferi*. In the absence of an established genetic system, the transient expression of a reporter gene in *B. burgdorferi* cells transformed by nonreplicative DNA provides a method to study promoter activity. A transient chloramphenicol acetyltransferase (CAT) expression system was previously developed to examine promoter activity in *B. burgdorferi* (99). Plasmids pGOΔ1 (promoterless), pGOΔ5 (*ospA*), and pGOΔ15 (*ospC*) (Fig. 19 and Table 2) were constructed by placing the *B. burgdorferi* promoters upstream of a promoterless *Streptococcus agalactiae* cat gene in an *E. coli* vector. The *ospA* promoter was found to be 20-fold more active than the promoterless construct or *ospC* promoters in *E. coli*, a more experimentally tractable system than *B. burgdorferi* (99). In *B. burgdorferi*, the *ospA* promoter was also 20-fold more active than the promoterless construct, but the *ospC* promoter was 7-fold more active than the promoterless construct (99). We analyzed these promoters both in *E. coli* and in *B. burgdorferi* after perturbing DNA supercoiling by either a temperature shift, addition of coumermycin, or a gyrase mutation.

**Minimum inhibitory concentrations (MICs).** Promoter activity was measured *in vivo* by MICs of chloramphenicol in *E. coli* containing plasmids pGOΔ1 (promoterless), pGOΔ5 (*ospA*), and pGOΔ15 (*ospC*) (Table 3). An increase in MIC...
reflects an increase in promoter activity as the up-regulation in cat expression allows the cells to grow on higher concentrations of chloramphenicol on the agar plates.

Pharmacological experiments were done using the DNA gyrase inhibitor coumermycin at concentrations that were subinhibitory in E. coli (0, 0.1 and 1 µg ml⁻¹). The IC₅₀ of coumermycin in E. coli is 10 µg ml⁻¹. The ospC promoter increased in activity with the addition of coumermycin and the relaxation of plasmid DNA. The promoterless construct and ospA promoter showed no appreciable change in activity. These results indicated that the ospC promoter was responsive to changes in DNA supercoiling, while the ospA and promoterless plasmids were not.

CAT ELISAs in E. coli. To further examine the regulation of the B. burgdorferi promoters by DNA supercoiling, we used a more quantitative assay to measure expression of the cat gene. The CAT ELISA employed is based on the sandwich ELISA principle. Antibodies to CAT are prebound to the surface of microtiter plate wells. Following lysis of the transfected cells, the cell extracts, which contain the CAT enzyme, are added to the wells. All CAT contained in the cell extracts binds specifically to the anti-CAT antibodies bound to the microtiter plate surface. Next, a digoxigenin-labeled antibody to CAT is added and binds to CAT. In the subsequent step, an antibody to digoxigenin conjugated to peroxidase is added and binds to digoxigenin. In the final step, the peroxidase substrate ABTS® is added. The peroxidase enzyme catalyzes the cleavage of the substrate yielding a colored reaction product. The absorbance of the sample is determined using a microtiter plate (ELISA) reader and is directly correlated to the level of CAT present in the medium supernatant (Boehringer Mannheim). CAT enzyme standards are prepared from an enzyme stock supplied with the kit in the concentration
range of 0 to 1.0 ng ml$^{-1}$. The plate reader uses Softmax software to calculate the standard curve and determine the CAT level of each sample using the curve. Two samples of each standard concentration were made and used in calculating the standard curve, and all unknown samples were measured in duplicate.

CAT ELISAs were performed in *E. coli* DH5α cells that had been transformed with plasmids containing *B. burgdorferi* promoters for *ospA* (pGOΔ5) and *ospC* (pGOΔ15) and a control promoterless plasmid (pGOΔ1) cloned upstream of a promoterless *Streptococcus agalactiae cat* gene. A mock transformation with no DNA was also performed. Selection and maintenance of transformants with these plasmids were carried out in Luria-Bertani (LB) broth with 10 μg ml$^{-1}$ of chloramphenicol.

The quantitative effect of DNA supercoiling on expression from the *ospA* and *ospC* promoters was tested in experiments using a temperature shift from 23°C to 37°C. The CAT activity from the *ospA* promoter with no temperature shift was 4-fold higher than from the promoterless plasmid or the *ospC* promoter. The temperature shift had little or no effect on the CAT activity from the promoterless construct and *ospA* promoters (Fig. 20). The CAT activity from the *ospC* promoter with no temperature shift was similar to that from the promoterless plasmid. The CAT activity from the *ospC* promoter increased 4-fold after a temperature shift, approaching that of the *ospA* promoter activity (Fig. 20).

