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Differential homeostatic supercoil regulation in Escherichia coli and Salmonella enterica serovar Typhimurium.

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DIFFERENTIAL HOMEOSTATIC SUPERCOIL REGULATION IN ESCHERICHIA COLI AND SALMONELLA ENTERICA SEROVAR TYPHIMURIUM

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DIFFERENTIAL HOMEOSTATIC SUPERCOIL REGULATION IN ESCHERICHIA COLI AND SALMONELLA ENTERICA SEROVAR TYPHIMURIUM

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BIOCHEMISTRY AND MOLECULAR GENETICS

ABSTRACT

Escherichia coli and *Salmonella enterica* serovar Typhimurium are closely related gram-negative bacterium. They share a high degree of homology among conserved genes and have very similar genetic maps. In addition, most defined processes are nearly indistinguishable between the two. However, several persisting and unexplained differences exist in the literature. Paramount among these is the observation that a deletion of the gene encoding topoisomerase I is lethal in *E. coli* without a compensatory mutation, but viable in *Salmonella*. To address this disparity, we examined the effect of the *Salmonella gyrB652* allele in *E. coli*. The *gyrB652* transversion has a complex phenotype in *Salmonella* as a result of an inefficient DNA gyrase enzyme. We found that this mutation, which is viable in *Salmonella*, is lethal in *E. coli*. We also found that *E. coli*, but not *Salmonella*, is very sensitive to altered DNA gyrase levels. This led us to compare the level of negative supercoiling in each species. Using two-dimensional chloroquine electrophoresis, we found that *E. coli* contains about 15% more unconstrained negative supercoiling than *Salmonella*. We confirmed these measurements using supercoil-sensitive chromosomal reporter cassettes. We further demonstrated this by showing phenotypic differences in physiological systems that have a supercoil component in their regulation. These include chromosome condensation via the SMC protein MukB, replication initiation via SeqA, and the lysis/lysogeny pathway of phage Mu. Further analysis showed that both species have a similar chromosomal

supercoil fluctuation pattern through the growth phase. However, the supercoil fluctuations of plasmid DNA in the two species exhibited a stark difference. This may indicate that, in addition to differences in global supercoiling levels, there may be differences in plasmid maintenance between the species.

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LIST OF ABBREVIATIONS

ATP	adenosine 5'-triphosphate
bp	base pairs
β-gal	beta galactosidase
CFU	colony forming units
dif	deletion induced filamentation
DNA	deoxyribonucleic acid
DAPI	4',6-diamidino-2-phenylindole
Fis	factor for inversion stimulation
GFP	green fluorescent protein
IHF	integration host factor
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase pairs
kDa	kilodalton
LB	Luria-Bertani broth
MB	megabase pairs
Mg	magnesium
μg	microgram
μl	microliter
μm	micrometer
μM	micromolar

ml	milliliter
my	million years
M	molar
OD	optical density
ONPG	orthonitrophenyl β-D-galactoside
PCR	polymerase chain reaction
REP	repetitive extragenic palindromic
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
SMC	structural maintenance of chromosome
TS	temperature sensitive
TBE	tris-borate-EDTA buffer
UV	ultraviolet
V	volt
WT	wild type
X-Gal	5-bromo-4-chloro-3-indolyl β-D-galactoside

INTRODUCTION

DNA Supercoiling

In 1963, while investigating the genome of the small polyoma virus, Weil & Vinograd made the startling discovery that the chromosome of this virus had three distinct sedimentation coefficients (Weil and Vinograd, 1963). Through electron micrographs, they established that form III consisted of linear DNA. However, forms I and II were both circular, and had identical compositions. Two years later in 1965, Vinograd made the landmark discovery that form I could be converted to form II via digestion with DNase I (Vinograd *et al.*, 1965). From this, he suggested that polyoma DNA existed in a "twisted circular form". To achieve this form, the ends of the double helix must be twisted opposite the normal winding pattern before joining. Vinograd believed these "tertiary turns" were incorporated during replication. Based on similar results in papilloma virus (Crawford, 1964), SV40 (Crawford and Black, 1964) and RF-ϕX (Jansz and Pouwels, 1965), he proposed that all circular duplex DNA existed in this state. All of Jerome Vinograd's hypotheses were correct, and bore the field of DNA supercoiling research.

The standard Watson-Crick double helix has about 10.5 base pairs per turn. This is the most accurate representation of DNA in its most thermodynamically favorable conformation, including linear or nicked circular DNA. However, DNA that is not free to rotate around the helical axis, such as closed circular DNA or chromosomal domains, becomes physiologically under-wound. This under-winding comes from processes that

separate the Watson-Crick strands, such as DNA replication and transcription, and from topoisomerase enzymes, which can increase or decrease the amount of winding. As a result, the Watson-Crick helix becomes torsionally strained in a high-energy form that assists in processes that require a denaturation of the complimentary strands. This torsional strain is compensated along the contour length by the DNA winding upon itself. This "coiling" of the helical "coil", which Vinograd termed the "twisted form", is called supercoiling. An over-winding of the helix results in positive supercoiling. Conversely, when the torsional strain is a result of under-winding, the supercoiling is said to be in a negative conformation. Every known organism on Earth maintains its genome in a negatively supercoiled state. The many functions of negative supercoiling are discussed below.

The Role of Supercoiling in DNA Condensation

Escherichia coli and *Salmonella enterica* serovar Typhimurium serve as informative model systems in many aspects of basic science. The role of negative supercoiling in DNA condensation has been especially well characterized using these organisms. These prokaryotes have genomes over 4 MB, which is equivalent to about 1 mm of DNA length. In order to fit this amount of DNA into the small cell space requires a compaction of over 1000-fold (Fig. 1). Brownian motion will introduce random bends into DNA about every 50 nm (or 150 bp), the persistence length, which can decrease the size to about 10 μm (Trun and Marko, 1998). However, this is still too large for the given space, and it produces a genomic structure that is completely random and

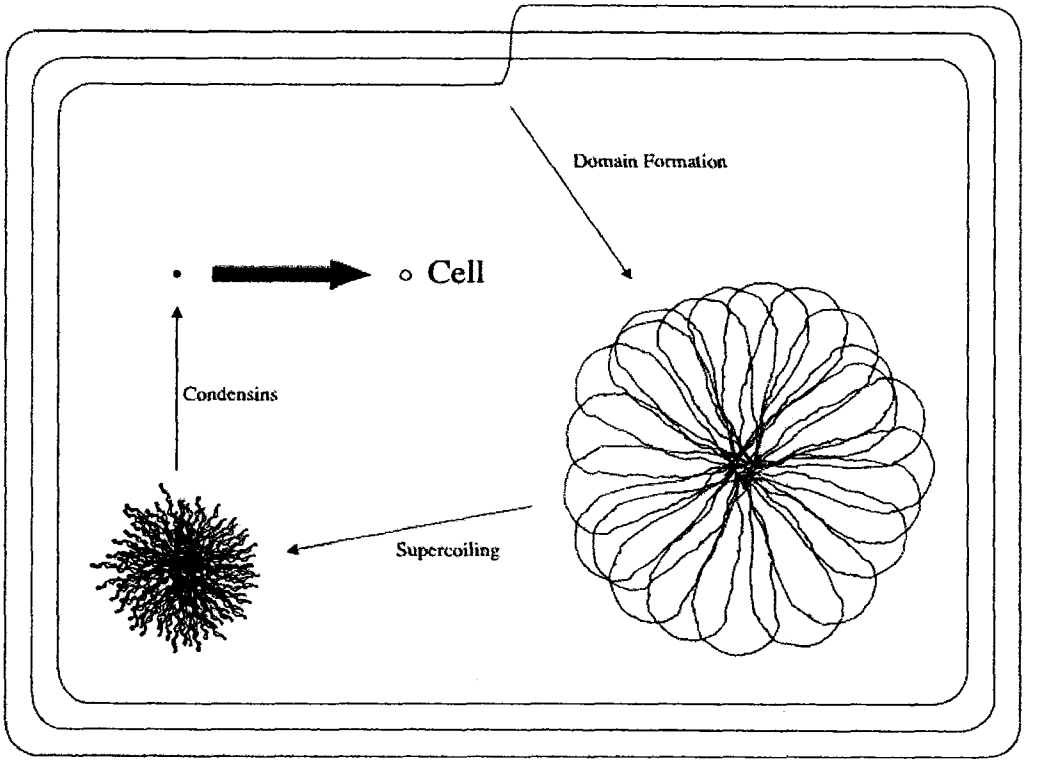

Figure 1. DNA Compaction. Illustration of the 1000-fold linear compaction required to fit the prokaryotic chromosome inside the cell. Organizing the circular molecule framing the figure into domains is one layer of compaction. Supercoiling the DNA provides a second. Additional organization and compaction are provided by condensin proteins. All illustrations are to scale.

disordered. Chromosomes must be packaged to an even greater degree, and must do so in an organized fashion to allow the condensed genome to be available to cellular machinery for processes such as replication, transcription, translation, and recombination. This ordered condensation is achieved through both constrained and unconstrained supercoils and the formation of supercoil domains.

Constrained vs. Unconstrained Supercoils

In eukaryotes, the major condensation step is the binding and packaging of the DNA by histones. This wrapping of DNA on the surface of proteins forms constrained supercoils that account for all eukaryotic supercoiling. In *E. coli*, the observation was made that, when DNA was nicked *in vivo*, only half of the supercoiling was lost. This was in contrast to the total relaxation seen *in vitro* with purified prokaryotic DNA, and the completely constrained content of *in vivo* eukaryotic DNA. This suggested that, in prokaryotes, about half of the supercoiling was constrained by DNA binding proteins *in vivo* (Pettijohn and Pfenninger, 1980). The other half exists in an unconstrained form called plectonemic supercoiling. This type is unique to prokaryotes, and allows the DNA to move upon itself in either a "slithering" (Marko, 2001) or "branch migration" (Staczek and Higgins, 1998) movement (Fig. 2). This formation not only helps in the compaction process, but also provides a mechanism that allows distant DNA sites to come into contract with each. The importance of plectonemic supercoiling will be discussed in detail later.

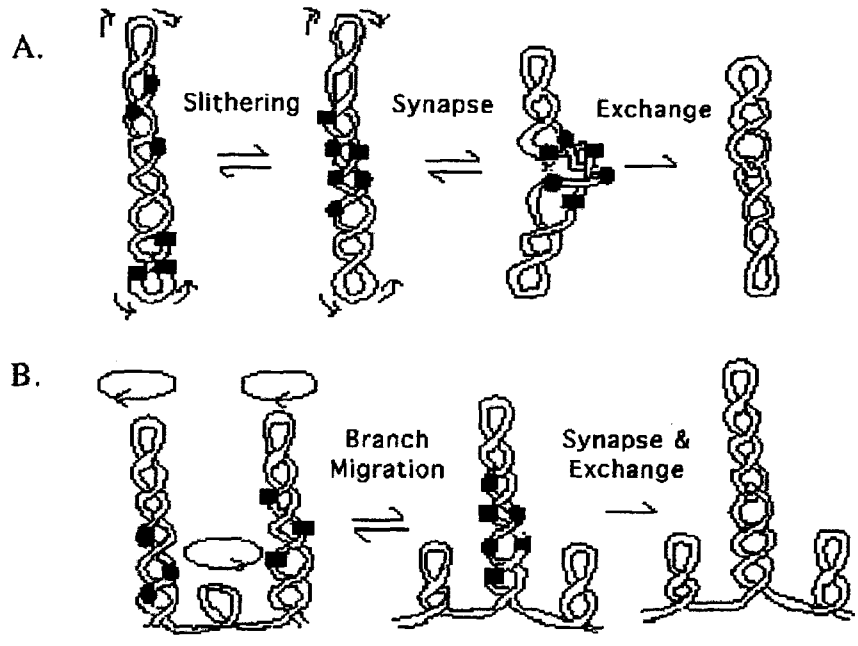

Figure 2. Slithering and Branch Migration. The plectonemic structure of unconstrained prokaryotic supercoils helps in compaction, and allows rapid movement against itself. This type of motion has two forms: (A) slithering and (B) branch migration. The figure shows how these motions can bring distant sites into close proximity. From "Gyrase and topo IV modulate chromosome domain size *in vivo*" by P. Staczek and N. Patrick Higgins, 1998, Molecular Microbiology 29(6), pp.1435-1448. Copyright 1998. Reprinted with permission from Blackwell publishing.

Though half of the supercoiling in prokaryotes exist in an unconstrained state, half are protein bound like eukaryotic DNA. In a rapidly growing *E. coli* cell, there are about 42,000 constrained supercoils (Higgins and Vologodskii, 2004). DNA footprinting has identified 55 DNA binding proteins potentially involved in their constraint (Robison *et al.*, 1998). A few of the major proteins known to be involved in supercoil constraint are discussed below.

RNA Polymerase. RNA polymerase is the tetrameric protein responsible for gene transcription, and is highly conserved in all organisms. In *E. coli* grown in rich media, about 1500 RNA polymerase molecules are actively transcribing at any given time (French and Miller, 1989). Each RNA polymerase can unwind and bind about 1.7 supercoils, which would compensate a ΔLk of about 2400 (Higgins *et al.*, 2005).

HU. HU is a heterodimeric DNA-binding protein consisting of HUα and HUβ subunits (Drlica and Rouviere-Yaniv, 1987). HU binding covers a 34 base pair site, and can bend DNA to angles over 150° (Johnson *et al.*, 2005). It is known to be a major component of the bacterial nucleoid (Rouviere-Yaniv and Gros, 1975), and in the presence of topoisomerase I, will introduce negative supercoils, suggesting that it constrains DNA *in vivo* (Bensaid *et al.*, 1996; Rouviere-Yaniv *et al.*, 1979). Exponential cells contain about 30,000 HU molecules (Azam *et al.*, 1999), and, at optimum *in vitro* binding conditions, 2.5 molecules restrain a single supercoil (Broyles and Pettijohn, 1986). Assuming the *in vivo* binding is similar, HU is responsible for constraining about 12,000 supercoils. This is equivalent to 15% of the negative supercoiling in the cell, and

correlates well with results from *hupAB* mutations in both *E. coli* and *Salmonella typhimurium* (Hillyard *et al.*, 1990).

H-NS. H-NS is a homodimeric protein that prefers binding to curved AT-rich DNA (Tendeng and Bertin, 2003). Exponential cells contain about 20,000 molecules of H-NS, with each binding to a 10 base pair site (Azam *et al.*, 1999; Lucht *et al.*, 1994; Rimsky, 2004). This is enough H-NS to constrain 3% of the supercoils in a cell. It is thought that H-NS first binds at a high-affinity site, and then spreads to form a filament on the DNA (Falconi *et al.*, 1993; Schnetz, 1995). H-NS is also known to affect the transcription of about 5% of the genes in *E. coli* (Hommais *et al.*, 2001).

Integration Host Factor. Integration Host Factor (IHF) is a heterodimeric protein, encoded by *ihfA* and *ihfB*, that is required for both Mu transcription (Higgins *et al.*, 1989) and λ integration (Miller and Friedman, 1980). IHF is highly homologous to HU, and also binds to a 34 base pair site (Johnson *et al.*, 2005). IHF bound to plasmid DNA in the presence of topoisomerase I does not show an increase in negative supercoiling, but is known to bend the DNA over 150° (Higgins *et al.*, 1989; Rice *et al.*, 1996). This bending is involved in the regulation of about 100 genes in *E. coli* (Arfin *et al.*, 2000). Assuming similar binding affinities as HU, IHF probably constrains about 6% of negative supercoils (Higgins and Vologodskii, 2004).

Factor for Inversion Stimulation. Factor for Inversion Stimulation (Fis) is a very abundant protein that is present at 30,000-60,000 copies in exponentially growing cells,

and decreases to approximately 100 copies in stationary phase (Ball *et al.*, 1992). Fis is important in regulating HU (Claret and Rouviere-Yaniv, 1996), and can repress the genes encoding DNA gyrase (Schneider *et al.*, 2001). Fis will weakly supercoil plasmid DNA in the presence of topoisomerase I. Primarily due to its abundance, Fis may account for 15% of the constrained negative supercoiling in *E. coli* (Higgins and Vologodskii, 2004).

Supercoil Domains

Both prokaryotic and eukaryotic DNA is segregated into chromosomal domains. This architecture not only helps to further condense the DNA, but also provides a layer of organization (Fig. 1). Separating DNA in this way allows processes in one domain to be unaffected by processes in neighboring domains. This also provides protection to the cell because a single break in the DNA will not cause global supercoiling loss.

Worcel & Burgi first demonstrated prokaryotic domains in 1972. Using chromosome sedimentation analysis, they demonstrated the need for more than one DNase I nick to completely relax the chromosome. By estimating the activity of DNase I in their reactions, they suggested the chromosome was divided into 12-80 domains. The authors also suggested a role for RNA in domain formation (Worcel and Burgi, 1972). A few years later, Pettijohn & Hecht supported the role of RNA in domain formation by showing that the genome becomes unfolded when treated with rifampicin (Pettijohn and Hecht, 1974). The first electron micrographs of the *E. coli* genome left no doubt as to the existence of domains, and showed a "rosette" pattern of genomic organization (Fig. 3).