To perturb the level of plasmid supercoiling, 0, 0.1, and 1 μg ml$^{-1}$ coumermycin was added to the growth media prior to incubation at 23°C. The activity of the *ospA* promoter was 5-fold greater than the promoterless plasmid or *ospC* promoter with no coumermycin added. Coumermycin had no appreciable effect on the activity of the
promoterless plasmid or ospA promoter (Fig. 21). The ospC promoter activity was similar to that from the promoterless plasmid with no coumermycin added. After the addition of 0.1 μg ml⁻¹ coumermycin, the activity of the ospC promoter increased 2.4-fold, and it increased 4-fold after the addition of 1 μg ml⁻¹ coumermycin, surpassing the activity of the ospA promoter (Fig. 21). This suggests that DNA supercoiling modulates transcription from the ospC promoter. There was no detectable CAT activity in the mock-transformed cells.

An isogenic pair of E. coli strains first described by Menzel and Gellert (70), whose gyrB genes were sequenced by Contreras and Maxwell (22), was used to evaluate the effect of gyrB mutations on promoter activity. E. coli N99 carries a wild-type gyrB, and strain N4177 has two gyrB mutations that together confer a coumermycin-resistant and temperature-sensitive phenotype. Growth of strain N4177 is permissive at 30°C but is restricted at 42°C. Plasmid supercoiling is reduced in N4177 compared to N99 (22). Experiments were carried out at 30°C for the temperature-sensitive N4177, and at 37°C for N99. The promoterless plasmid and ospA promoter had the same activity in the two strains, with the ospA promoter 10.5-fold more active than the promoterless plasmid (Fig. 22). The ospC promoter in strain N4177 was over twice as active as in strain N99 (Fig. 22), even though N99 was grown at a higher temperature where ospC would be expected to have a higher activity. ospC promoter activity in N99 was 5-fold higher than that of the promoterless plasmid, while ospC promoter activity in N4177 was 10.6-fold higher than that of the promoterless plasmid.

Taken together, the results of the CAT ELISAs in E. coli demonstrate that the ospC promoter is regulated by DNA supercoiling changes due to temperature, a DNA
gyrase inhibitor (coumermycin), or a mutation in the DNA gyrase gene. The \textit{ospA} promoter and the promoterless plasmid lack this regulatory response.

\textbf{CAT ELISAs in \textit{B. burgdorferi}.} We transformed the plasmids by electroporation into HB19 (92) and performed CAT ELISAs similar to those performed in \textit{E. coli} to measure the quantitative effect of DNA supercoiling on expression from the \textit{ospA} and \textit{ospC} promoters in their natural environment.

Temperature shift experiments were done with cells grown at 23°C and 35°C. The CAT activity from the promoterless plasmids at both temperatures and the \textit{ospC} promoter at 23°C was at the same level as the low background for the assay. The activity of the \textit{ospA} promoter was 14.3-fold higher than background at 23°C and decreased by 71% at the higher temperature (Fig. 23). The \textit{ospC} promoter had a 19-fold increase in activity at 35°C from its background level at 23°C, exceeding the activity of the \textit{ospA} promoter at 23°C (Fig. 23). There was no detectable CAT activity in the mock-transformed cells.

Pharmacological experiments were done using coumermycin concentrations of 0, 10, and 100 ng ml\(^{-1}\), and a growth temperature of 23°C. The IC\(_{50}\) of coumermycin in HB19 is 14 ng ml\(^{-1}\) (Fig. 8). We used these higher concentrations due to the short term nature of the experiment and the transient expression of these plasmids in this organism. The CAT activity from the promoterless plasmid at both coumermycin concentrations and with no coumermycin added was at low background levels as was the CAT activity from the \textit{ospC} promoter with no coumermycin added. \textit{ospA} promoter activity was 10.6-fold higher than background with no coumermycin added, decreased 18% after the addition of 10 ng ml\(^{-1}\) coumermycin, and decreased by 71% after the addition of 100 ng

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ml⁻¹ coumermycin (Fig. 24). *ospC* promoter activity increased 4.2-fold over the background level with the addition of 10 ng ml⁻¹ coumermycin, and increased 22-fold at the highest coumermycin concentration (Fig. 24). There was no detectable CAT activity in the mock-transformed cells.

The results of the CAT ELISAs in *B. burgdorferi* strain HB19 demonstrate that both the *ospA* and *ospC* promoters respond to changes in DNA supercoiling due to a temperature shift or the addition of coumermycin.

**Analysis of other *B. burgdorferi* promoters.** The activity of other *B. burgdorferi* promoters was analyzed to determine if they were regulated by DNA supercoiling (Table 2). Since the *gyrB* promoter is regulated by DNA supercoiling in *E. coli* (37, 67-70), we explored how it would respond to changes in DNA supercoiling in our CAT ELISA assays. We also examined its activity in *B. burgdorferi* since it is carried on a linear molecule in that organism. The artificial system constructed to measure promoter activity alters the DNA carrying the *gyrB* promoter from a linear molecule in its native state into a circular plasmid that constrains torsional stress. The *gyrB* promoter was cloned into the promoterless pGOΔ1 by Scott Knight and Corbin Schwanke (58).