These famous pictures show intact supercoiled domains adjacent to relaxed domains. From these micrographs, it was estimated that *E. coli* contained between 50-100 domains. These micrographs also included a matrix material at the center of the "rosette" thought to be important in domain organization. It was shown that this matrix contained RNA (Kavenoff and Bowen, 1976; Kavenoff and Ryder, 1976). The idea of RNA as a scaffold material for domains remained the favored explanation until the work of Sinden & Pettijohn in 1981. They used trimethylpsoralen binding to DNA to determine torsional strain, and demonstrated that more than one γ-irradiation induced nick was required for total genomic relaxation much like the conclusion of Worcel & Burgi nearly 10 years earlier. Their work also showed that each genome contained 43 ± 10 domains, a hypothesis that would remain for nearly 15 years. However, Sinden & Pettijohn went on to show that cells treated with rifampicin contained the same number of domains as those with normal RNA content. This proved that RNA was not solely responsible for partitioning, and forced a rethinking of domain formation. The persisting approximation of 43 ± 10 domains per genome equivalent suggested that each domain was roughly equal, and contained about 100 kilobase pairs of DNA (Sinden and Pettijohn, 1981). However, using a non-invasive supercoil-dependent γδ-recombination system, Higgins *et al.* showed that domain intervals are random, and the average domain is around 17 kb. This would suggest nearly 200 chromosomal domains (Higgins *et al.*, 1996). Later, using this same system with shorter half-life enzymes, Stein *et al.* demonstrated over 400 domains with a medial size of 9 kb (Stein *et al.*, 2005). Recently the Cozzarelli lab has confirmed these results using two independent methods. First, using a gentle lysis protocol and electron microscopy, they showed a "rosette" pattern with a medial domain

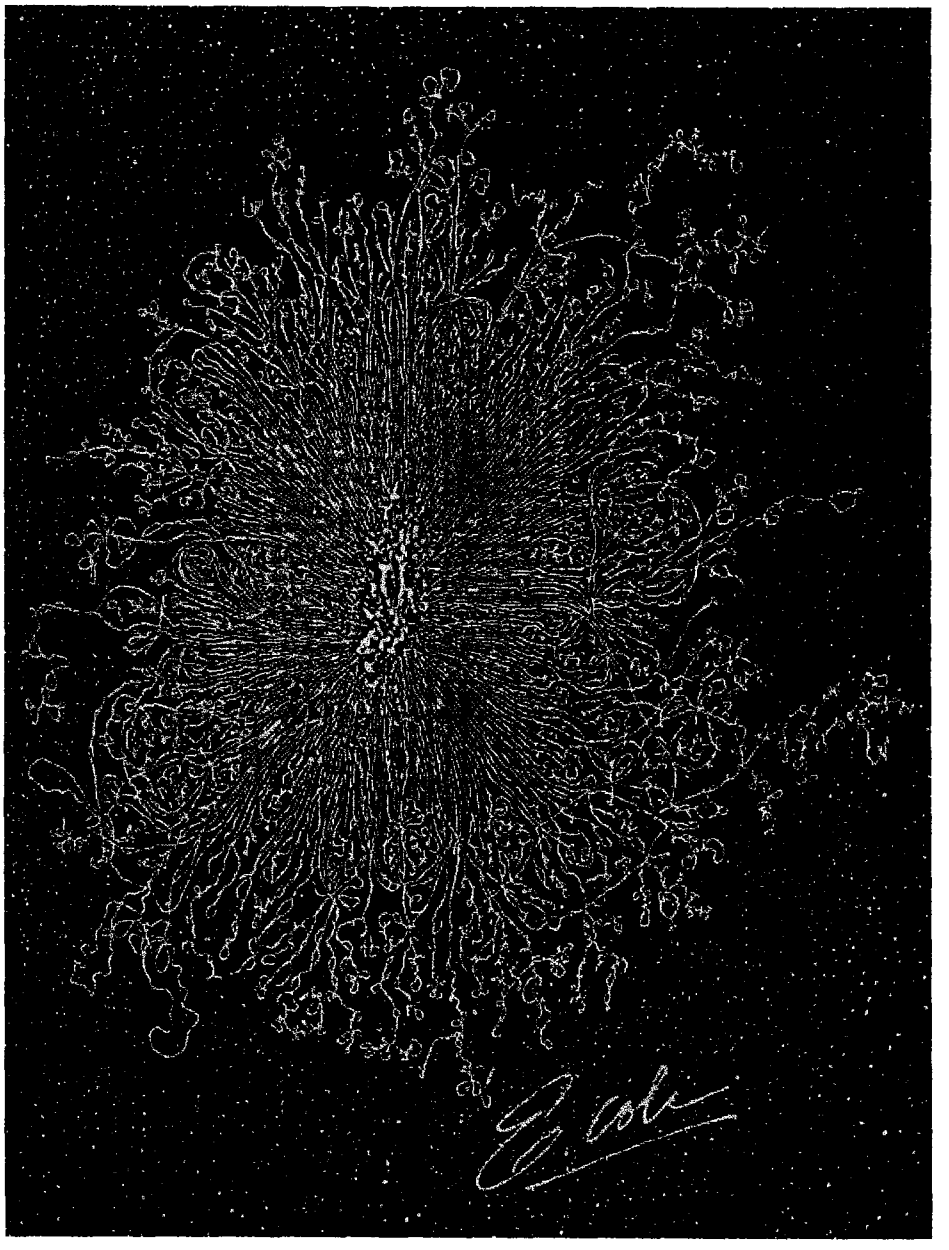

Figure 3. The *E. coli* Chromosome. This electron micrograph of a lysed *E. coli* cell shows the "rosette" pattern. The picture shows tightly supercoiled chromosomal domains adjacent to relaxed ones indicating domain barriers. From this micrograph, it was estimated that *E. coli* contains between 50-100 supercoil domains.

size of 11 kb. Next, by monitoring the expression of over 300 supercoil-sensitive genes in concert with site-directed restriction digests, they were able to determine how far relaxation could spread. These experiments showed a medial domain size of 9 kb (Postow *et al.*, 2004).

The precise components of supercoil domain barriers are still a matter for debate though three models dominate the literature. In 1987, Liu & Wang proposed the Twin Domain Theory (Fig. 4). Knowing that the RNA polymerase must rotate when transcribing DNA, they suggested that if the RNA polymerase is anchored and unable to rotate, then the DNA topology must change: positive supercoils will accumulate in front of the RNA polymerase, and negative supercoils will accumulate behind it (Liu and Wang, 1987). Using this theory as a guide, it was shown that co-transcriptional translation of membrane proteins could provide this anchoring of RNA polymerase (Lodge *et al.*, 1989). Later, many exported or membrane proteins were shown to be viable anchor points for plasmid DNA in *E. coli*. It was suggested that there were approximately 100 sites on the *E. coli* chromosome sufficient to provide this type of domain barrier (Lynch and Wang, 1993). Binenbaum *et al.* provided further evidence by showing that antibiotics that inhibit either transcription or translation change the viscosity of the membrane. They also showed that changes in membrane dynamics could "freeze" a domain in place. They use the term "transertion" to describe this co-transcriptional-translation-membrane insertion model (Binenbaum *et al.*, 1999). Mathematical analyses have shown that transertion could explain the size, number and spacing of domains (Woldringh, 2002).

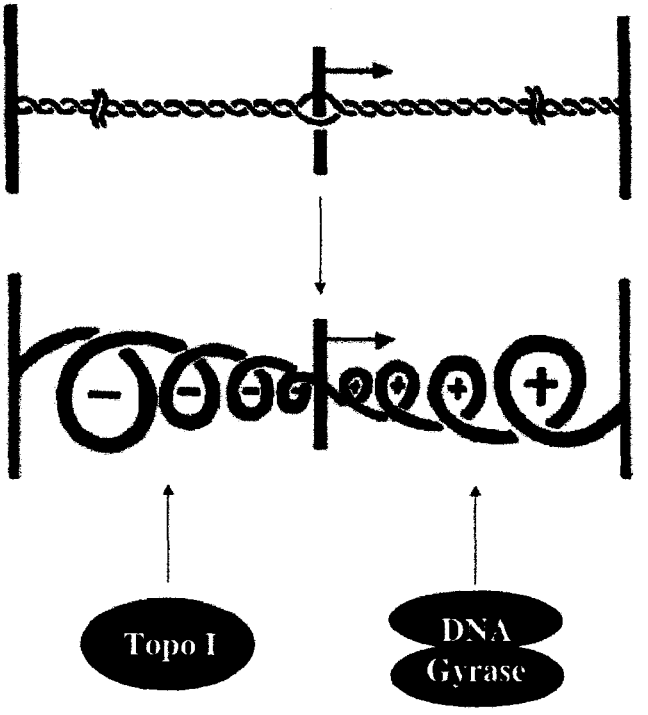

Figure 4. The Twin Domain Theory of Supercoiling. The Twin Domain Theory of Liu & Wang (Liu & Wang, 1987) describes the topological events resulting from processes that separate the Watson and Crick strands such as transcription and replication. As an example, when an RNA polymerase transcribes, it must rotate with the DNA unless the enzyme complex is anchored. In this case, the separating of strands creates a wave of positive supercoils in front of the molecule, and a wake of negative supercoils. Topoisomerase I removes the excess supercoiling behind RNA polymerase while DNA gyrase works to remove the torsional problems in front of the molecule.

DNA gyrase-DNA interactions have also been suggested as an explanation for chromosomal domains. This idea was first introduced when it was shown that chromosomes treated with oxolinic acid, which cleaves at active DNA gyrase sites, produced a similar number of breaks as did the current estimate of domains (Snyder and Drlica, 1979; Worcel and Burgi, 1972). From this, it was suggested that there was one strong DNA gyrase binding site per domain. Such sites, termed repetitive extragenic palindromic (REP) sites were soon found, and suggested a higher order of organization (Yang and Ames, 1988). However, it was later shown that DNA gyrase does not cleave at all REP sites, but at more infrequent sites called toposites (Condemine and Smith, 1990). Also, using more current predictions on the size and number of domains, it is obvious that there is no high-affinity DNA gyrase site for every domain. Even though a role for DNA gyrase in domain formation is commonly suggested, its precise role is uncertain.

The third commonly accepted model for domain formation involves partitioning machinery. These anchor points work by binding a protein or protein complex at a DNA site, and adhering that site to the cell membrane. Examples of this are the SpoOJ complex in *Bacillus subtilis*, which localizes the DNA to specific sub-cellular locations (Lin and Grossman, 1998), the SeqA protein in *E. coli*, which binds hemi-methylated DNA at mid-cell (Hiraga *et al.*, 1998), and the SopB protein of the F plasmid, which binds the *sopC* site and localizes the plasmid to the quarter-cell positions (Hiraga, 1992; Kim and Wang, 1998). Any such system, which uses a protein to bind the DNA to the membrane, could act as a domain barrier.

The finding of separate DNA supercoil domains with independent activities begged the question as to whether each domain had a different level of supercoiling. This was addressed specifically in both *E. coli* and *Salmonella* with similar results. Miller & Simons fused the supercoil sensitive promoter of *gyrA*, one subunit of DNA gyrase, to the *lacZ* gene. Then, using a modified Tn*10* transposon, they randomly integrated the module into 91 sites along the *E. coli* chromosome. By measuring β-galactosidase expression, they determined that all domains had an equal level of negative supercoiling (Miller and Simons, 1993). A weakness of this study is the assumption that the Tn*10* transposition was random. At the current time, the best estimate of the number of domains was 43 ± 10, established by Sinden & Pettijohn (Sinden and Pettijohn, 1981). Statistically, the insertion of 91 elements was enough to insert in each domain twice. Since more recent experiments put the proper domain number at over 400 (Stein *et al.*, 2005), a bias to similarly supercoiled domains could explain their uniform results. Also, of the 11 insertion sites they map, most are near the origin and terminus, further allowing the possibility of chromosomal site preference. At the same time, Pavitt & Simons did a similar experiment in *Salmonella.* They fused the promoter for *proU* to the *luxAB* genes as a reporter system, and used a directed recombination system to insert this cassette specifically into pre-mapped Tn*10* insertion sites. In agreement with the *E. coli* results, they found similar *luxAB* expression regardless of chromosomal location, suggesting a homogeneity in supercoil levels throughout the chromosome (Pavitt and Higgins, 1993). The directed insertion avoids the potential problems with the *E. coli* experiments, however, there has been some evidence that the *proU-luxAB* reporter system responds to potassium glutamate levels instead of supercoiling (Gowrishankar and Manna, 1996).

Regardless of the possible complications of these two studies, the idea that all domains are equally supercoiled is widely accepted, and believed to be an indicator of the effectiveness of the cellular topoisomerases in maintaining a correct level of global supercoiling.

The Role of Supercoiling in Physiological Processes

Not only does supercoiling provide a means for DNA condensation, but it is involved in many aspects of important cellular processes. As mentioned above, the under-winding of the double helix causes the DNA to be in a high-energy state. This provides the energy needed to separate the strands to allow access for complexes such as RNA polymerase or the replisome. The initiation of replication is an example of this. *E. coli* contains 4 DnaA boxes and 3 AT-rich 13mers in the *oriC*. The DnaA proteins bind to the DnaA boxes, and induce a bend into each. This binding causes the AT-rich cluster to become partially unwound, and form an open complex. The DnaB helicase can then bind, and allow access for the replication machinery (Fig. 8*A*). Correct negative supercoiling is of vital importance to this step (Messer *et al.*, 2001). Several *gyrB* mutations have shown initiation-defective phenotypes due to insufficient negative supercoiling (Filutowicz, 1980; Filutowicz and Jonczyk, 1981; Orr *et al.*, 1979). Along these lines, several temperature-sensitive *dnaA* mutants, which are defective in replication initiation, are suppressed by a *topA* deletion. The explanation is that the *topA* deletion causes a hyper-negative substrate that contains enough stress to allow melting of the AT-rich region without the aid of DnaA protein (Louarn *et al.*, 1984).

In addition, it has been shown that an under-wound molecule is essential in nearly all aspects of transcription. Promoter strength has shown the ability to be dramatically influenced by supercoiling. The precise effects are promoter specific (Record *et al.*, 1996), as demonstrated by *in vitro* experiments, showing that increases in supercoiling cause a 160-fold increase in the *lacUV5* promoter (Buc and McClure, 1985), a 50-fold increase in $lacP^S$ (Stefano and Gralla, 1982), a 20-fold increase in TAC16 and about a 4-fold increase in TAC17 (Su and McClure, 1994). The binding of RNA polymerase (Malan *et al.*, 1984; Wang *et al.*, 1974), RNAP elongation (Borowiec and Gralla, 1987; Hsieh and Wang, 1978), promoter clearance (Menzel and Gellert, 1987a, 1987b), transcriptional pausing (Krohn *et al.*, 1992) and termination (Carty and Menzel, 1989) have also been shown to be influenced by negative supercoiling.

A negatively supercoiled substrate is also required for many site-specific recombination systems. This is in addition to the plectonemic structures created by negative supercoiling addressed below. Lambda integrative recombination depends on proper supercoiling of the *attP* site. The supercoiled substrate enhances Int protein binding, and promotes recombination (Richet *et al.*, 1986). Negative supercoiling also determines the activation energy barrier for Tn*21* resolvase, and the reaction becomes very rare with lowered supercoiling (Castell and Halford, 1989).

Another aspect of DNA supercoiling is the plectonemic association of the helices. Since all eukaryotic DNA is wrapped around histone proteins, plectonemic supercoils are prokaryotic specific. In addition to condensation, this structure can undergo a "slithering" motion that allows distant DNA sequences to come into close contact with each other very quickly (Fig. 2) (Staczek and Higgins, 1998). This attribute has been

shown to be vitally important for DNA recombination, and for the regulation of many transcriptional promoters. In the Phage P1 Cre-*loxP* site-specific recombination system, it has been shown that a supercoiled DNA template produces significantly more recombinants than a nicked circular substrate. This is due to the plectonemic structure of supercoiled DNA, and the ability of distant *loxP* sites to come in contact (Abremski and Hoess, 1985). The initial DNA strand transfer event for Mu transposition requires a negatively supercoiled template that aligns the Mu ends in an inverted repeat orientation (Craigie and Mizuuchi, 1986).. Site-specific recombination catalyzed by Tn*3* has been shown to require plectonemic supercoils that adopt a specific structure for both the forward and reverse reactions (Stark *et al.*, 1989). Furthermore, it has been shown that the Tn*3* resolvase is able to scan along large lengths of DNA to find suitably orientated *res* sites, and bring them into proper alignment through plectonemic supercoiling (Benjamin *et al.*, 1985). Flagellin synthesis in *Salmonella* is controlled by an inversion reaction between *hin* sites. Proper supercoiling is required for the formation and maintenance of the *hin* sites in a complex with the DNA binding proteins Fis and HU (Johnson and Bruist, 1989). Plectonemic supercoiling can also be involved in transcriptional regulation. For example, the expression of *gal* is controlled by a structure involving GalR and HU binding to the O_E and O_I repressors, and forming a nucleoprotein complex with a looped out region of DNA (Aki and Adhya, 1997).