Knight and Samuels first described a 34 kDa DNA-binding protein representing the GyrA C-terminal domain (Gac) (59). A 246-bp fragment (1213-1459) encoding the *gac* promoter containing all three transcriptional start sites, and a truncated 205-bp fragment (1213-1418) containing only one transcriptional start site were cloned into pGOΔ1 (58). The *gac* promoter contains two direct repeats and the 3' transcriptional start site at nucleotide 1435 is 39 nucleotides upstream of the ribosome binding site located on
pGOΔ1. The \textit{gac.2} promoter contains only one direct repeat, and the transcriptional start site at nucleotide 1412 is positioned 18 nucleotides upstream of the ribosome binding site. Based on primer extension data, nts 1430 and 1435, which are separated by one turn of the helix, were predicted to be the two predominant transcriptional start sites (58).

The resulting plasmid constructs, pGOΔGyrB, pGOΔGac, and pGOΔGac.2 were transformed into \textit{E. coli} and \textit{B. burgdorferi}, and temperature shift and pharmacological experiments were performed to analyze the promoters response to changes in DNA supercoiling.

The CAT activity from the \textit{gac} promoter in \textit{E. coli} was 6-fold greater than that from the promoterless plasmid at both 23°C and 37°C. The \textit{gac} promoter was unaffected by the temperature shift. The \textit{gac.2} promoter was 3-fold more active than the promoterless plasmid at both temperatures, although it had half the activity of the \textit{gac} promoter. The \textit{gac.2} promoter was also unaffected by the temperature shift. \textit{gyrB} promoter activity was 1.4-fold higher than the promoterless plasmid at 23°C, and increased 2-fold at the higher temperature (Fig. 25).

Pharmacological experiments were performed in \textit{E. coli} using coumermycin at concentrations of 0, 0.1, and 1 μg ml\(^{-1}\). The coumermycin had no effect on \textit{gac} or \textit{gac.2} promoter activity. \textit{gyrB} promoter activity was 1.4-fold higher after the addition of 0.1 μg ml\(^{-1}\) coumermycin and 2-fold higher after the addition of 1 μg ml\(^{-1}\) coumermycin than the cells with no coumermycin added (Fig. 26).

Temperature shift experiments in HB19 showed that the CAT activity from the \textit{gac} promoter was 25-fold higher than the background level of the promoterless plasmid and was unaffected by the temperature shift. The CAT activity from the \textit{gac.2} promoter
was 14-fold higher than that of the promoterless plasmid and was also unaffected by the temperature shift. However, gyrB promoter activity was 7-fold higher than that of the promoterless plasmid, and increased by 2.4-fold at the higher temperature (Fig. 27).

Pharmacological experiments were done in HB19 using coumermycin concentrations of 0, 10, and 100 ng ml⁻¹ as described earlier. The CAT activities from the gac and gac.2 promoters were similar to those of the temperature shift experiments and were unaffected by the addition of coumermycin. gyrB promoter activity increased 1.3-fold with the addition of 10 ng ml⁻¹ coumermycin and it increased 2-fold after the addition of 100 ng ml⁻¹ coumermycin (Fig. 28).

The results of the CAT ELISAs of the gac, gac.2, and gyrB promoters demonstrate that the gac and gac.2 promoters do not respond to changes in DNA supercoiling while the gyrB promoter does respond to changes in DNA supercoiling due to either a temperature shift or the addition of coumermycin. In fact, in all experiments, the gyrB promoter activity doubles upon treatment. The gac.2 promoter has approximately half the activity of the gac promoter in all experiments, consistent with the prediction that the two transcriptional start sites absent from the gac.2 promoter (nts 1430 and 1435), are the predominant transcriptional start sites (58).
Table 3. MIC of chloramphenicol in *E. coli*.