The Role of Supercoiling in Gene Expression

As mentioned above, negative supercoiling influences all aspects of transcription. It is also involved in forming correct structural complexes at promoter sites via plectonemic supercoiling. In addition to these influences, the actual level of supercoiling influences the regulation of many genes. A recent analysis of the *E. coli* chromosome showed that 7% of the genes were both directly and significantly affected by supercoiling levels (Peter *et al.*, 2004). When the downstream effects of supercoiling are included, an even greater number of genes can be considered supercoil-sensitive. Some of the best examples of this are the regulation of the topoisomerase genes. The genes for *gyrA* and *gyrB*, the subunits for DNA gyrase, are controlled by negative supercoiling in a feedback mechanism that allows the cell a way to regulate the total supercoiling level. The production of both subunits can undergo a 10-fold increase upon blockage of DNA gyrase activity. Conversely, the production can be decreased by increased negative supercoiling (Menzel and Gellert, 1983). Promoter fusions have shown that this regulation happens at the level of transcription (Menzel and Gellert, 1987b). An increase in supercoiling can allow easier strand separation, and explains how increases in supercoiling lead to increases in gene transcription. However, this does not explain how a decrease in supercoiling leads to an increase in transcription. Even more amazing is that the minimal control region for these genes is a 20 base pair sequence containing the –10 consensus region, the transcriptional start site, and the first few bases of the coding region (Menzel and Gellert, 1987a). The amount of supercoiling in the molecule is detected in the promoter region by the helical twist. These σ^{70} promoters are very sensitive to the relative orientation of the –10 and –35 regions, and the 17 base pair

spacer. When supercoiling is low, these regions lie on the same face of the DNA helix, and the gyrase genes are transcribed. When supercoiling is increased, the helical twist changes, and the regions are no longer simultaneously available to the RNA polymerase (Wang and Syvanen, 1992).

In the above study, which found that 7% of all *E. coli* genes were directly influenced by supercoiling, many gene classes were represented. As expected, genes involved in DNA replication and genome maintenance were found, but also found were genes feeding into many regulatory networks (Peter *et al.*, 2004). As the following examples demonstrate, supercoiling allows the cell a way to monitor the environment and adjust accordingly. Increases in extra-cellular osmolarity cause an increase in the degree of negative supercoiling. In turn, this supercoil change stimulates the expression of the *proU* gene up to 100-fold. This gene encodes a glycine betaine transport system that allows the cell to balance the external osmolarity and restore turgor. To emphasize the importance of negative supercoiling in this reaction, *topA* deletions can induce *proU* in normal osmolarity conditions (Higgins *et al.*, 1988). As another example, a lack of oxygen causes cells to switch to anaerobic growth that increases the degree of torsional strain. This change causes a repression in the *tonB* gene, which is involved in energy-coupled membrane processes, and is responsible for uptake of iron during aerobic growth. During anaerobic growth, this role, as well as the other roles of *tonB*, are unnecessary and repressed. In addition to *tonB*, it has been shown that over 50 genes are turned on during the switch to anaerobic growth while many more are turned off (Dorman *et al.*, 1988). Experiments also show that salt shock (McClellan *et al.*, 1990), anaerobic shock (Hsieh *et al.*, 1991), nutrient up-shift or down-shift (Balke and Gralla,

1987), growth phase (Dorman *et al.*, 1988), sporulation (Nicholson and Setlow, 1990) and temperature shifts (Dorman *et al.*, 1988) all influence the level of supercoiling, and are in turn affected by supercoiling through various regulatory networks (Drlica, 1992; Peter *et al.*, 2004).

Perhaps the most famous supercoil-sensitive promoter is *leu-500*. The *leu-500* mutation was first described in *S. typhimurium* (Mukai and Margolin, 1963), but has since been used and described in a variety of organisms. The *leu-500* promoter differs from the wild type *leu* promoter by an adenine to guanine mutation in the –10 region that makes the formation of the open complex more energetically expensive. Therefore, this promoter requires a high level of negative supercoiling for expression. This extra supercoiling can be provided by certain *topA* mutations (Chen *et al.*, 1992; Chen *et al.*, 1994).

The Level of Supercoiling

Closed circular DNA has intrinsic topological properties that must be defined and quantitated to numerically describe supercoiling. The first topological property to consider is the linking number (Lk). Lk is defined as the number of times one DNA strand crosses the other DNA strand when the molecule is made to lay flat (Fig. 5*A*) (Sinden, 1994). Lk will always be an integer value, and cannot be changed without cleavage of the phosphodiester backbone. Lk has two components to consider: twist (Tw) and writhe (Wr), which are not integer values, and can change in an uncleaved molecule. The Tw is defined as the number of helical turns. In a relaxed molecule,

where there is no Wr, Tw will always be equal to Lk. However, Wr is always present in free supercoiled DNA, and describes the value of the supercoiling in space. The combination of these three values define the topology of DNA in the equation (White, 1969):

$$Lk = Tw + Wr \qquad \text{(Eq.1)}$$

Therefore, in a closed circular molecule, changes in the Tw can only result in changes in Wr since Lk is a fixed value. This means that any stress to the molecule will either change the number of base pairs per turn, or cause spatial deformation of the helical axis (Vologodskii, 1999). A relaxed molecule has no Wr. The Lk of this relaxed molecule, Lk_0, can be determined by the simple equation

$$Lk_0 = N / \gamma \qquad \text{(Eq.2)}$$

where N is the number of base pairs in the molecule, and γ represents the number of base pairs per Watson-Crick helix. In normal B-form DNA, γ equals approximately 10.5. As an example, a 210 base pair plasmid will have a Lk_0 of 20 (Fig. 5*A*). In a supercoiled molecule, the Lk will not be equal to Lk_0, and the difference is called ΔLk. The helix in this supercoiled molecule will be under-wound, and will result in more base pairs per Watson-Crick helix (γ). This will create torsional strain that will change Tw. Since this is not the most thermodynamically favorable conformation, the change in Tw will be absorbed in Wr. Consider the following example with the 210 base pair plasmid (Sinden, 1994). During physiological processes, the backbone is cleaved, and two Lk are removed. The Lk and Tw value will initially change to 18, and there will be no Wr (Fig. 5*B*). However, the strain is unfavorable, and will be compensated by two negative supercoils (Wr) to allow the helical strain (Tw) to return to its most favorable value (Fig.

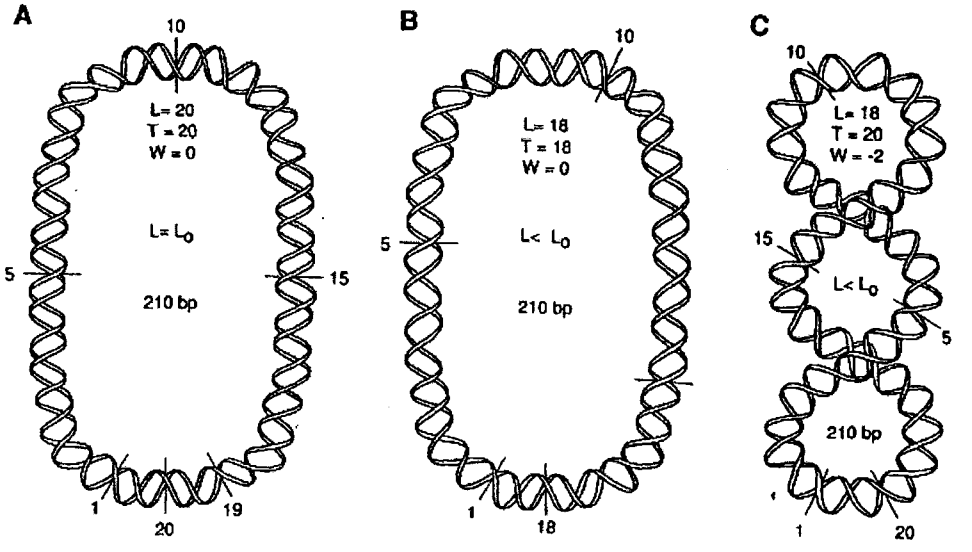

Figure 5. Quantitation of Negative Supercoils in a Closed Circular Molecule. (A) A planar (i.e. Wr = 0) 210 base pair plasmid has a Lk of 20 (210 base pairs / 10.5 bp/turn), and, therefore, a Tw of 20. (B) Cleaving the phosphodiester backbone allows the removal of 2 Lk giving a total of Lk = 18. Assuming the molecule is held planar (i.e. Wr = 0) means the Tw is also reduced to 18. (C) However, this form is not thermodynamically favorable, and the molecule writhes out of the plane to take up the excess stress and reset the Tw to 20. The Lk, unable to change without backbone cleavage, remains at 18. Since the Tw is reset to a favorable 20, the Wr now has a value of –2, and represents the 2 negative supercoils in the molecule. This is explained in the equation Lk = Tw + Wr. From "DNA Supercoiling" in *DNA Structure and Function.* San Diego, CA: Academic Press, pp.95-113. Copyright 1994. Reprinted with permission from Elsevier.

5*C*). At this point, the Lk remains 18, but the Tw has returned to 20. This means that Wr is equal to –2, which represents the 2 negative supercoils.

As a matter of convenience, ΔLk values are normalized to the length of the molecule, and given as supercoil density (σ). The following equation illustrates how to use ΔLk, and the known parameters of a molecule to determine σ :

$$\sigma = \Delta Lk / Lk_0 = \gamma (\Delta Lk / N) \qquad \text{(Eq.3)}$$

All known physiological DNA is negatively supercoiled, and thus has a negative σ. Normal σ values can range anywhere from –0.03 to –0.09, but are usually around –0.06 (Bauer *et al.*, 1980). A σ of –0.06 translates to a ΔLk of -25 for a plasmid the size of pBR322, or a ΔLk of over -25,000 for the *E. coli* genome. To emphasize the strain compensated by supercoiling, if the *E. coli* genome were not allowed to writhe, all the strain would be taken up by changes in Tw. This would decrease Tw by 25,000, and cause the Watson-Crick helix to accommodate 11.2 base pairs per turn instead of the normal 10.5. It has been shown that in physiological conditions, about 75% of ΔLk is compensated through Wr, and 25% is compensated through changes in Tw (Vologodskii and Cozzarelli, 1994).

Maintaining a normal level of supercoiling is vital for the cell. Through topoisomerase mutant analysis, it has been estimated that, for proper growth, supercoiling must be maintained within 15% of the median value (Drlica, 1992). Increases in supercoiling beyond the normal range can cause the formation of alternative DNA structures such as cruciform extrusion, left-handed Z-DNA, intermolecular triplexes with RNA (R-loops) and intramolecular triplexes (H-DNA) with single-stranded DNA from the same molecule (Higgins and Vologodskii, 2004). All of these structures may act to

block the DNA replication machinery and RNA polymerases. They may also become substrates for recombinational proteins such as Holliday Junction resolving enzymes. A decrease in negative supercoiling beyond the typical amount has its own set of potential problems. Most of these involve defects with proper DNA condensation, which can lead to impaired genome segregation, septum formation and cell division. In fact, the earliest partition defect phenotype, *parA*, which formed anucleate and guillotined cells, mapped to DNA gyrase (Hirota *et al.*, 1968; Kato *et al.*, 1989). It was also found that certain DNA gyrase mutations caused an increase in sedimentation rate that reflected an inability of daughter cells to separate from each other (Steck *et al.*, 1984). Along these lines, it has recently been shown that a *gyrB652* mutation in *Salmonella* can cause the loss of supercoil dynamics near the replication terminus that will cause fork collapse at high initiation rates (Pang *et al.*, 2005). These examples highlight the need for normal supercoiling levels to maintain proper genome partitioning.

DNA Topoisomerases

DNA topoisomerases are enzymes that break the phosphodiester backbone of DNA, and allow either strand passage or rotation, thus changing the linking number. They are involved in maintaining a proper level of supercoiling in the cell, and in decatenating daughter chromosomes after replication. They also work in front of and behind replication forks and RNA polymerase to prevent the accumulation of hyper-negative or relaxed DNA (Fig. 4). In prokaryotes, there are 4 topoisomerases: two each of Type I and Type II. The function of each is discussed below:

Type I Topoisomerases

Type I topoisomerases work by breaking a single DNA strand, and allowing a rotation of one strand around the other to change the linking number in increments of 1 (Fig. 6*A*). This family of topoisomerases can be further divided into Type IA and Type IB. Type IA includes prokaryotic topoisomerase I and topoisomerase III, as well as the eukaryotic topoisomerase III. These enzymes work by forming a 5'-phosphotyrosine linkage to the cleaved DNA in the presence of Mg^{2+} to partially relax negative substrates. Type IB, which includes eukaryotic topoisomerase I, works very similarly, but forms a 3'-phosphotyrosine linkage, and has no requirement for Mg^{2+}. These enzymes promote the complete relaxation of both positively and negatively supercoiled substrates (Sinden, 1994; Tse-Dinh, 1998). For our discussion, we only need consider the prokaryotic topoisomerases of Type IA.

Topoisomerase I. Topoisomerase I, encoded by the *topA* gene, was first discovered as the omega protein in *E. coli*. Topoisomerase I represents the major relaxing activity in the cell to counteract the supercoiling activity of DNA gyrase. As mentioned above, topoisomerase I does not relax DNA to completion. This may be due to the preference of topoisomerase I for more negatively supercoiled DNA over less negatively supercoiled DNA (Wang, 1971). This can be explained by the propensity of very negatively supercoiled DNA to provide easier strand separation to allow enzyme access. A good example of the importance of topoisomerase I is provided by the Twin Supercoiling Domain Model (Fig. 4). In this model, a translocating replication fork or RNA polymerase will cause a wave of positive supercoils in front while causing hyper-

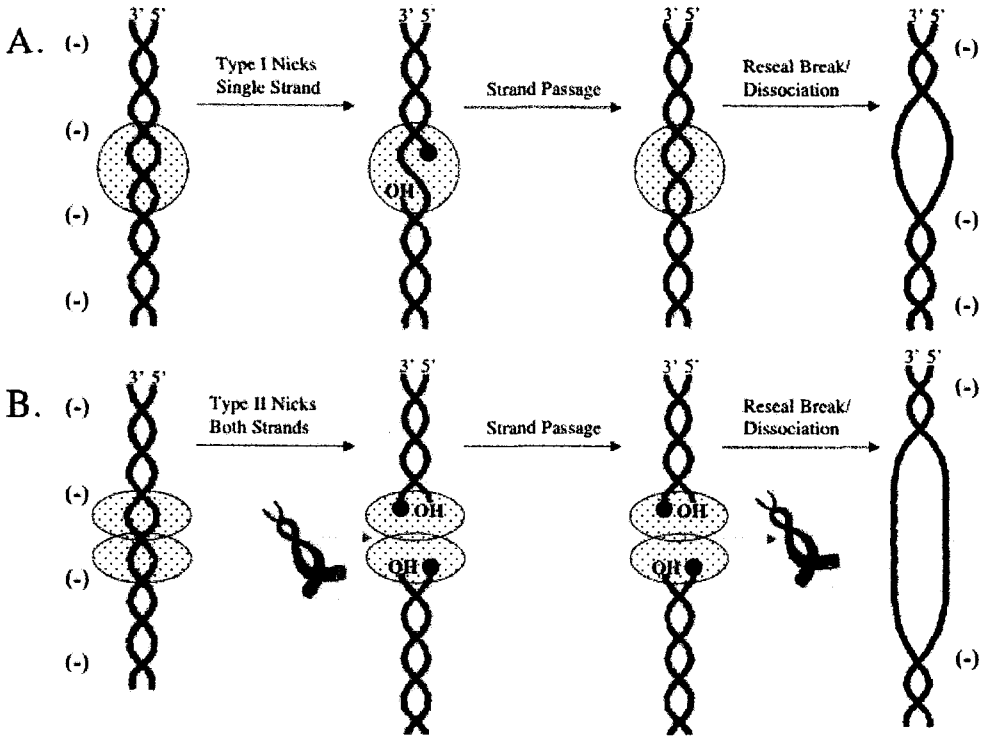

Figure 6. Type I and Type II Topoisomerases. (A) Type I topoisomerases work by cleaving a single strand, and allowing strand passage. In the figure, this is shown by the movement of the red strand from the foreground to the background. This process changes the linking number in increments of 1. (B) Type II topoisomerases cleave both strands, and promote the passage of another segment of the same molecule through the break. This process changes the linking number in increments of 2. The example shows the removal of 2 negative supercoils, however, the Type II enzyme DNA gyrase can actively introduce negative supercoils in increments of 2.

negative supercoiling in its wake. Topoisomerase I can act behind the fork to relax this hyper-negative supercoiling while DNA gyrase actively supercoils the DNA before the fork to prevent relaxation (Liu and Wang, 1987). This view of topoisomerase I and DNA gyrase working together to set the supercoiling level emerged through early *topA* deletion experiments. Initially, it was thought that a *topA* deletion was viable in *E. coli* (Sternglanz *et al.*, 1981). However, it was later shown that this deletion was only allowed when the cell acquired a compensatory mutation. This inability of *E. coli* to tolerate a Δ*topA* allele without compensatory mutation was confirmed through several independent experiments. DiNardo *et al.* showed that a Δ*topA* mutation could not be moved to a wild type background with ϕP1 transduction (DiNardo *et al.*, 1982). Pruss *et al.* showed that certain *topA* point mutations caused the expected increase in negative supercoiling, but, when the locus was repaired, the resulting strain became under-supercoiled (Pruss *et al.*, 1982). Zumstein & Wang created many *topA* constructs, and tested their ability to suppress a temperature sensitive *topA* strain to determine what regions of topoisomerase I were essential. They found that if the new construct did not suppress the temperature sensitive phenotype, the strain was dead without a compensatory mutation (Zumstein and Wang, 1986). The most common compensatory mutations mapped to the genes for DNA gyrase. This suggested an importance in the level of negative DNA supercoiling, and implied the involvement of topoisomerase I and DNA gyrase to establish this level. Importantly, it was later shown that a *topA* deletion could survive without a compensatory mutation in *S. typhimurium* (Richardson *et al.*, 1984) and in *Shigella flexneri* (Bhriain and Dorman, 1993). These results suggest a difference in the supercoiling requirement between these species.

A nice example of the consequences of hyper-negative supercoiling in the absence of topoisomerase I is given by the formation of R-loops. Using *in vitro* reactions that contained DNA gyrase without topoisomerase I, it was shown that hyper-negative pBR322 molecules are formed which contain an intermolecular triplex with a nascent RNA molecule, a structure termed an R-loop (Drolet *et al.*, 1994). A subsequent experiment showed that the addition of topoisomerase I to the above reaction prevented R-loop formation. Examples of this *in vivo,* using *topA* mutations, have also been demonstrated, and a link between R-loop formation and serious growth problems has been established. It was also shown that overproduction of RNase H (*rnhA*), which will degrade the RNA in an R-loop, would partially suppress the *topA* mutation. In addition, defects in the genes encoding DNA gyrase can correct some phenotypes associated with improper RNase H production, and a *topA rnhA* double mutant is lethal (Drolet *et al.*, 1995).

Topoisomerase III. Topoisomerase III is a dispensable enzyme encoded by the *topB* gene. It was first detected as a DNA relaxing enzyme in strains deficient in topoisomerase I (Dean *et al.*, 1983). Structurally, topoisomerase III is very similar to topoisomerase I, but is missing a Zn-finger domain near the C-terminal end (Tse-Dinh, 1998). There is no known role for topoisomerase III, and the only known phenotype of a Δ*topB* mutation is a 5-fold increase in RecA-independent recombination between direct repeats (Schofield *et al.*, 1992). However, it has been shown *in vitro* that topoisomerase III is a very efficient decatenase, and can promote DNA replication elongation through a weak relaxing activity. The decatenase activity is enhanced in the presence of small gaps

in the DNA suggesting a requirement of single-stranded DNA for binding (DiGate and Marians, 1988). This activity may explain the observation that high copy expression of *topB* will suppress a *parC1215* mutation (Nurse *et al.*, 2003). Because of this activity, it has been proposed that topoisomerase III acts as a backup system to the decatenating activity of topoisomerase IV (Hiasa *et al.*, 1994; Tse-Dinh, 1998).

Type II Topoisomerases

Type II topoisomerases are found and conserved in all organisms (Huang, 1994). They share very little amino acid identity with Type I topoisomerases (Huang, 1996), and their mechanism of action is quite different. Type II topoisomerases break both strands of DNA, and change the linking number in increments of 2 in an ATP-dependent reaction (Fig. 6*B*). All Type II's are heterotetrameric complexes with conserved ATPase and DNA-binding domains (Champoux, 2001). The heterotetramer consists of two dimers whose interface forms a "gate" region that is responsible for DNA passage (Schoeffler and Berger, 2005). The DNA binds to this "gate", or "G-segment", and is cleaved by the enzyme. Another region of DNA then binds to form a "transport", or "T-segment", which is passed through the "G-segment" within the enzyme, and exits through an opening on the other side of the molecule (Levine *et al.*, 1998; Mizzuuchi *et al.*, 1980). Because Type II enzymes form transient double-stranded breaks, they have become a valuable target for both antibacterial and chemotherapy drugs (Schoeffler and Berger, 2005).

Prokaryotes contain two essential Type II topoisomerases, DNA gyrase and topoisomerase IV. As discussed in detail below, these two enzymes are highly

homologous, but have very different cellular functions. DNA gyrase works to actively introduce negative supercoils into the chromosome while topoisomerase IV contains the primary decatenating activity. A structural explanation for such varied activities in highly homologous proteins has recently been explained (Corbett *et al.*, 2004; Hsieh *et al.*, 2004). The subunits for DNA gyrase, GyrA and GyrB, and topoisomerase IV, ParC and ParE, can be aligned over their entire lengths including the C-terminal domain (CTD) of GyrA/ParC, a region that is unstructured in eukaryotic enzymes. Corbett *et al.* have shown that the CTD of DNA gyrase forms a unique 6-propeller β-pinwheel structure that is held tightly closed by a region termed the GyrA Box (Corbett *et al.*, 2004). Hsieh *et al.* then showed that the CTD of topoisomerase IV also forms a 6-propeller β-pinwheel structure, but is left in a more broken state due to the absence of a GyrA Box (Hsieh *et al.*, 2004). This difference may explain how DNA gyrase is able to wrap DNA and introduce negative supercoiling while topoisomerase IV is unable to wrap DNA, and therefore functions best as a decatenase.

DNA Gyrase. DNA gyrase is unique in its ability to introduce negative supercoils into DNA. It was discovered in 1976 in a screen to identify elements responsible for λ integration (Gellert *et al.*, 1976). Later the genes encoding the two subunits were mapped to *nalA* and *cou*, named such because of their known susceptibility to naladixic acid and coumermycin, and were renamed *gyrA* and *gyrB* (Reece and Maxwell, 1991a). These genes encode the GyrA and GyrB subunits, which form an active A_2B_2 tetramer (Krueger *et al.*, 1990) that binds to a 140 base pair region of DNA (Klevan and Wang, 1980). The GyrA subunit is responsible for DNA breakage and ligation (Sugino *et al.*, 1977), and the

GyrB subunit houses the ATPase domain (Mizuuchi *et al.*, 1978). The expression of *gyrA* and *gyrB* is under homeostatic control such that when supercoil levels are high, the expression is repressed, and when supercoil levels fall, the genes are turned up (Menzel and Gellert, 1983). It was shown that if you added DNA gyrase without ATP to nicked circular plasmid DNA in the presence of DNA ligase, the plasmid is converted into positively supercoiled DNA (Liu and Wang, 1978a). This suggested that DNA gyrase was wrapping DNA. This was confirmed with nuclease degradation experiments, which also showed a wrapping action involving 140 base pairs (Liu and Wang, 1978b). The linking difference caused by this wrapping has been measured at approximately 0.6 to 0.8 suggesting that DNA gyrase wraps DNA around itself nearly a full turn (Liu and Wang, 1978a; Reece, 1990). This wrapping action can explain the mechanism of DNA supercoiling by DNA gyrase. DNA gyrase binds DNA, and wraps it into a positive node. This creates a positive supercoil that is compensated by a negative supercoil elsewhere in the molecule. The enzyme then cleaves the DNA, allows strand passage, and religates the ends together. This action has increased the supercoiling by two, leaving the molecule with one positive supercoil caused by DNA gyrase binding, and three negative plectonemic supercoils. When the enzyme releases, the positive supercoil and one negative supercoil are relieved, leaving a net supercoil change of two (Reece and Maxwell, 1991b). As with all Type II enzymes, this reaction requires ATP and Mg^{2+}. The ATP molecule is required to reopen DNA gyrase, when one reaction has completed, to "reset" the enzyme (Roca and Wang, 1994). Because of this, DNA gyrase in the absence of ATP has a slight relaxation activity 20 to 40-fold less efficient than the supercoiling reaction (Reece and Maxwell, 1991b). The enzyme is also highly processive

which allows elongation of the replication forks and RNA polymerase. In *in vitro* reactions, DNA gyrase can catalyze 100 reactions per minute. This calculates to one ΔLk/second (Higgins *et al.*, 1978).

As mentioned above, one of the major roles of DNA gyrase in the cell is to allow both replication fork and RNA polymerase progression (Fig. 4). The Twin Domain Theory suggests that both of these protein complexes force a wave of positive supercoils in front of them that must be removed by DNA gyrase (Liu and Wang, 1987). As an indication of this, the DNA gyrase inhibitors, nalidixic acid and coumermycin, were originally discovered as replication inhibitors (Drlica *et al.*, 1980). In addition, DNA gyrase serves the critical role, along with topoisomerase I, of maintaining a suitable level of negative supercoiling in the cell. As discussed in detail previously, this level is important for gene expression and proper nucleoid condensation. In addition to these major roles, DNA gyrase also has the ability to remove knots (Shishido *et al.*, 1987), bend and fold DNA, and provide a low level of decatenation.

The *gyrB652* allele of *Salmonella typhimurium* results from a single base pair transversion that causes an arginine to serine change at residue 436 (R436S). Since this mutation lies near the GyrA-GyrB interface, it may affect the interaction of the subunits. Gari *et al.* originally reported this mutation, as well as the complex phenotype of these strains. They observed that these cells stop growing at temperatures above 42°C. In addition, at lower temperatures, the cells were induced for the SOS response, had RecBCD-dependent DNA degradation, and were killed when combined with a *recA* or *recB/C* mutation. Their explanation for this set of phenotypes was that *gyrB652* formed a temperature sensitive enzyme that caused fork collapse, and led to double strand breaks

which RecBCD then degraded (Gari *et al.*, 1996). Later, Staczek & Higgins showed that strains with the *gyrB652* mutation contained twice as many domain barriers as wild type cells (Staczek and Higgins, 1998). Recently, Pang *et al.* demonstrated that enzyme formed with the GyrB652 subunit was not temperature sensitive *in vivo* or *in vitro*. However, the enzyme had a very low catalytic efficiency, k_{cat}, at all temperatures. They showed that these strains have frequent replication fork collapse, and that the topological problems increase closer to the replication terminus. With the increase in domain number in these cells, they noted that these strains would have more domains than DNA gyrase molecules. To exacerbate this, the GyrB652 molecules are inefficient. To explain the temperature sensitive phenotype, as well as the other observations, they proposed that the increase in replication initiation at high temperatures was responsible. These cells contain more domains, and have inefficient DNA gyrase, and are therefore prone to replication fork stalling due to increased positive supercoiling in front of the replication machinery. In these cells, if a fork is stalled or broken, the next fork will overtake it and produce double strand DNA ends. These would then induce the SOS system, be degraded by RecBCD, and explain the temperature sensitivity of these strains (Pang *et al.*, 2005).

Topoisomerase IV. Topoisomerase IV contains the cells major decatenase activity needed to unlink daughter chromosomes from each other as a result of homologous recombination during replication. In 1988, Kato *et al.* discovered a partition mutant, *parC*, that was unrelated to DNA gyrase or other known partitioning mutants (Kato *et al.*, 1988). Later *parC* was sequenced, and found to be homologous to *gyrA*.

Subsequently, a *gyrB* homolog that also had a partitioning phenotype, *parE*, was found upstream. They found a complex of ParC/ParE in crude extracts, and demonstrated that it could relax negative supercoils. They named this new enzyme topoisomerase IV (Kato *et al.*, 1990). Soon after, topoisomerase IV was found in *Salmonella* (Luttinger *et al.*, 1991), and it was shown to be a heterotetramer made up of two subunits of ParC and two subunits of ParE much like the composition of DNA gyrase (Peng and Marians, 1993). The enzymes role in the cell began to be elucidated when it was discovered that cells with inhibited topoisomerase IV accumulated catenated plasmids (Adams *et al.*, 1992). A model of the replication fork soon emerged showing that it forms positive torsional strain of two types as it progresses: positive supercoiling in front of the fork, and right-handed catenane windings, or precatenanes, behind the fork. With DNA gyrase working in front of the fork, it was hypothesized that topoisomerase IV would work behind the fork to remove the precatenanes. This decatenation activity is of elevated importance in the terminal stages of DNA replication when daughter chromosomes must be untangled for chromosome partitioning to take place (Hiasa and Marians, 1996). This terminal activity was further progressed by the finding, through quinolone-induced cleavage, that topoisomerase IV preferentially worked at the *dif* site in the replication terminus (Hojgaard *et al.*, 1999). The finding that topoisomerase IV physically interacts with FtsK, a septal-binding protein, strengthened this idea. It was also shown that this interaction with FtsK actually stimulates topoisomerase IV activity (Espeli *et al.*, 2003). In addition to its decatenation role, it has recently been suggested that topoisomerase IV contributes to maintaining the supercoil level of the cell. This contribution was minor, compared to topoisomerase I and DNA gyrase, but important. This role may be

explained by the observation that topoisomerase IV can relax DNA at a rate 6-fold less than topoisomerase I, but unlike topoisomerase I, can relax DNA to completion (Zechiedrich *et al.*, 2000).

Biological Systems Affected by Supercoiling

MukB

Proper partitioning of the newly replicated daughter chromosomes is of critical importance. Partitioning abnormalities can cause cell filamentation, anucleate cells and cell death. By the early 1990's, several genes had been identified whose deletions resulted in the *par* phenotype. However, most of these mapped to topoisomerase genes that caused the defect because of replication problems. Hiraga *et al.* developed a screen that allowed them to identify non-lethal mutations that produced frequent anucleate cells. From this screen, they found a mutant that formed distinct nucleoids, but had frequent anucleate cells and the corresponding diploid cells. They termed this mutant *muk* from the Japanese work "mukaku" meaning anucleate (Hiraga *et al.*, 1989). The gene product, MukB, was later shown to be a large 177 kDa protein with DNA-binding and ATPase domains (Fig. 7*A*). The dimer formed a rod-like structure with globular ends and a hinge domain (Fig. 7*B*) (Niki *et al.*, 1991), and was found to associate with the MukE and MukF proteins (Yamazoe *et al.*, 1999). From its unique structure, and its involvement with condensation and segregation, MukB was classified as a structural maintenance of chromosome (SMC) protein (Britton *et al.*, 1998). SMC proteins are present in nearly all organisms, and are involved in chromosome condensation (Fig. 1), segregation and sister

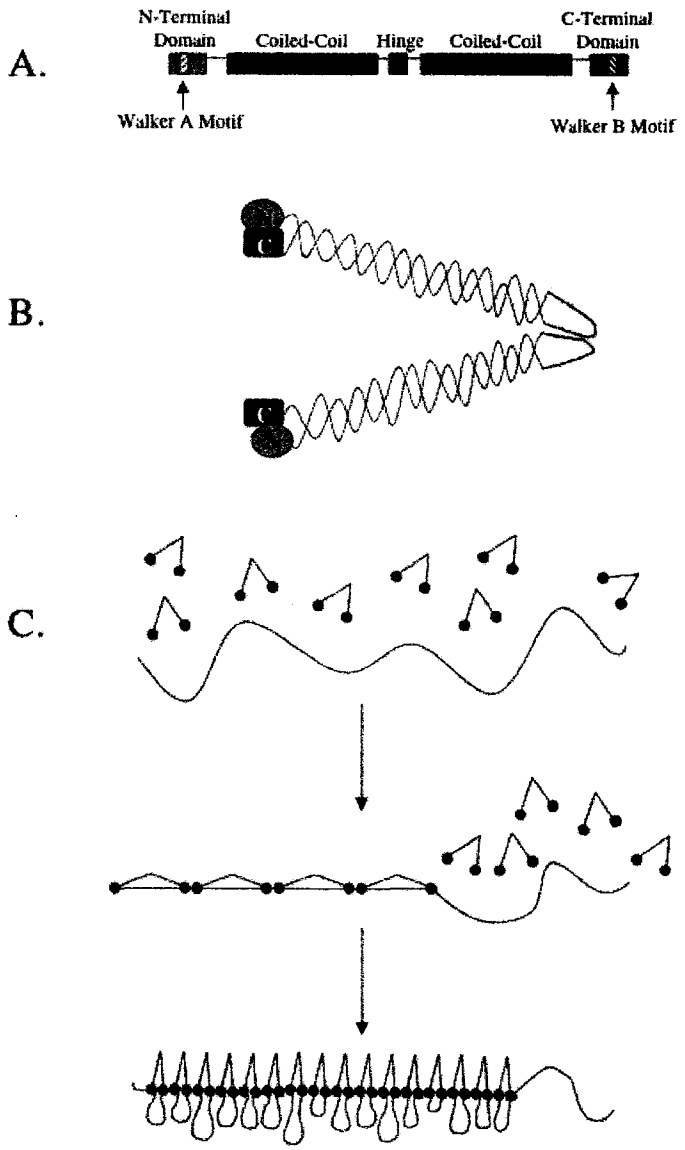

Figure 7. Structural Maintenance of Chromosome (SMC) Proteins. (A) SMC proteins are highly conserved with globular domains at each end separated by two coiled-coil regions and a hinge. (B) The active enzyme forms a dimer that can accommodate angles from 0° to 180°. (C) The illustration shows how SMC proteins in the open conformation bind unordered DNA, and organize it into repetitive loops.

chromatid cohesion. Eukaryotic condensin and cohesin are well known SMC proteins (Graumann, 2001). In addition to the anucleate cell formation, *muk* mutants exhibit a slow sedimentation rate, and a spread-out appearance upon DAPI staining (Weitao *et al.*, 1999). More recently, it has been shown that chromosomes in *muk* mutants no longer segregate to their correct sub-cellular location (Niki *et al.*, 2000). These phenotypes have given rise to a model in which the MukBEF complex binds and condenses newly replicated DNA coming out of the replication fork (Fig. 7*C*), and delivers it to the correct cellular location (Graumann, 2001). It has been demonstrated that a mutation in the *topA* gene will partially suppress the *muk* phenotype. The observation that increased levels of negative supercoiling can alleviate the *par* phenotype confirms the role of MukBEF in DNA condensation (Sawitzke and Austin, 2000). Similarly, it was shown that mutations in the *seqA* gene would also suppress the Δ*muk* phenotype (Crooke, 1995). The reasons for this will be discussed below in the SeqA section.