<table>
<thead>
<tr>
<th>plasmid</th>
<th>0 μg ml⁻¹ coumermycin</th>
<th>0.1 μg ml⁻¹ coumermycin</th>
<th>1 μg ml⁻¹ coumermycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGOΔ1 (promoterless)</td>
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<td>40</td>
<td>40</td>
</tr>
<tr>
<td>pGOΔ5 (ospA)</td>
<td>640</td>
<td>640</td>
<td>640</td>
</tr>
<tr>
<td>pGOΔ15 (ospC)</td>
<td>80</td>
<td>160</td>
<td>640</td>
</tr>
</tbody>
</table>
Fig. 19. Structure and selected restriction sites of recombinant plasmids containing *B. burgdorferi* promoters and the *cat* gene. In pGOΔ1, the *cat* gene is oriented opposite the *bla* and *lac* promoters and lacks its own promoter. The solid arrow indicates the *cat* gene, and the cross-hatched arrow indicates the partially deleted *bla* gene. The short open arrow shows the *lac* promoter (93).
Fig. 20. *B. burgdorferi* promoter activity in *E. coli* following temperature shift as assayed by CAT ELISA. CAT ELISAs were performed on cell lysates of *E. coli* cells transformed with plasmids containing no promoter, the ospA promoter, or the ospC promoter cloned upstream of a cat gene. The cells were grown either at 23°C (open bars), or at 37°C (solid bars). CAT activity is measured in ng CAT per μg protein × 10⁻⁴. Assays were done in duplicate. Error bars indicate standard errors of the mean.
Fig. 21. *B. burgdorferi* promoter activity in *E. coli* following addition of coumermycin as assayed by CAT ELISA. CAT ELISAs were performed on cell lysates of *E. coli* cells transformed with plasmids containing no promoter, the ospA promoter, or the ospC promoter cloned upstream of a *cat* gene. The cells were grown in the presence of 0 (open bars), 0.1 (dashed bars), or 1 (solid bars) μg ml⁻¹ coumermycin at 23°C. CAT activity is measured in ng CAT per μg protein × 10⁴. Assays were performed in duplicate. Error bars indicate standard errors of the mean.
Fig. 22. *B. burgdorferi* promoter activity in *E. coli* with or without *gyrB* mutations as assayed by CAT ELISA. CAT ELISAs were performed on cell lysates of *E. coli* cells with a wild type (cross-hatched bars), or a mutated (open bars) gyrase transformed with plasmids containing no promoter, the *ospA* promoter, or the *ospC* promoter cloned upstream of a *cat* gene. N4177 cells were grown at 30°C (permissive temperature of this temperature-sensitive strain), and N99 cells were grown at 37°C. CAT activity is measured in ng CAT per μg protein × 10^4. Assays were performed in duplicate. Error bars indicate standard errors of the mean.
Fig. 23. *B. burgdorferi* promoter activity in *B. burgdorferi* following a temperature shift as assayed by CAT ELISA. CAT ELISAs were performed on cell lysates of *B. burgdorferi* cells transformed with plasmids containing no promoter, the *ospA* promoter, or the *ospC* promoter cloned upstream of a *cat* gene. The cells were grown either at 23°C (open bars), or at 35°C (solid bars). CAT activity is measured in ng CAT per μg protein × 10⁻⁴. Assays were performed in duplicate. Error bars indicate standard errors of the mean.
Fig. 24. *B. burgdorferi* promoter activity in *B. burgdorferi* following addition of coumermycin as assayed by CAT ELISA. CAT ELISAs were performed on cell lysates of *B. burgdorferi* cells transformed with plasmids containing no promoter, the *ospA* promoter, or the *ospC* promoter cloned upstream of a *cat* gene. The cells were grown in the presence of 0 (open bars), 10 (dashed bars), or 20 (solid bars) ng ml⁻¹ coumermycin at 23°C. CAT activity is measured in ng CAT per μg protein x 10⁴. Assays were performed in duplicate. Error bars indicate standard errors of the mean.
Fig. 25. *B. burgdorferi* promoter activity in *E. coli* following temperature shift as assayed by CAT ELISA. CAT ELISAs were performed on cell lysates of *E. coli* cells transformed with plasmids containing no promoter, the *gac* promoter, the *gac.2* promoter, or the *gyrB* promoter cloned upstream of a *cat* gene. The cells were grown either at 23°C (open bars), or at 37°C (solid bars). CAT activity is measured in ng CAT per μg protein × 10^{-4}. Assays were performed in duplicate. Error bars indicate standard errors of the mean.
**Fig. 26.** *B. burgdorferi* promoter activity in *E. coli* following addition of coumamycin as assayed by CAT ELISA. CAT ELISAs were performed on cell lysates of *E. coli* cells transformed with plasmids containing no promoter, the gac promoter, the gac.2 promoter, or the gyrB promoter cloned upstream of a cat gene. The cells were grown in the presence of 0 (open bars), 0.1 (dashed bars), or 1 (solid bars) μg ml⁻¹ coumamycin at 37°C. CAT activity is measured in ng CAT per μg protein × 10⁴. Assays were performed in duplicate. Error bars indicate standard errors of the mean.
Fig. 27. *B. burgdorferi* promoter activity in *B. burgdorferi* following temperature shift as assayed by CAT ELISA. CAT ELISAs were performed on cell lysates of *B. burgdorferi* cells transformed with plasmids containing no promoter, the gac promoter, the gac.2 promoter, or the gyrB promoter cloned upstream of a *cat* gene. The cells were grown either at 23°C (open bars), or at 35°C (solid bars). CAT activity is measured in ng CAT per μg protein × 10⁴. Assays were performed in duplicate. Error bars indicate standard errors of the mean.
Fig. 28. *B. burgdorferi* promoter activity in *B. burgdorferi* following addition of coumermycin as assayed by CAT ELISA. CAT ELISAs were performed on cell lysates of *B. burgdorferi* cells transformed with plasmids containing no promoter, the gac promoter, the gac.2 promoter, or the gyrB promoter cloned upstream of a cat gene. The cells were grown in the presence of 0 (open bars), 10 (dashed bars), or 20 (solid bars) ng ml⁻¹ coumermycin at 23°C. CAT activity is measured in ng CAT per µg protein × 10⁻⁴. Assays were performed in duplicate. Error bars indicate standard errors of the mean.
Chapter 6

Over-expression of groEL in coumermycin-resistant gyrB mutants

Thirty coumermycin-resistant gyrB mutants and thirty wild type clones of B. burgdorferi strain B31 were screened for OspC production, as described in chapter 4. Differences in OspC synthesis were noted between the gyrB mutants and wild type cells. These experiments did show some other differences in the protein profiles of the mutants as compared to the wild type clones. A protein of approximately 68 kDa appeared to be greatly upregulated in the mutants. In addition, a protein of approximately 25 kDa also appeared to be upregulated. We grew a large culture of the mutant in order to isolate the proteins. However, we were unable to re-create this protein profile with subsequent cultures of the mutants inoculated from frozen cells. The 68 kDa protein was not upregulated to the same extent in these cultures. Another protein of approximately 68 kDa (but not upregulated to the same extent) and the 25 kDa protein were isolated by column chromatography using a BioLogic automated protein purification system (Bio-Rad) from 1.5 liter cultures of HB19 (Fig. 29A). Proteins were N-terminally sequenced by Joan Strange (Murdock Molecular Biology Facility, The University of Montana) using an Applied Biosystems 373A DNA Sequencer.

A basic local alignment tool (BLAST) search was performed for the 68 kDa protein resulting in the identification of GroEL, a heat shock protein (Fig. 29B).
Inhibitors of DNA gyrase induce heat shock proteins in *E. coli* (16, 71). The 25 kDa protein was found to be N-terminally blocked.

An immunoblot for GroEL was performed using cell lysates from B31 wild type cells, heat-shocked B31 cells, and cells from the gyrB mutant cultures in order to assay if GroEL synthesis was upregulated in the gyrB mutant (Fig. 30). The blot showed that GroEL protein levels were increased in the heat-shocked cells as compared to the wild type cells and the protein levels in the gyrB mutant cells were increased even more so.

Northern blots were performed to assess the levels of steady-state groEL mRNA transcript for the three culture conditions (Fig. 31). The results correlated with the immunoblot results. The groEL mRNA transcript was 2.3-fold (± 0.4 SEM) higher in the gyrB mutants as compared to the B31 wild type cultures and 1.6-fold (± 0.2 SEM) higher as compared to the B31 heat-shocked cultures.

We wanted to determine why groEL gene expression was increased in the gyrB mutant. We speculated that the increase was due to DNA relaxation since gyrB mutants have more relaxed DNA than wild type cells (22, 71). One-dimensional agarose gels with and without the addition of chloroquine were run as previously described in chapters 3 and 4 with a small reporter plasmid, pGOΔ15, inserted in B31 wild type cells and in the gyrB mutant cells.