SeqA

SeqA was initially identified as a protein involved in the negative regulation of DNA replication initiation (Lu *et al.*, 1994). The *oriC* region of the chromosome contains 4 unique 9mers, called DnaA boxes, that have a high affinity for the ATP-bound form of DnaA (Fig. 8A) (Fuller *et al.*, 1984). When DnaA binds these sequences, it forms a complex along with IHF or HU (Hwang and Kornberg, 1992). With sufficient negative supercoiling, this causes the strands of 3 AT-rich 13mers nearby to separate, and allow access to the DnaB helicase to begin replication (Fig. 8*A*) (Baker *et al.*, 1986;

Bramhill and Kornberg, 1988; Funnell *et al.*, 1986). The *oriC* region also contains many GATC sequences. The adenine N^6 in this sequence is the preferred substrate for *dam* methylase (Bakker and Smith, 1989). When both strands are methylated, the region is competent for replication. After replication, the new strand is unmethylated leaving the GATC in a hemi-methylated state, the preferred substrate for SeqA (Fig. 8*B*). The SeqA protein binds to two hemi-methylated GATC sites, and adheres them to the membrane to prevent re-initiation until *dam* has the opportunity to methylate the newly synthesized strand thus preventing over-initiation (Brendler *et al.*, 1995; Brendler and Austin, 1999; Shakibai *et al.*, 1998).

Several subsequent experiments indicated that SeqA had an activity in the cell separate from its role in replication initiation. SeqA has the potential to bind any hemi-methylated GATC site in the chromosome (Slominska *et al.*, 2001), and SeqA-GFP foci localize to mid-cell even in Δ*oriC* strains. After replication, they move to the cell quarter-sites, and this localization is dependent on MukB (Hiraga *et al.*, 1998). In addition, cells with Δ*seqA* mutations exhibit a drastic change in their transcriptional domain pattern (Lobner-Olesen *et al.*, 2003). The Δ*seqA* strain was also shown to filament, have asymmetric septation, produce anucleate cells and form compact nucleoids (Nordstrom and Dasgupta, 2001). Surprisingly, the mutant was shown to suppress the Δ*mukB* phenotype (Weitao *et al.*, 1999). This is partially explained by the SeqA and MukB influence on supercoiling levels. It was shown that Δ*seqA* cells have increased levels of negative supercoiling while Δ*mukB* cells have decreased levels. This is visible in the diffuse nucleoids of Δ*mukB* cells, and the compact nucleoids of Δ*seqA* cells. This pattern was present, but not nearly as dramatic in plasmid DNA, suggesting that they play

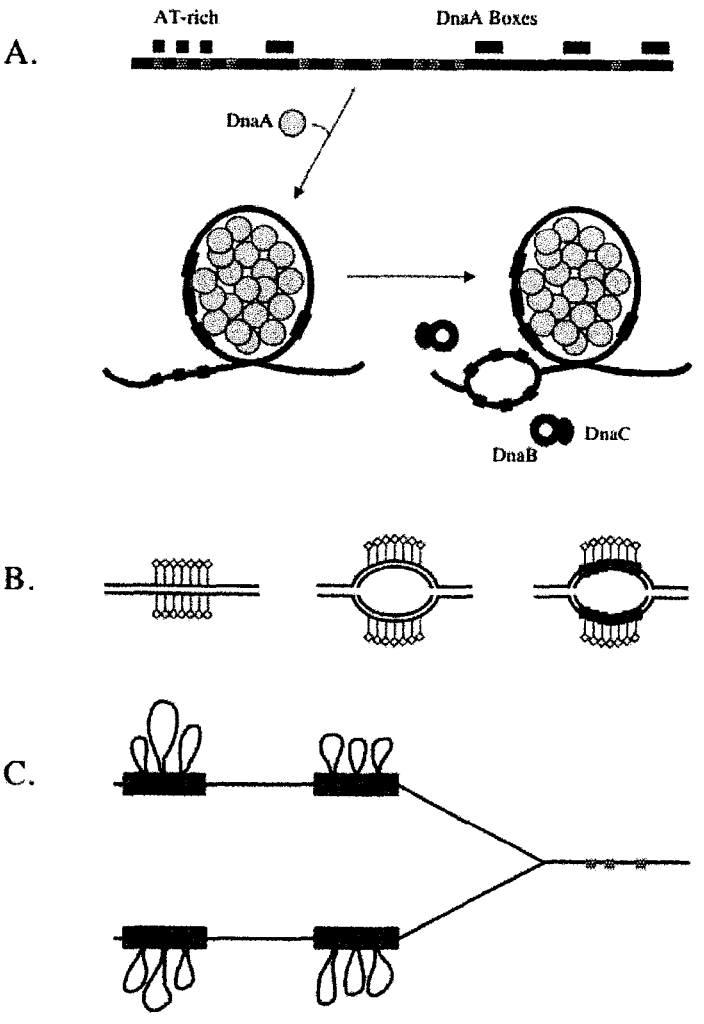

Figure 8. The SeqA Protein. (A) The *oriC* region contains 4 9mer DnaA boxes (blue boxes) and 3 AT-rich 13mers (red boxes). This region also contains many GATC sequences (gray boxes). When DnaA binds it forms a structure that, with sufficient negative supercoiling, causes the AT-rich regions to form an "open complex" to allow access to DnaB and DnaC to initiate replication. (B) When GATC sequences are fully methylated (diamonds), they do not bind the SeqA protein (orange boxes), and replication initiation can occur. After replication, the GATC sequences are in a hemi-methylated state, which is the preferred substrate for SeqA. SeqA binds hemi-methylated *oriC* to the membrane, and prevents further replication initiation until the origin becomes fully methylated by *dam*. (C) SeqA also binds to GATC sequences at the replication fork. It is proposed that they organize newly replicated DNA immediately after replication.

roles, not only in setting supercoil levels, but also in domain organization (Weitao *et al.*, 2000). It has been proposed that SeqA binds newly replicated DNA at the replication fork (Fig. 8*C*), and tethers it to the division plane (Brendler *et al.*, 2000). This binding will protect the DNA from topoisomerase I (Torheim and Skarstad, 1999) until methylation causes a change from SeqA to MukB binding. The MukBEF complex will then recondense the DNA, and segregate it to its proper address in the cell (Datsenko and Wanner, 2000).

Bacteriophage Mu

Bacteriophage Mu is a 36 kb element that infects many different gram-negative bacteria. It replicates via replicative transposition, and thus acts as a fully active transposon as well as a bacteriophage. Once Mu inserts into the genome, it can opt for one of two pathways. By repressing the lytic P_E promoter, it can exist as a latent prophage. Otherwise, it can de-repress the P_E promoter, and enter the lytic cycle where it will undergo replicative transposition until 100-200 copies are produced (North and Nakai, 2005).

This pathway decision is modulated by the Mu repressor (Rep), encoded by the *c* gene, along with host IHF and the negative supercoil level of the cell. It has been demonstrated that Mu can infect ΔIHF strains, but cannot produce a lytic infection (Miller and Friedman, 1980; Yoshida *et al.*, 1982). It has also been shown that mutant *gyrB* alleles, which cause a decrease in negative supercoiling, only allow the lysogenic state (Ross *et al.*, 1986). This link to negative supercoiling was directly shown as increases in superhelical density caused a shift in IHF that repressed transcription from

the *c* gene. This caused a de-repression of the lytic phase, and increased lysis by 40 fold (Higgins *et al.*, 1989).

Experimental Procedures Used

Two-Dimensional Gel Electrophoresis

There are several methods to determine the amount of supercoiling in a closed plasmid molecule. Early experiments attempted to quantitate this amount by counting cross-over events they could see in electron micrographs (Liu and Wang, 1975). However, this method was highly unreliable. Bauer & Vinograd developed a method that depended on predictable changes in the sedimentation coefficient in sucrose gradients to determine supercoil levels. The sedimentation coefficient of a plasmid is maximal for highly supercoiled DNA, and becomes less with unwinding. They predicted that adding the intercalating dye, ethidium bromide, would cause an unwinding of the helix, and a corresponding decrease in sedimentation coefficient. By applying plasmid DNA to a series of sucrose gradients with increasing amounts of ethidium bromide, they were able to determine a sedimentation minimum corresponding to relaxed DNA (Bauer and Vinograd, 1968). Any subsequent binding will cause the DNA to become over-wound, result in positive supercoils, and thus cause an increase in the sedimentation coefficient. This relationship is defined by the equation

$$\nu = 360^{\circ} (\Delta Lk) / (\varphi N) \qquad \text{(Eq.4)}$$

where ν is the number of molecules bound per base pair, which can be determined by spectral methods, and φ is the degree change in angle caused by binding of a single

intercalating molecule, which for ethidium bromide is 26° (Sinden, 1994; Vologodskii, 1999).

Walter Keller proposed a simpler, more accurate method in 1975. Since ΔLk can only change by integers of one, he proposed that, under the optimum electrophoresis conditions, bands corresponding to individual topoisomers could be distinguished, and an average ΔLk could be determined (Fig. 9). He found that SV40 DNA ran in a Gaussian-like distribution around a mean value of ΔLk (Keller, 1975). Soon after this, Shure & Vinograd used the Keller electrophoresis method to determine absolute Lk instead of ΔLk. By running a supercoiled population next to a relaxed population, they were able to count back from the relaxed band to determine what they termed titratable or potential Lk. This is now called the band counting method (Shure and Vinograd, 1976). Later, this technology was advanced again by Lee *et al.* by running samples in two dimensions (Fig. 9). They ran the samples in the first dimension as usual, then turned the gel 90°, and ran the gel in a second dimension with a calculated amount of intercalating agent. Using this technique, they were able to more clearly resolve topoisomers near the nicked circular band, and resolve positive from negative supercoils (Lee *et al.*, 1981). This technology is now routinely used to detect alternative DNA structures such as Z-DNA formation (Wang *et al.*, 1983). Conditions for two-dimensional gels depend on the plasmid used, the intercalating agent, and vary according to investigator. Using chloroquine as the intercalating agent, a common strategy is to use low amounts in the first dimension to bring the distribution into the resolving power of agarose electrophoresis, and a saturating amount in the second dimension to resolve positive from negative supercoils as well as to detect alternative structures (Fig. 9).

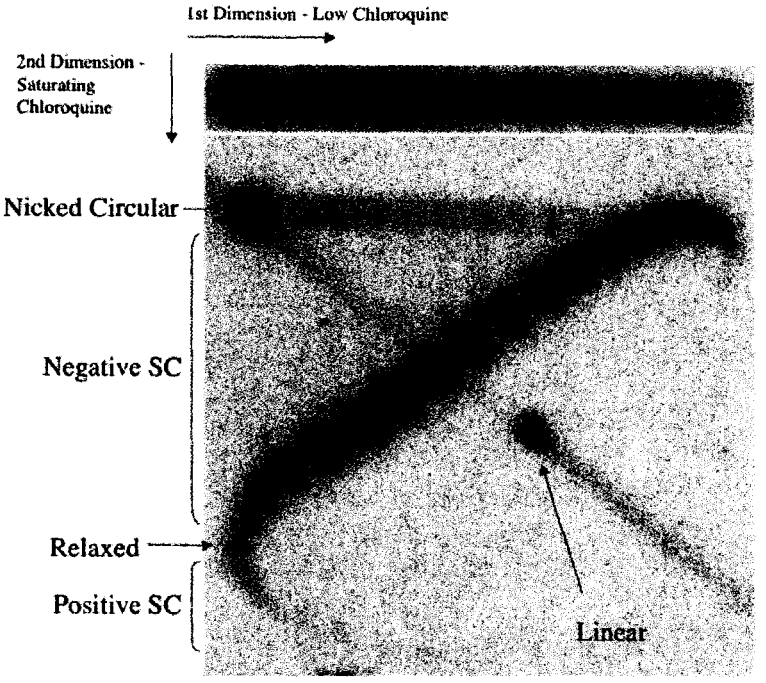

Figure 9. Two-Dimensional Chloroquine Agarose Gels. Plasmid DNA is run in a standard agarose gel containing a low level of the intercalator chloroquine. After topoisomer separation in the first dimension, the DNA set is rotated 90°, and run in a second dimension in a new agarose gel containing a saturating amount of chloroquine. In this protocol, more highly supercoiled DNA runs toward the bottom of the gel in the first dimension, and in the top right hand quadrant in the second dimension. Nicked circular DNA, linear DNA, the relaxed topoisomer, and both positive and negative topoisomer sets are indicated.

Lambda RED Recombination

To make chromosomal modifications we used the λ RED recombination system, or "recombineering". The basis for this technology was first discovered by Kenan Murphy in 1998. By replacing the *recBCD* genes in *E. coli* with the λ RED genes, he was able to recombine short linear fragments of DNA into the chromosome at highly elevated rates (Murphy, 1998). This RecA-independent recombination is mediated via the three λ proteins: Gam, Exo and Beta. Gam works to inhibit the RecBCD nuclease complex that normally degrades linear DNA (Karu *et al.*, 1975). Exo is a 5'-exonuclease that works on linear DNA and leaves 3'-overhangs (Carter and Radding, 1971). Beta is a single-strand DNA binding protein that acts much like RecA in that it binds to the 3'-overhangs produced by Exo, protects them from degradation, and anneals them to the target site for homologous strand invasion (Carter and Radding, 1971; Yu *et al.*, 2000). Two groups enhanced this system to make the λ genes inducible to integrate linear DNA made with PCR (Fig. 10) (Datsenko and Wanner, 2000; Yu *et al.*, 2000). In one system, the Gam, Exo and Beta genes were made inducible by placing them under the control of the λp_L promoter that is regulated by the temperature sensitive λcI_{857} repressor. When grown at 30°C, the repressor is active, and keeps the promoter repressed. When heated to 42°C, the λcI_{857} repressor is denatured, and the system is de-repressed (Yu *et al.*, 2000). Each group used a PCR-based strategy to create double-stranded linear DNA with 40-50 base pair homologies. They achieved this by making PCR primers with the 3' 20 bases homologous to the region to be amplified and inserted, and the 5' 40 bases homologous to the chromosomal target (Fig. 10*A*). Electroporating this PCR product into cells that had been induced for λ RED genes (Fig. 10*B-C)* allowed recombination into the

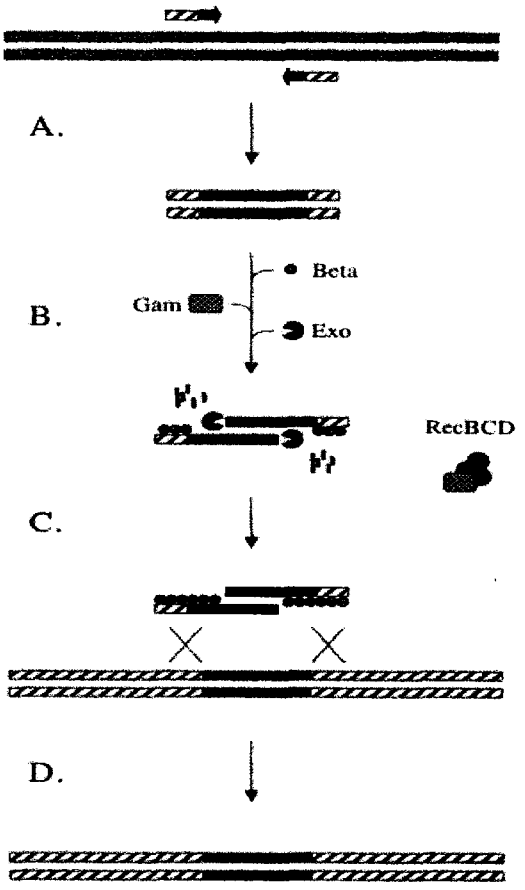

Figure 10. Lambda RED Recombination. A recombination strategy using a PCR product and lambda RED recombination proteins. (A) PCR is performed on the DNA cassette to be integrated using 60mer oligonucleotides with the 3' 20 base pairs homologous to the integration cassette (red), and the 5' 40 base pairs homologous to the target sequence (striped). (B) The resulting PCR is electroporated into a cell induced to express the lambda RED recombination proteins. Gam inhibits cellular RecBCD. Exo is a 5' to 3' exonuclease that produces single strand overhangs that Beta binds and prepares for strand invasion. (C) The resulting DNA with the bound Beta proteins finds the region of homology, and recombination occurs between the target sequence and the targeting homology from the PCR product. (D) The resulting product contains the insert DNA (red) in the place of the native DNA (green).

chromosome at precise locations (Fig. 10*D*). Recombinants were identified through antibiotic markers, phenotypic markers, or even PCR strategies. The efficiency was shown to be as high as 1 in 1000 surviving cells (Datsenko and Wanner, 2000; Yu *et al.*, 2000).

FLP-FRT Recombination

Another recombination system we used was the FLP-FRT system from the *Saccharomyces cerevisiae* 2μm plasmid. FLP is the recombination protein, and is a member of the Integrase family. Each FRT site consists of two 13 bp inverted symmetry elements flanking an 8 bp core region. For recombination, a FLP monomer binds to each of two FRT sites. This forms complexes that bend the DNA by over 140°. The FLP protein cleaves the FRT site, and links the 3'-DNA via an active site tyrosine residue. This creates a free 5'-hydroxyl group that attacks the other FLP-FRT complex, which forms a Holliday Junction that is then resolved by normal Holliday Junction-resolving enzymes. This type of recombination occurs by random collision of the FRT sites, and has no requirement for supercoiling (Luetke and Sadowski, 1998).

Recently, Pang *et al.* used FLP-FRT recombination to create a system to move a large DNA complex into various positions on the chromosome (Fig. 11). Through PCR, they made a product that contained the entire *lacIZYA* operon and a gentamicin resistance cassette flanked by FRT sites. They cloned this insert into an Invitrogen pCR-XL-TOPO vector creating plasmid pPM1 (Pang *et al.*, 2005). On the chromosome, they inserted a FRT-kanamycin-FRT marker at a specific location, then used the FLP recombinase to remove the kanamycin cassette, leaving a "naked" chromosomal FRT site (Datsenko and

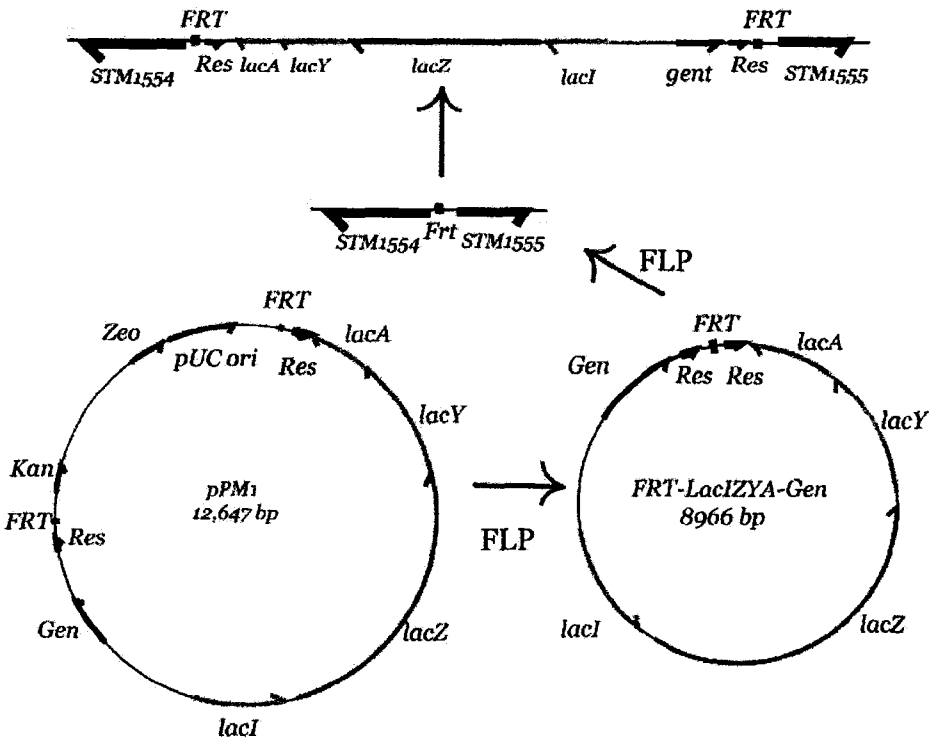

Figure 11. FLP-FRT System of Genomic Modification. This describes a strategy to move large regions of DNA using the FLP-FRT recombination system. A PCR product containing the *lacIZYA* operon and a gentamicin resistance gene flanked by FRT sites was cloned into an Invitrogen pCR-XL-TOPO vector creating plasmid pPM1. This vector was electroporated into a strain containing an inducible FLP recombinase gene and a single FRT sequence at the target site. Upon FLP recombinase expression, the FRT sites on pPM1 recombine to create a FRT-*LacIZYA-Gen* circle. The single FRT site on this circle can then recombine with the genomic FRT site, and allow integration into the chromosome. From "A gyrase mutant with low activity disrupts supercoiling at the replication terminus" by Z. Pang, R. Chen, D. Manna and N. Patrick Higgins, 2005, Journal of Bacteriology 187(22), pp.7773-7783. Copyright 2005. Reprinted with permission from ASM.

Wanner, 2000). Into this stain they introduced the pPM1 vector, and induced for recombination. They were able to screen for recombination products in which the FRT-flanked cassette from pPM1 was inserted into the chromosomal FRT site. By this method, they were able to move a large reporter cassette (nearly 10 kb) easily (Pang *et al.*, 2005).

HOMEOSTATIC SUPERCOIL LEVELS DIFFERENTIATE ESCHERICHIA COLI FROM SALMONELLA ENTERICA

by

KEITH CHAMPION AND N. PATRICK HIGGINS

In preparation for *Molecular Microbiology*

Format adapted for dissertation

ABSTRACT

Escherichia coli and *Salmonella enterica* serovar Typhimurium share a high degree of DNA and amino acid identity in 65% of the homologous genes shared by the two genomes. Yet there are different phenotypes for many homologous genes that control chromosome dynamics. A mutant form of the GyrB DNA gyrase subunit, which causes a TS phenotype in *Salmonella*, is lethal in *E. coli*. Mutant forms of *mukB*, *topA*, and *seqA* also show quite different phenotypes in *E. coli* and *Salmonella*. A unifying hypothesis for how similar mutations lead to different physiology focuses on negative supercoiling. In mid-log phase, *E. coli* generates 15% more negative supercoiling than *Salmonella*. This difference in torsional strain explains a complex set of single gene phenotypes, and provides mechanistic insight into how supercoiling modulates epigenetic effects on prophage behavior *in vivo*.

INTRODUCTION

All organisms store DNA in a supercoiled conformation. Eukaryotic organisms organize supercoils on the surface of highly conserved nucleosomes, whereas bacteria use the ATP-dependent enzyme DNA gyrase to supercoil the chromosome and place it under torsional stress (Cozzarelli and Wang, 1990; Miller and Simons, 1993; Pavitt and Higgins, 1993; Wang, 1985). Supercoil strain causes DNA to wind upon itself in superhelices that condense the chromosome. Bacteria must compact DNA over 1000 fold while allowing transcription and replication to proceed with minimal topological stress (Holmes and Cozzarelli, 2000; Trun and Marko, 1998). Compaction is facilitated by negative supercoil torsional strain that causes DNA to wind upon itself in superhelices and by structural maintenance of chromosome (SMC) proteins which are ATP-binding pincer-like proteins that are conserved from bacteria to humans (Cobbe and Heck, 2000).

Negative supercoiling is also a source of stored energy. An underwound double-helix is easily converted to the single strand state, which is necessary for initiating DNA replication (Baker *et al.*, 1986; Ryan, 1976) and RNA transcription (McClure, 1985; Sanzey, 1979; Smith *et al.*, 1978). In prokaryotes, the interwound supercoil structure is in constant motion. "Slithering" (Marko, 2001) and "branching" (Staczek and Higgins, 1998) causes plasmid DNA to adopt an enormous ensemble of interwound conformations (Vologodskii, 2001). Supercoil-driven strand movement underpins processes such as homologous and site-specific recombination (Gellert and Nash, 1987; Hays and Boehmer, 1978; Mizuuchi and Nash, 1976), cooperative repressor binding by looping (Bellomy and Record, 1990; Schleif, 1992), and transposition (Craigie and Mizuuchi, 1986). More than 300 *E. coli* genes are regulated by negative supercoiling; at least 200

genes decrease output and 100 increase output when the DNA is relaxed (Peter *et al.*, 2004).

The central importance of supercoiling requires bacteria to maintain DNA supercoil levels within a narrow range (± 15% of the normal value) (Drlica, 1992). Supercoil density is controlled by three topoisomerases - gyrase, topoisomerase I, and topoisomerase IV (Menzel and Gellert, 1983; Zechiedrich *et al.*, 2000). These three enzymes generate a balance of supercoiling with sufficient torsional strain to condense DNA, but not enough to trigger formation of alternative DNA structures such as Z-DNA, R-loops, cruciforms, or intramolecular triplexes. The alternative non-B-DNA conformations stop replication forks, stall transcription complexes, and cause introduction of single and double strand chromosome breaks by several mechanisms (Higgins *et al.*, 2005).

Gari *et al.* discovered the *Salmonella gyrB652* mutation in a genetic screen designed to identify gyrase mutants that cause DNA damage at 42°C (Gari *et al.*, 1996). The complex phenotype associated with *gyrB652* included: 1) temperature sensitive (TS) growth in which cell division in LB stops at 42°C, 2) resistance to low levels of nalidixic acid (5 μg/ml), 3) induction of the RecA-dependent SOS response at 37°C, 4) "*rec*-less" degradation of chromosomal DNA at temperatures above 37°C, and 5) synthetic lethality when *gyrB652* was combined with *recA* or *recB/C* mutations. Recently the complex phenotype was studied using *in vitro* and *in vivo* assays (Pang *et al.*, 2005). DNA gyrase containing the GyrB652 subunit is not a classic TS enzyme. It has decreased supercoiling catalytic activity at temperatures from 30°C to 42°C. At 30°C, the GyrB652 gyrase can resolve all topological problems before cell division takes place. Rapid

growth at 42°C triggers frequent initiation of DNA replication at *oriC* and leads to chromosomes with multiple replication forks. The sluggish GyrB652 gyrase can not resolve all topological problems in time for cell division, which leads to topological chaos near the terminus of DNA replication, replication fork failure, and cell death (Pang *et al.*, 2005).

GyrB is one of the most conserved proteins in *E. coli* and *Salmonella*. 96% of the 804 GyrB amino acids are identical and most substitutions are located in the C-terminal region of the protein (21 of 31 substitutions are beyond H567.) To study the *gyrB652* (R436S) allele in *E. coli*, an oligonucleotide was designed to introduce a single bp substitution (Fig. 1).

The *gyrB652* mutation proved to be lethal in *E. coli*. Moreover, ectopic expression of the *Salmonella* WT GyrB or GyrB652 protein from a plasmid was toxic for *E. coli* whereas both *E. coli* and *Salmonella* tolerated over-expressed *E. coli* GyrB. This effect is reminiscent of *topA* mutations that are lethal in *E. coli* strains but are tolerated in *Salmonella* strains (Bhriain and Dorman, 1993; Margolin *et al.*, 1985). One hypothesis is that *E. coli* and *Salmonella* have adapted to different supercoil levels. Using reporter plasmid pBR322, the average supercoil density of mid-log cultures of WT *Salmonella* (σ = -0.059) was observed to be 15% lower than *E. coli* (σ = -0.069). Analysis of plasmids with 56 bp of alternating GC sequence showed that *E. coli* DNA was also under a significantly greater level of torsional strain. These two observations explain numerous phenotypic differences between the organisms.

RESULTS

GyrB652* in *E. coli. The *Salmonella gyrB652* mutation (Gari *et al.*, 1996) results from a single C-A transversion that substitutes serine for arginine at residue 436 (R436-S) (Pang *et al.*, 2005). In the three-dimensional model of gyrase, this residue is near a GyrB-GyrA interface (Cabral *et al.*, 1997; Kampranis *et al.*, 1999; Reece and Maxwell, 1991). The GyrB652 subunit has a lower than normal catalytic efficiency, k_{cat}, and displays a TS phenotype that is not caused by enzyme inactivation at 42°C (Pang *et al.*, 2005). Growth stops due to a rapid DNA initiation frequency, which leads to topological chaos at the terminus of replication (Pang *et al.*, 2005). To determine if terminal chaos happens in *E. coli*, we tried to introduce the *gyrB652* mutation into two strains of *E. coli* derived from W3110, NH3423 and NH3612.

Bacteriophage λ "recombineering" technology (Yu *et al.*, 2000) allows one to modify bacterial chromosomes efficiently using short single-stranded DNA substrates with >30 bp of targeting homology (Ellis *et al.*, 2001). To introduce the *gyrB652* mutation, a synthetic 70-mer (oligo #1) was made with 35 nucleotides of 5'- flanking homology followed by a single mismatch (the *gyrB652* C-A transversion mutation), and 34 nucleotides of 3'- flanking homology (Fig. 1)(see Table 3 for oligo #1 sequence). Because the *E. coli* and *Salmonella gyrB* sequence is identical for this segment, we used λ recombineering and oligo #1 to modify two *E. coli* strains (NH3423 and NH3612) and *Salmonella* (NH3702). Cells were thermo-induced at 42°C for 15 min to express λ RED-recombination proteins, oligo #1 was introduced by electroporation, and the cultures were incubated over night in LB before plating onto selective media.

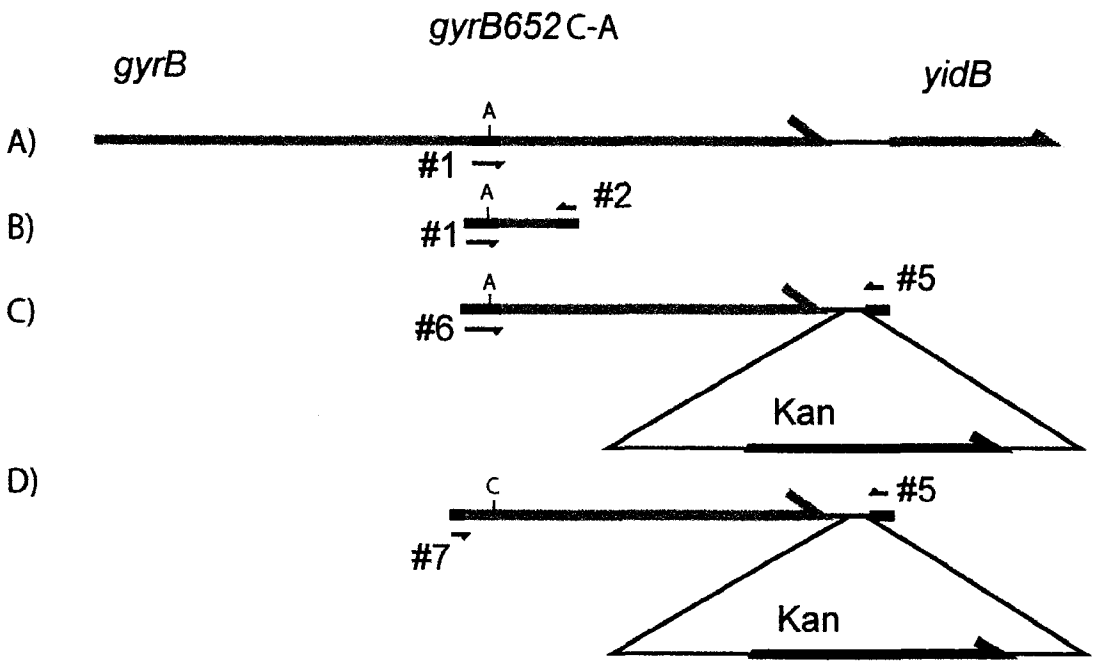

Figure 1. Strategies for introducing the *gyrB652* C-A transversion mutation into the *E. coli* chromosome. Map of the *gyrB-yidB* region of *E. coli* and the *gyrB652* mutation (marked by an A.) Strategy A (A) A synthetic single stranded 70-mer (oligo #1) with the C-A transversion at position 36. Strategy B (B) A PCR product made with oligo #1 and #2 to generate a 339 bp fragment with 35 bp of homology upstream and 303 bp downstream of the C/A substitution. Strategy C (C) Oligo's #5 and #6 make a 2824 bp PCR fragment with the *gyrB652* mutation at bp 54, 1160 bp of further *gyrB* homology followed by a selectable Kan^R module and 133 bp of intergenic homology. The control PCR (D) Oligo #5 and #7 make a 2916 bp PCR product with 1306 bp of WT *gyrB* homology with the C marking the WT *gyrB* sequence, the Kan^R module, followed by 133 bp of downstream homology. Arrow tips of genes indicate the transcription direction.

The *gyrB652* mutation was easily introduced into *Salmonella* NH3702. The phenotype includes TS growth at 42°C and resistance to 5 μg/ml of nalidixic acid (Nal) (Gari *et al.*, 1996). After spreading 100 μl of the 1 ml NH3702 electroporation culture onto Nal plates, an average 125 Nal^R colonies per plate were detected. Re-streaking 10 independent colonies on LB agar at 30°C and 42°C showed that all 10 Nal^R strains were TS for growth at 42°C and sequence analysis of *gyrB* DNA PCR amplified from four of the *Salmonella* NH3702-derived Nal resistant TS colonies showed each had the *gyrB652* C-A transversion mutation. However, the substitution was not successful in *E. coli*. The same volume of the NH3423 or NH3612 yielded an average of 6 colonies on Nal plates, but none were TS upon re-streaking. The number of Nal^R colonies arising from the *E. coli* electroporation experiment was the same as the control. 100 μl of an unmodified *E. coli* or *Salmonella* culture gave an average of 7 Nal^R colonies, of which none were TS at 42°C. Mutations in *gyrA* and *tolC* also give Nal^R phenotypes, which accounts for the background resistance. Sequence analysis of four Nal^R colonies from *E. coli* NH3612 electroporation plates all had WT *gyrB* sequences.

The difficulty of creating the *gyrB652* mutation in *E. coli* could be due to intolerance of this variant protein. However, it could also be caused by a species difference in the *gyrB652* phenotype or differences in the λ recombineering efficiency in *E. coli* and *Salmonella*. To increase target homology, a second strategy (*B* in Fig. 1) combined oligo #1 with a downstream 20-mer (oligo #2) to generate a double-stranded 339 bp PCR product. This PCR product contains the *gyrB652* mutation with 35 bp of upstream *gyrB* homology and 303 base pairs of homology down stream of the *gyrB652* transversion. Strains NH3702 and NH3612 were thermo-induced and electroporated with

this PCR product. After plating 100 µl of the recombineering culture, an average of 100 Nal^R colonies per plate were found for *Salmonella* NH3702, but *E. coli* NH3612 produced no more colonies than control plates, and none had a TS phenotype.