The plasmid DNA from the wild type B31 was negatively supercoiled, and the plasmid DNA from the gyrB mutant was relaxed (data not shown). This was the same pattern of DNA supercoiling as seen in Chapter 4 for the B31 wild type and the gyrB mutant CR9C (Fig. 18).
The relaxation of DNA supercoiling has previously been shown to be associated with the induction of heat shock proteins (71). Furthermore, DnaK plays a role in protecting negative DNA supercoiling in *E. coli* against thermal stress (77). We observed an over-expression of GroEL in a gyrB mutant of *B. burgdorferi*. DNA supercoiling is more relaxed in cells with a defective DNA gyrase (22). Our question was whether the over-expression of GroEL is induced by relaxed DNA, or was the GroEL protein induced by the presence of a mutant DNA gyrase. The GroEL in this latter case could have stabilized DNA gyrase, resulting in the return of DNA supercoiling levels to those seen in the wild type cells. The GroEL protein in this scenario would be acting in a similar fashion as DnaK observed by Ogata et al. (77) in maintaining the negative supercoiling in the cell in response to a stress in the cell. This stress could have been the mutation in the *gyrB* gene. However, our results suggest that the over-expression of GroEL is merely a response to relaxation of DNA. Notably, *groEL* is on the chromosome (38), which is a linear DNA molecule in *B. burgdorferi*. The demonstration that this gene is regulated by DNA supercoiling suggests that this linear molecule in *B. burgdorferi* is subject to superhelical torsion and the ends must therefore be constrained, perhaps by binding to the cell membrane or to a higher-order nucleoprotein complex.
Fig. 29. Purification of GroEL from a coumermycin-resistant gyrB mutant of *B. burgdorferi*. (A) The 68 kDa protein (later identified as GroEL) was purified by column chromatography. Lane 1 is the cell lysate loaded onto the first (heparin) column. Lane 2 is the flow through from the heparin column where most of the proteins, including the 68 kDa protein of interest, eluted. Lane 3 is the pooled fractions containing the 68 kDa protein after being passed over a phenyl superose column. Lane 4 is the pooled fractions containing the 68 kDa protein after being passed over a Mono-Q column. The cell lysate and pooled fractions were resolved by 12.5% SDS-PAGE and the proteins visualized by silver staining. Molecular weight markers are in kDa. (B) N-terminal sequence of the 68 kDa protein and alignment with GroEL.
Fig. 30. Synthesis of GroEL by a coumermycin-resistant gyrB mutant of B. burgdorferi. Cell lysates from B31 wild type (wt), experimentally heat-shocked B31 wild-type (hs), and a gyrB mutant were resolved by 12.5% SDS-PAGE, blotted to a PVDF membrane, and probed with an anti-GroEL antiserum.
Fig. 31. Over-expression of \textit{groEL} by a coumermycin-resistant \textit{gyrB} mutant of \textit{B. burgdorferi}. RNA was isolated from culture of B31 wild type (wt), experimentally heat-shocked B31 wild-type (hs), and the \textit{gyrB} mutant. Equal amounts of RNA (15 \(\mu\)g) were separated on a formaldehyde-agarose gel, blotted to a nylon membrane, and hybridized with a \textit{groEL} probe.
Chapter 7

Discussion

Infection by a bacterium necessitates adaptation to the host environment(s). Responding to these environments usually requires the coordinated expression of a number of bacterial genes. We are interested in understanding how *B. burgdorferi* senses the temperature change associated with the environmental transition from tick vector to mammalian host, and how the signal is transduced to affect gene expression. We postulated that these roles were played by DNA supercoiling as in several other systems (27-30, 35, 45, 49, 52, 53, 56, 69, 70, 75, 87, 111, 116). Therefore, we examined the relationship between DNA supercoiling and gene expression through three approaches: temperature shift, pharmacological perturbation of DNA supercoiling, and genetic manipulation of DNA supercoiling. We found that an increase in temperature results in a decrease in DNA supercoiling, which results in an increase in the expression of the *ospC* gene and the synthesis of OspC, and a decrease in the expression of the *ospA* gene and the synthesis of OspA. We have shown that OspC levels are increased by artificially perturbing DNA gyrase pharmacologically or genetically, and that OspA levels are decreased by perturbing DNA gyrase pharmacologically. Taken together, our results suggest that *ospC* and *ospA* are environmentally regulated in *B. burgdorferi* by temperature-induced changes in DNA supercoiling (Fig. 32).

A self-regulating or homeostatic mechanism balancing the activities of DNA gyrase and topoisomerase I has been proposed to control DNA supercoiling in bacteria.
The promoters of the topA, gyrA, and gyrB genes are themselves sensitive to DNA supercoiling, with increases in negative supercoiling activating topA (111), and decreases activating gyrA and gyrB (68). Changes in growth temperature alter plasmid supercoiling (43), but the direction of change depends on the species of bacterium (30, 43, 45, 72, 86). Analysis of reporter plasmid DNA supercoiling in vivo has shown the level of DNA supercoiling to be in the state of constant flux (29), theoretically providing the cell with a highly pleiotropic environmental sensor to elicit a physiological response. There is some evidence from studies of supercoiling-sensitive promoters in enteric bacteria that fluctuating levels of DNA supercoiling provide an overlap of responses to different environmental stimuli (75). Thus, DNA supercoiling as an agent of gene regulation provides a coordinated response to environmental change by affecting numerous genes that affect different aspects of metabolism and physiology.

The cellular mechanism whereby temperature effects topological changes in DNA structure has not been elucidated. A mechanism can be proposed based on the response of bacterial cells to other environmental changes. A change in the [ATP]/[ADP] ratio occurs in E. coli after exposure to salt shock (53), or a switch from aerobic to anaerobic growth (52). These changes were also associated with changes in DNA supercoiling (52, 53). Cellular energetics may influence DNA supercoiling since ATP is required for DNA gyrase to introduce negative supercoiling into DNA and since DNA gyrase removes negative supercoils in the absence of ATP (41, 105). In vitro, the ratio of [ATP] to [ADP] strongly influences the level of supercoiling attained in the presence of DNA gyrase, regardless of whether DNA gyrase is removing or introducing negative supercoils (114). A temperature shift may also effect a change in DNA supercoiling by changing
the cellular energetics as metabolic rate and free energy changes are a function of temperature. A test of this hypothesis would be to measure the [ATP]/[ADP] ratio in bacterial cultures grown at 23°C, and after a shift to 35°C. ATP and ADP could be assayed using a luciferase assay as previously described (19).