To lower the Nal^R background, a Kan^R module was introduced into the intergenic region between *gyrB* and *yidB* (NH3703 and *C* in Fig. 1). Using chromosomal DNA from NH3703 as a template, and oligo #6 which has the *gyrB652* transversion at position 54 of 74 nucleotides with oligo #5 lying down stream of the Kan gene, a 1391 bp PCR product was made with 53 bp of homology upstream of the C-A transversion and 133 bp of homology beyond the Kan^R inset (Fig. 1*C*, Table 3). *E. coli* strain NH3702 was electroporated with the new PCR product and 20 Kan^R recombinants were recovered from 100 µl of the electroporation culture. None of the Kan^R colonies were Nal^R. The *gyrB* region of three pools of 6 Kan^R strains was PCR amplified and sequenced. No indication of the C-A mutation was found in any pool of Kan^R recombinants.

The failure to isolate a *gyrB652* mutation in *E. coli* in Kan^R recombinants could mean that mismatch repair machinery had corrected the single base pair mismatch to the WT sequence in all recombinants, or that homology beyond position 56 was used for the cross-over point during recombination. To test these possibilities, strain NH3704 was constructed with a *mutS*::Tn*10* allele that eliminates mismatch repair (Marti *et al.*, 2002). The recombination efficiency of the mutant PCR product of oligos #5 and #6 was compared to a nearly identical PCR product made with oligos #5 and #7 (Table 3) with a WT *gyrB* sequence (Fig. 1*D*). In both of these PCR products, there is more than 1200 bp of *gyrB* homology upstream of the Kan module and 139 bp beyond the Kan module. In NH3704, 100 µl aliquots of a culture treated with the WT PCR product averaged 150

Kan^R colonies per plate. However, no plate spread with 100 µl of NH3704 electroporated with the *gyrB652* PCR product (oligos #5 and #6) gave a Kan^R colony. These results indicate that Kan^R resistant colonies generated with the *gyrB652*-containing PCR product in the Mut^+ background (NH3702) arose from MutS correction of the A/C heteroduplex to the WT C *gyrB* sequence. This strong bias provides evidence that the *gyrB652* mutation is lethal for *E. coli.*

Salmonella* GyrB protein is toxic for *E. coli. The final attempt to study GyrB652 in *E. coli* involved plasmids with *Salmonella gyrB* genes. Two pGem-vectors (pRC05 and pRC03) contain a copy of the *Salmonella* WT *gyrB* or the *gyrB652* alleles, respectively, cloned adjacent to a bacteriophage T7 promoter. These plasmids were used previously to express and purify *Salmonella* WT and GyrB652 proteins in BL21::DE3, an *E. coli* strain that harbors a Lac-inducible T7 RNA polymerase (Pang *et al.*, 2005). However, the T7 promoter is slightly "leaky" in strains lacking T7 RNA polymerase. pRC03 can be maintained by continuous selection on ampicillin, but these strains grew more slowly than strains with the pGem vector alone or pGem vectors with other cloned inserts (data not shown).

To measure the *gyrB652*-associated penalty, overnight cultures of NH3706 (with pRC03) were diluted 1:100 into LB plus ampicillin and cultures were grown into stationary phase at 30°C. 3 µl aliquots of serially diluted samples were spotted onto both LB and LB-Amp plates (Fig. 2). A 4 order of magnitude difference separated the number of colony forming units (CFU) on the LB-Amp and LB plates; pRC03 was lost from 99.99% of the CFU in culture. The same experiment was also carried out with NH3707, which contains a pGem plasmid with the WT *gyrB* gene from *Salmonella.*

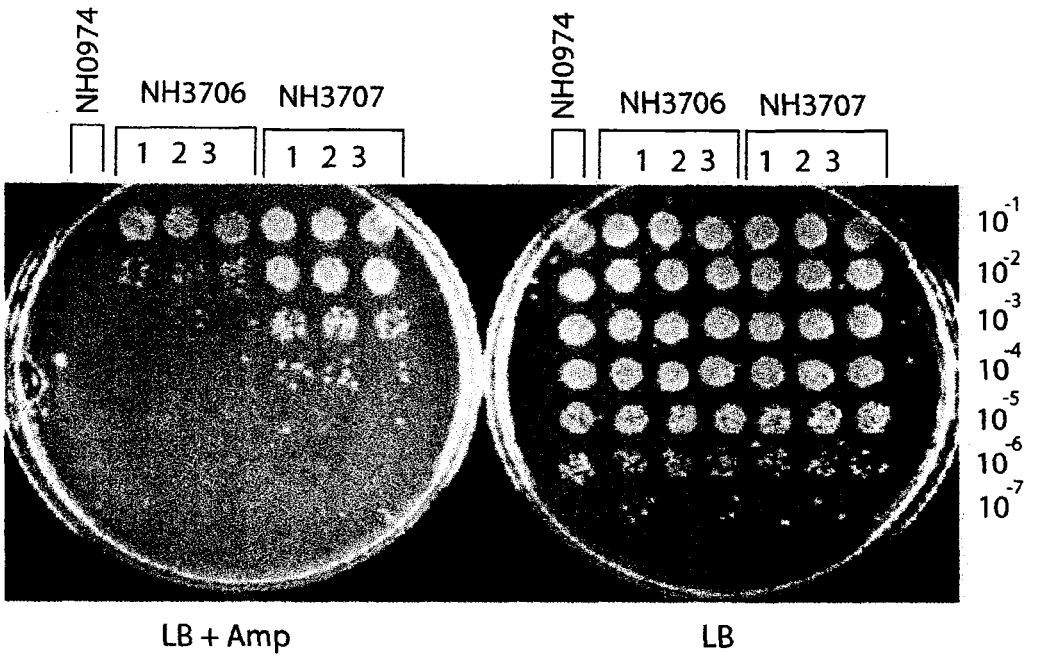

Figure 2. Stability of plasmid pGem vectors carrying the *gyrB652* or WT *gyrB* gene of *S.* Typhimurium in *E. coli.* LB media containing 50 μg/ml ampicillin was inoculated 1:100 in triplicate with overnight cultures of *E. coli* containing a pGem plasmid with *Salmonella gyrB652* gene (NH3706) or a WT *Salmonella gyrB* gene (NH3707). Cultures were grown at 30°C to stationary phase in a shaking incubator. A portion of culture (labeled 1-3) was serially diluted into 96-well micro-titer plates and 3 μl aliquots were spotted onto LB plates with and without 50 μg/ml ampicillin. The dilution factor is shown on the right side. Pictures were taken after 24 h incubation at 30°C.

Even a WT *Salmonella gyrB* gene was toxic in *E. coli*. pRC05 was eliminated from 99% of the CFU. Control experiments with pGem vector or pGem vectors with other cloned genes showed that >99% of all cells retained these plasmids under the same experimental protocol. This provides insight into why attempts to introduce the *gyrB652* gene into *E. coli* failed. *Salmonella* GyrB is toxic, even in the presence of a WT chromosomal copy of *gyrB*.

To determine if the *E. coli gyrB* gene is toxic, plasmid pAG111 was introduced into both *E. coli* and *Salmonella*. In pAG111, the WT *E. coli gyrB* sequence is cloned in the pTTQ-18 vector under Lac promoter control. Cells inoculated at a 1:100 dilution in fresh LB-Amp medium containing the indicated amounts of IPTG were grown for 6 h at 30°C. Each culture was serially diluted and 3 μl aliquots were spotted onto LB-Amp plates (Fig. 3). In *E. coli*, there was no significant plasmid loss if no IPTG was added to growth medium. Over the range from 10 μM to 1 mM there was a 1000-fold plasmid destabilization noted, but at the high IPTG level GyrB protein is over-produced up to 40% of the total soluble cell protein (by about 500-fold) (Hallett *et al.*, 1990). The *E. coli* GyrB protein is not nearly as toxic to *E. coli* as either the *Salmonella* WT GyrB or the GyrB652 protein. In *Salmonella*, there was no plasmid loss associated with expressing *E. coli* GyrB, except at 1 mM IPTG. Thus, *E. coli* is more sensitive to increases in GyrB protein level than *Salmonella* and is extremely sensitive to *Salmonella* GyrB proteins.

***E. coli* and *Salmonella* have different supercoil set points.** The phenotypic differences described above led us to consider whether an intrinsic difference in mean supercoil density (σ) exists between these organisms. To compare values of σ, the linking difference of pBR322 was analyzed in strains with a WT set of topoisomerases.

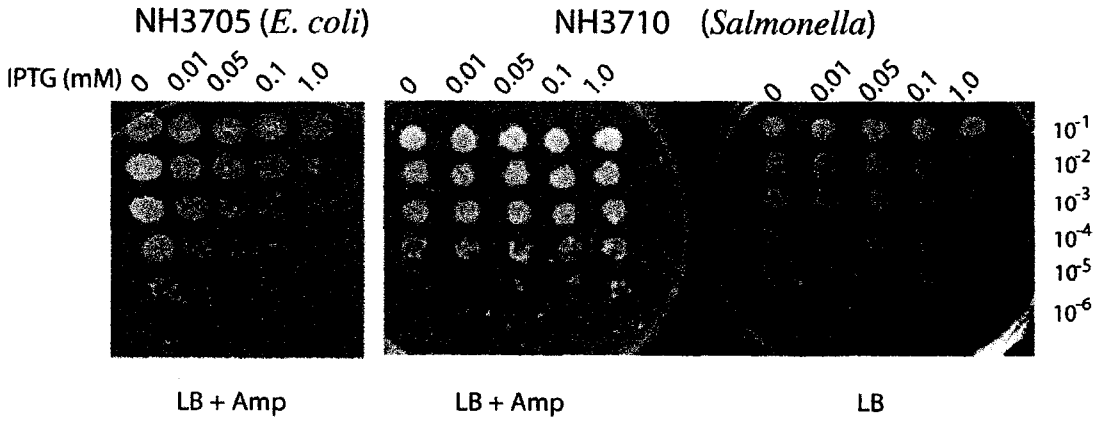

Figure 3. Stability of plasmid pAG111, which expresses a WT *E. coli gyrB* gene, in *S.* Typhimurium and *E. coli.* LB media containing 50 μg/ml ampicillin was inoculated with overnight cultures 1:100 and grown at 30°C for 4 h in a shaking incubator with the indicated amounts of IPTG. A portion of each culture was serially diluted into 96-well micro-titer plates and 3 μl aliquots were spotted onto LB plates as indicated. The dilution factor is shown at the right. Pictures were taken after 24 h incubation at 30°C.

E. coli JTT1 (NH0572) has been used in several important topological analyses (DiNardo *et al.*, 1982; Margolin *et al.*, 1985; Sternglanz *et al.*, 1981). In *Salmonella,* experiments were carried out with LT2 (NH0742) and SL4213 (NH0405), a strain with WT topoisomerases that carries a restriction mutation, which made it possible to move plasmids from *E. coli* directly to *Salmonella.* Actively growing cultures at mid log phase (Klett 50-70) were harvested and plasmid DNA was purified. Topoisomers were separated in 2-D 1.0% agarose gels, and DNA was blotted to nylon membranes for quantitation. Remarkably, the mean σ of pBR322 in *Salmonella* was significantly lower than *E. coli* (Fig. 4). With DNA isolated in mid-log phase, a 3-4 topoisomer difference exists between these two organisms. Using the band counting method in multiple experiments (Shure and Vinograd, 1976), the mean supercoil density of plasmid DNA from *E. coli* was $\sigma = -0.069$ and from *Salmonella* , $\sigma = 0.059$, a 15% difference. This difference places *Salmonella* in the *E. coli* toxic zone. The *gyrB652* mutation of *Salmonella* lowered the Lk number of pBR322 by ~1.5 topoisomers relative to WT (Pang *et al.*, 2005; Staczek and Higgins, 1998), representing an additional ~ 5% loss of supercoil density. This level is in the non-viable range for *E. coli.* If the GyrB protein is a determinant of the mean supercoil density, this would account for the dramatic toxicity of the GyrB652 protein in *E. coli.*

In bacteria, supercoiling is partitioned into constrained (S_C) and unconstrained (S_U) conformations *in vivo* ($\Delta Lk = S_U + S_C$) (see Jaworski et al., 1991). Constrained supercoiling (S_C) is created by the unwound regions of DNA located within the 3000 transcribing RNA polymerases and writhe associated with binding of abundant DNA binding proteins like HU, H-NS, and Fis

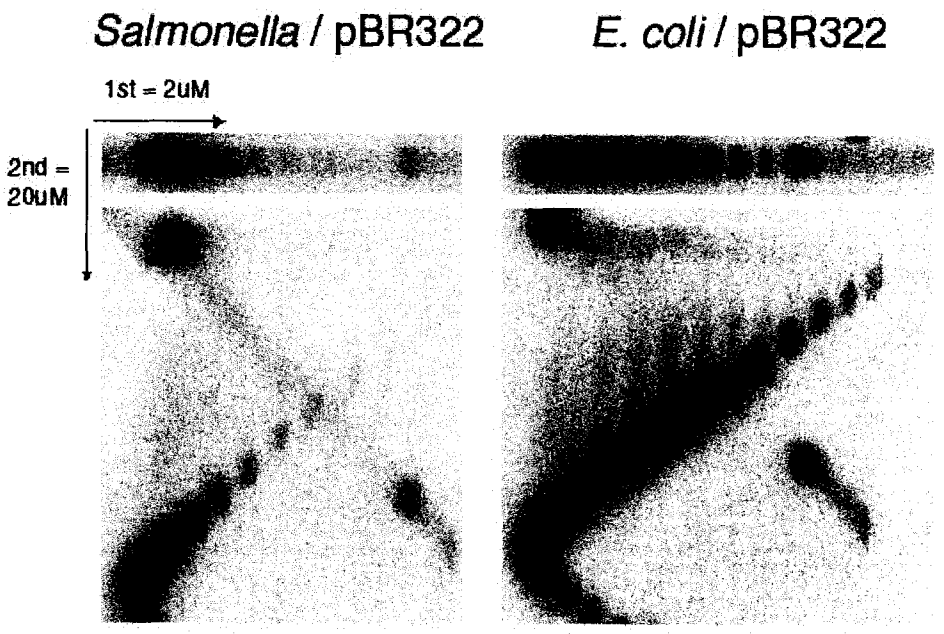

Figure 4. *E. coli* and *Salmonella* supercoiling analysis of pBR322 on 2-D chloroquine gels. *Salmonella* (NH3715) and *E. coli* (NH3718) with WT topoisomerases were grown to mid-log phase (Klett 70). Plasmid pBR322 DNA was purified, concentrated, and then separated in a 25 cm 1.0% agarose gel. Electrophoresis was for 46 hours at 2.0 V/cm in 2 μM chloroquine for the first dimension, and 46 hours at 2.0 V/cm containing 20 μM chloroquine for the second dimension (Peck and Wang, 1983). The supercoil density determined by the band counting method was -0.059 for *Salmonella* and -0.069 for *E. coli.*

(Higgins and Vologodskii, 2004; Johnson *et al.*, 2005). In *E. coli* S_U and S_C distribute roughly 50:50 (Bliska and Cozzarelli, 1987; Jaworski *et al.*, 1991; Pettijohn and Pfenninger, 1980). S_U causes torsional strain that can stabilize inter- and intramolecular triplex structures, left-handed Z-DNA, and cruciforms at palindromic sequences (Higgins and Vologodskii, 2004). To compare the S_U in *E. coli* and *Salmonella*, the Z-DNA forming potential for plasmid pRW478 was measured in both organisms. pRW478 has a 56 bp repeating (CG) insert at the *EcoRI* site of pBR322 and 50% of the plasmid adopts a left-handed Z-DNA form in vitro when $S_U = -0.025$ (Zacharias *et al.*, 1988). pRW478 isolated from *E. coli* NH3716 and *Salmonella* NH3713 was compared on 2-D gels (Fig. 5). Relative to *Salmonella*, the *E. coli* pRW478 gel pattern showed a striking difference. Most of the plasmid existed in a hyper-supercoiled state with bands migrating in the upper right sector of the gel. This pattern signifies the formation of Z-DNA *in vivo*, because after the 56 bp segment flips to the left-handed conformation, gyrase can introduce about a dozen additional negative supercoils into plasmid DNA (Jaworski *et al.*, 1991). Most of the *E. coli* plasmid population (>70% of total) was hyper-supercoiled relative to the 2-D profile for pBR322 (Figs. 4 & 5). For *Salmonella*, some plasmids adopted the left-handed conformation, but the fraction was substantially less than *E. coli* (<40% of total). These results show that the average σ and the torsional strain in *E. coli* DNA were both higher than in *Salmonella*.