Other temperature-sensitive DNA switches have been described that are influenced by small DNA-binding proteins (35), or the melting of intrinsic bends in DNA (87). B. burgdorferi lacks an H-NS homolog (38). H-NS and HU are bacterial analogs of eukaryotic histone proteins responsible for compaction of DNA (10). There are two small DNA-binding proteins in B. burgdorferi: Hbb (109), an HU homolog, and Gac (59). The B. burgdorferi ospC promoter also contains several poly (dA) tracts and a large inverted repeat, so there is likely some effect of protein-induced or intrinsic bending on DNA supercoiling and the expression of ospC. Experiments to examine the effects of DNA-binding proteins on DNA supercoiling could be done in E. coli by transforming the small reporter plasmid used in this study into a cell with a H-NS mutant background, and comparing the levels of DNA supercoiling to wild type cells. Similar experiments could be done in B. burgdorferi using Hbb and Gac mutants.

DNA gyrase is likely to be the major influence on DNA supercoiling in B. burgdorferi. The CAT activity from the ospC promoter was increased by a temperature shift by an amount comparable to coumermycin, a DNA gyrase inhibitor, in both E. coli and B. burgdorferi.

DNA supercoiling was shown to be necessary and sufficient to regulate the ospC promoter in a heterologous system, E. coli, as well as in its native environment, B. burgdorferi. However, the promoter analysis experiments demonstrate that the ospA
promoter responds to DNA supercoiling in B. burgdorferi but not in E. coli. This suggests the presence of a trans-acting repressor protein in B. burgdorferi involved in the regulation of ospAB transcription that is not present in E. coli. The presence of a protein with sequence-specific binding to the ospAB operon promoter region has previously been described (66) (Janet Alverson, Craig Kuchel, and Michele Kresge, unpublished data). The binding activity co-elutes with a 23 kDa protein (66). We speculate that the repressor protein that binds the ospAB operon promoter is a nonlipidated form of OspC (not associated with the membrane), which is increasing in synthesis coincidentally with the decrease in OspA synthesis. The identity of the repressor protein of the ospAB operon promoter is an area of on-going research in the laboratory. Genetic and biochemical experiments testing the hypothesis of OspC as the repressor protein for ospA involve the promoter constructs described here and DNA-protein assays, respectively.

Our studies have shown a decrease in negative supercoiling in E. coli with an increase in temperature, which is in contrast to a previous work that showed that plasmid linking number decreases with increasing temperature in E. coli, resulting in more negatively supercoiled DNA (43). The artificial system we created with plasmid constructs containing B. burgdorferi promoters cloned into three different E. coli strains consistently supported a pattern of relaxation of DNA with an increase in temperature, inhibition of gyrase with coumermycin, or gyrase mutations. In our system, E. coli DNA supercoiling behaved similarly to DNA supercoiling in other bacteria as previously described (30, 45, 72). Our plasmid may be responding to the melting of intrinsic bends in the DNA at higher temperatures (87), which allows for increased expression of specific genes. An experiment to address this problem is to transform the small reporter plasmid...
(with and without the *ospC* promoter) into *E. coli* and assay DNA supercoiling to determine if differences exist between the two bacteria.

The background promoter activity for the promoterless plasmid, pGOAl, is higher in *E. coli* than in *B. burgdorferi*. This is likely due to a cryptic promoter that does not function in the *B. burgdorferi* system (99). The changes in *ospC* promoter activity were much more dramatic in *B. burgdorferi* than in *E. coli*. Again, this may reflect an overall relaxation of supercoiled DNA in *B. burgdorferi* as opposed to only a melting of intrinsic DNA bends in *E. coli*. DNA with areas of intrinsic bending may be recognized by DNA-binding ligands such as H-NS, which is lacking in *B. burgdorferi*, but present in *E. coli*. DNA topology may be stabilized by the histone-like proteins. As the bacterium is shifted to a higher temperature, the intrinsic DNA bends are melted, dislodging the DNA-binding proteins, locally unwinding the DNA, and affecting gene expression.

DNA supercoiling acts as a signal transducer allowing the spirochete to react to the environment, which, in this case, is a change from a free-living tick vector to either a tick attached and feeding or a mammalian host. Temperature, a major environmental factor that differs between ticks and mammals, directly influences DNA structure (35, 43, 86, 87). Other environmental differences between ticks and mammals that may influence DNA supercoiling and gene expression include pH, nutrient availability, oxygen availability, and osmotic pressure (4, 17, 27-30, 32, 44, 49, 52, 53, 56, 72, 116). In fact, a decrease in pH, which accompanies the natural transmission from ticks to mammals, induces synthesis of OspC (17); the effects of pH and temperature may be additive. Experiments to examine the effect of pH on DNA supercoiling, and the combined effect of pH and temperature on DNA supercoiling could be done to explore this relationship.
The relaxation of DNA supercoiling has previously been shown to be associated with the induction of heat shock proteins (71). The same laboratory also showed that DnaK plays a role in maintaining the negative supercoiling in *E. coli* against thermal stress (77). We observed an over-expression of GroEL in a coumermycin-resistant *gyrB* mutant of *B. burgdorferi*. The DNA supercoiling in the cells with a defective DNA gyrase gene would be expected to have more relaxed DNA. Our question was whether the over-expression of GroEL was induced by the more relaxed DNA, or was the GroEL protein induced by a need to maintain DNA supercoiling homeostasis in the cell. The GroEL in this latter case could have stabilized the gyrase enzyme and returned DNA supercoiling levels back to those seen in the wild type cells. The GroEL protein in this scenario would be acting in a similar fashion to that of DnaK observed by Ogata et al. (77) in maintaining the negative supercoiling in the cell in response to a stress in the cell. In our case the stress was a mutation in the DNA gyrase gene. Our results suggest that the over-expression of GroEL is merely a response to relaxation of DNA. However, like *gyrB*, *groEL* is on the chromosome, which is a linear DNA molecule in *B. burgdorferi*. The demonstration that both promoters are regulated by DNA supercoiling suggests that the linear molecule in *B. burgdorferi* is subject to superhelical torsion and the ends therefore must somehow be constrained, perhaps by binding to the cell membrane or to a protein complex.

The upregulated 68 kDa protein observed in the original coumermycin-resistant *gyrB* mutant cultures (chapter 6) may be GyrB. In *E. coli*, *gyrB* mutants exhibit less negative supercoiling because the mutant DNA gyrase is defective (22). This would then be expected to induce *gyrB* expression by homeostatic regulation of supercoiling and
increase production of GyrB. We are not sure why this phenomenon is suppressed upon passage of the mutant from the frozen stocks. There may be a compensatory decrease in the activity of topoisomerase I. This would lower the need for DNA gyrase by lowering the relaxing activity that balances the level of supercoiling. Alternatively, upregulation of heat-shock proteins may stabilize the mutant enzyme.

The growth stimulation seen in *B. burgdorferi* strain HB19 with low concentrations (0.2 ng ml\(^{-1}\) to 0.5 ng ml\(^{-1}\)) of coumermycin (chapter 4) is provocative, although the mechanism is not understood. Perhaps the slight relaxation in DNA supercoiling at these low levels of coumermycin concentrations allows for improved polymerase tracking and gene expression before the higher concentrations of antibiotic begin to inhibit growth.

A Northern blot of the coumermycin time course showed a band migrating at approximately 2.4 kb (the *ospA* transcript migrates at 1.77 kb) after 8 h (chapter 4, Fig. 13). The identity of this larger transcript is unknown. Possible explanations include that the *ospA* transcript is larger after coumermycin treatment: a promoter upstream may be activated or a termination signal may be repressed. Another possibility is that there is an additional transcript homologous to *ospA* transcribed in response to coumermycin treatment. A BLAST search revealed a periplasmic protein (BB1363) with 51.6% nucleotide identity to *ospA* in a 624 nt overlap. The length of the gene encoding this periplasmic protein is 2010 nt, which could correspond to a mRNA transcript migrating at 2.4 kb.

DNA supercoiling can globally affect gene expression, upregulating or downregulating large groups of genes. This allows for a coordinated response to an
environmental stimulus (27, 28). Adapting to a changing environment is an obvious advantage for a bacterium, and essential for maintenance in an arthropod vector-mammalian host cycle. The evolutionary success of bacteria depends on an efficient system of environmental sensing and response, including coordination at the transcriptional level. Our results show that the DNA of spirochetes grown at increased temperatures is less negatively supercoiled than when grown at lower temperatures and that decreased supercoiling induces an increase in OspC synthesis and a decrease in OspA synthesis. Therefore, DNA supercoiling appears to be a sensor and signal transducer for environmental regulation of gene expression in \textit{B. burgdorferi}.
Fig. 32. Model of environmental gene regulation by DNA supercoiling in *B. burgdorferi*. The spirochete alters its program of gene expression in response to environmental changes, especially temperature. Transcriptional repression and activation of certain genes, predominantly *ospA* and *ospC* which encode outer surface proteins OspA and OspC, respectively, is correlated with relaxation of supercoiled DNA, which in turn, is correlated with the environmental temperature shift.
Chapter 8

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