Species-specific phenotypes for chromosome dynamics proteins. The differences in average σ and S_U in *E. coli* and *Salmonella* predict different regulatory response thresholds in each organism. For example, a supercoil-sensitive promoter tuned to the *E. coli* S_U level would be inappropriately expressed in *Salmonella* unless a re-

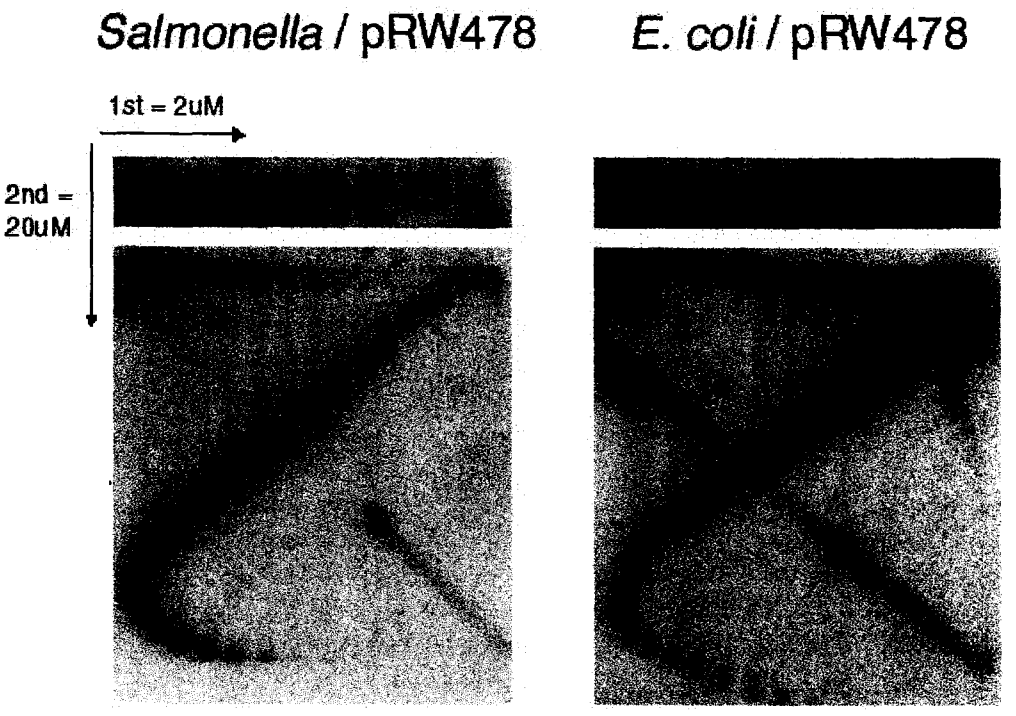

Figure 5. *E. coli* and *Salmonella* supercoil comparison of a plasmid containing 56 bp of alternating GC repeats (pRW478) on a 2-dimensional chloroquine gel. Strains of *Salmonella* (NH3713) and *E. coli* (NH3716) with WT topoisomerases and pRW478 were grown to mid-log phase (Klett 70). Plasmid DNA was purified and topoisomers were resolved in a 25 cm 1.0% agarose gel containing 2 μM chloroquine for 46 h at 2.0 V/cm in the first dimension and for 46 h in 20 μM chloroquine for the second dimension. More negatively supercoiled topoisomers migrate to the upper right region of this gel system. The pattern for *E. coli* shows a second distribution of highly supercoiled molecules with a break in the distribution. The second very highly supercoiled distribution indicates the formation of Z-DNA *in vivo* (Jaworski *et al.*, 1991).

tuning mechanism altered the system. *E. coli* responded in a very negative way to the *gyrB652* mutation, but how do other chromosome dynamics proteins behave? Null mutation in three genes, *topA*, *seqA*, and *mukB,* showed substantially different phenotypes when these two organisms were compared. The *topA* gene encodes *E. coli* topoisomerase I (ω protein) which is the predominant negative supercoil-relaxing enzyme (Sternglanz *et al.*, 1981). The discovery of *topA*'s genetic location was followed quickly by the finding that *topA* null mutants are lethal in WT strains of *E. coli* (DiNardo *et al.*, 1982; Zumstein and Wang, 1986). Compensatory mutations that can suppress the *topA* null lethality include chromosomal duplications of the region containing topoisomerase IV (Raji *et al.*, 1985), mutations in *gyrA* and *gyrB* (DiNardo *et al.*, 1982) and mutations in *tolC* (Dorman *et al.*, 1989). However, *topA* null mutations of the same type that are lethal in *E. coli* are healthy in *Salmonella* and *Shigella flexneri* WT backgrounds without compensatory mutations (Bhriain and Dorman, 1993; Dubnau *et al.*, 1973; Margolin *et al.*, 1985; Richardson *et al.*, 1984).

The *seqA* gene encodes an A-methylation-sensitive DNA binding protein that recognizes the sequence GATC (Lu *et al.*, 1994; Slater *et al.*, 1995; von Freiesleben *et al.*, 1994). SeqA binds preferentially to hemi-methylated GATC sites and, *in vivo*, SeqA sequesters the *oriC* region and blocks DnaA-dependent re-initiation of DNA synthesis for several minutes after its replication (Boye *et al.*, 1996). *E. coli seqA* deletions are sensitive to growth in rich medium and they fail to plate efficiently on rich LB plates at 30°C. A *Salmonella seqA* deletion plates efficiently on LB at 30°C (Fig. 6) and at temperatures up to 40°C (not shown). Cell cytometry showed that *Salmonella seqA*

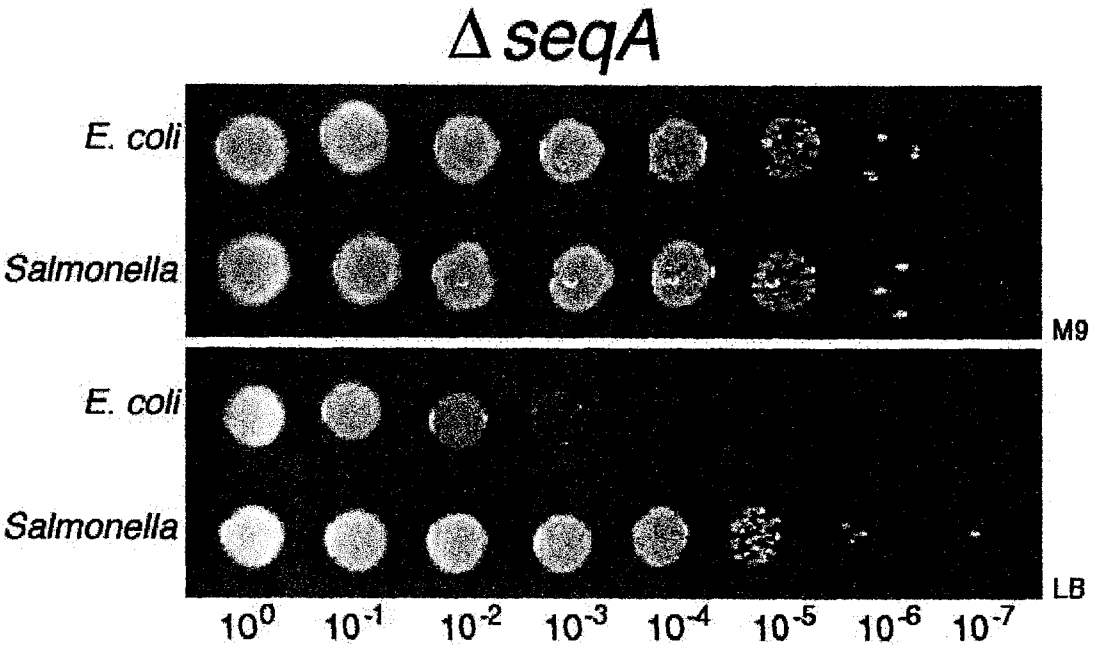

Figure 6. Species-specific phenotypes for Δ*seqA* strains grown in rich media. M9 media containing 0.2% glucose, 1 μg/ml thiamine, and 40 μg/ml L-proline was inoculated with single colonies and grown overnight at 30°C in a shaking incubator. Cell density was measured and cultures were diluted to an OD_{600} of 1.0. A portion of each culture was serially diluted (1:10) into 96-well micro-titer plates and 3 μl aliquots were spotted onto both LB plates and M9 plates with the above additives. Pictures were taken after 24 h incubation at 30°C.

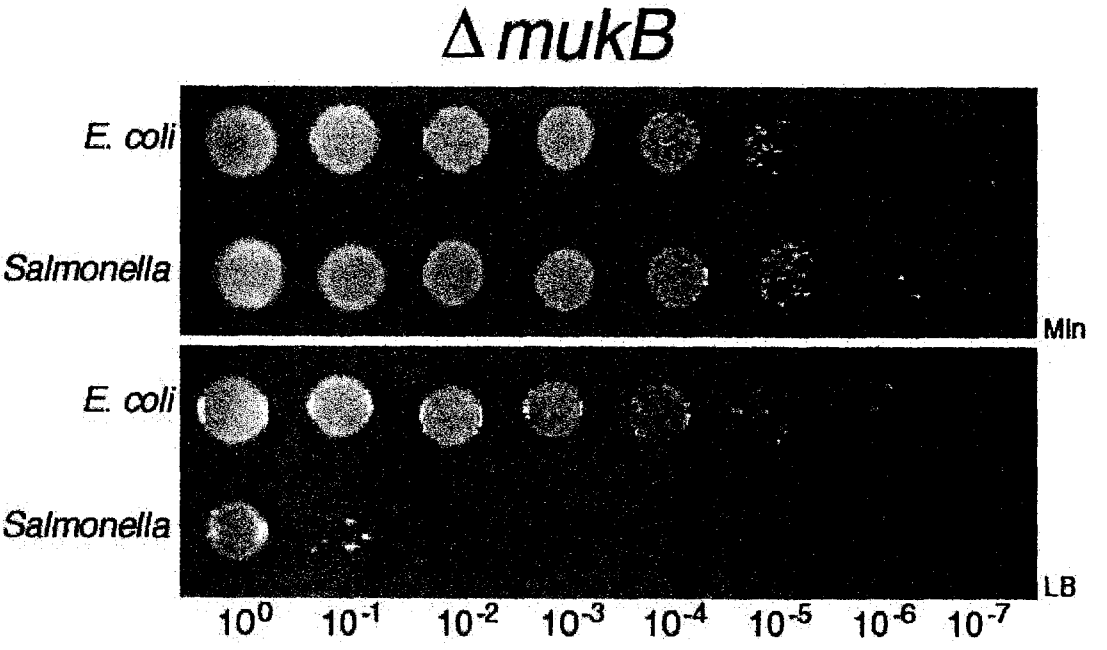

Figure 7. Species-specific phenotypes for Δ*mukB* strains grown in rich media. Minimal media containing 0.2% glucose was inoculated with single colonies and grown 48 h at room temperature in a shaking incubator. Cell density was measured and cultures were diluted to an OD_{600} of 1.0. A portion of each culture was serially diluted (1:10) into 96-well micro-titer plates and 3 µl aliquots were spotted onto both LB and Minimal-glucose plates. Pictures were taken after 48 h incubation at 30°C.

mutants grown in LB at room temperature do not have the filamentation phenotype that is characteristic of *E. coli seqA* mutants (data not shown). The third case is the bacterial "condensin" (Hirano, 2002), a protein composed of MukB, MukE, and MukF (Niki *et al.*, 1991; Niki *et al.*, 1992). *E. coli* deletions of *mukB*, *mukE*, or *mukF* exhibit the same phenotype, which is growth rate toxicity, frequent loss of F-plasmids, and segregation of DNA-less cells (Niki and Hiraga, 1997; Weitao *et al.*, 2000). *E. coli mukB* deletions were essentially 100% viable in minimal medium, and they plated efficiently on LB medium, as long as the temperature was 30°C or lower (Sawitzke and Austin, 2000) (Fig. 7.) *Salmonella mukB* deletion mutants plated efficiently on minimal medium at 30°C, but did not form colonies on LB plates at room temperature (Fig. 7). Growth in concentrated cell spots (10^0 dilution in Fig. 7 bottom panel) is due to a slow growth rate caused by competition for nutrients, because spreading the same number of cells across a single plate leads to only a few colonies that appear to have suppressor mutations. We interpret these results to indicate that WT *Salmonella* is more dependent on MukBEF for condensing and segregating chromosomes than *E. coli* (Sawitzke and Austin, 2000).

An altered Mu lysogenic state in *Salmonella*. In addition to altering the roles and efficiencies of proteins under selection for organizing and regulating chromosome movement, a low supercoil level should be distinguishable when supercoil-sensitive gene expression determines cell fate. The lysis-lysogeny development of phage Mu is an example of such a system. Two convergent promoters regulate the Mu lysis-lysogeny decision. The only Mu gene required for lysogeny is the *c* repressor, which is regulated by the leftward p_{Cm} promoter. Replication, transposition, and late gene lytic functions are controlled by the convergent rightward p_E promoter (Krause and Higgins, 1986).

Both promoters are supercoil-sensitive. At low supercoiling levels, p_{Cm} /p_E output is roughly equal. However, with negatively supercoiled DNA substrate, the p_E promoter is many fold stronger, and the lytic pathway is strongly favored (Higgins *et al.*, 1989; Yoshida *et al.*, 1982).

To test the influence of different supercoil levels on lytic/lysogenic behavior, Mu monolysogens were isolated for *E. coli* (NH1126) and *Salmonella* (NH742), which also carries the *musA* allele that changes the surface polysaccharide structure to permit Mu adsorption (Faelen *et al.*, 1981). Both strains harbored Mu *cts62* pAp1, which carries the TS *cts62* repressor allele (Vogel *et al.*, 1991). At 30°C, Mu *cts62* repressor binds to the operator and blocks p_E promoter activity, but a shift to 42°C induces conformation change in the C-terminal domain of repressor causing immediate release of DNA and initiation of the lytic transcription pathway (Vogel *et al.*, 1996). Duplicate overnight cultures were diluted 100-fold into fresh LB broth and incubated at 30°C to an O.D. of 0.6, which corresponds to approximately 5 x 10^8 colony forming units (CFU) /ml. Cultures were shifted to a 42°C shaking water bath and the O.D. as well as the number of CFU that plate on LB agar at 30°C were sampled at 10 min. intervals for 80 min. The O.D. of *E. coli* cultures dropped 30 min. after temperature shift while *Salmonella* cultures started lysis 10 min. later (Fig. 8). Thus, the eclipse phase of phage development was longer in *Salmonella* than in *E. coli*. More dramatically, the number of cells surviving the shift to 42°C were different. In *E. coli*, the CFU number fell immediately after shift, and rapidly declined. By 50 min. the CFU had dropped over 8 logs and no survivors were detectable. In *Salmonella*, the CFU also dropped immediately after the temperature

shift, but then leveled off at 20 min. and the viable cell count remained about 10^7 CFU/ml for the next hour. Whereas both species were sensitive to the Mu lytic cycle, *Salmonella* survived thermo-induction 7 orders of magnitude better than *E. coli*.

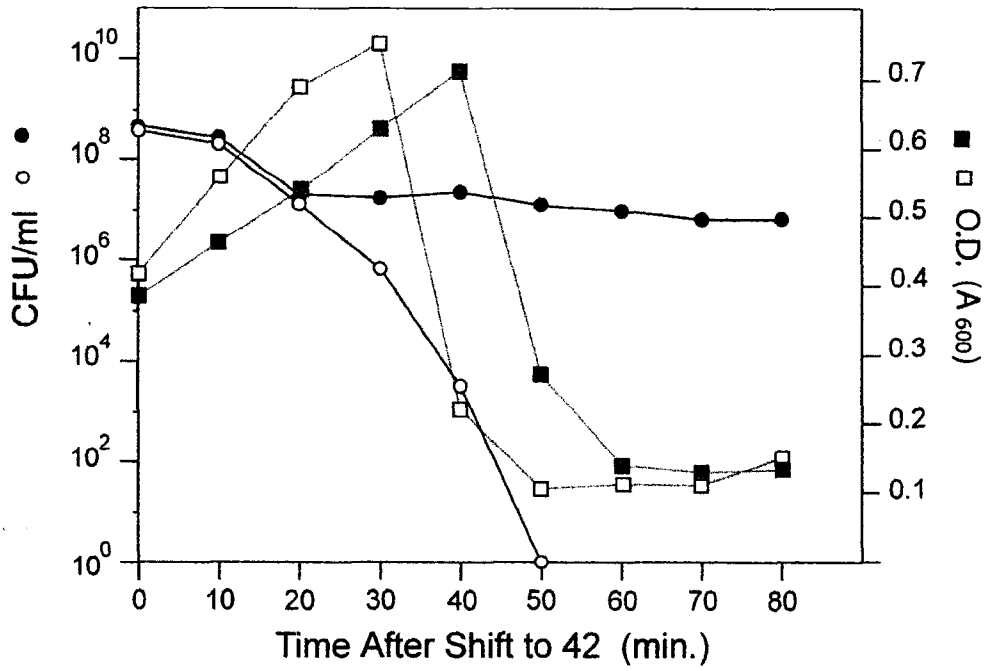

Figure 8. Different Mu lytic profiles for *E. coli* and *Salmonella*. Mu lysogens of *E. coli* (NH1126 open symbols) and *Salmonella* (NH742 closed symbols) were diluted 1:100 in duplicate LB cultures and grown in a 30°C incubator to log phase (OD_{600} 0.4). The cultures were shifted to a 42°C shaking water bath and the OD_{600} and CFU were measured at 10 min. intervals. The *E. coli* cultures (open squares) began lysis at 30 min. and the *Salmonella* cultures (filled squares) began lysis at 40 min. The number of viable cells in *Salmonella* cultures (filled circles) dropped 50-fold, while for *E. coli* (open circles) viability dropped by 8 orders of magnitude.

DISCUSSION

Species-specific *gyrB* mutations. An A-C transversion that causes the R436S substitution in GyrB is non-viable in an otherwise WT strain of *E. coli.* The R436S coding change could not be introduced into *E. coli* using 3 different genetic strategies (Fig. 1). The strongest evidence of a severe *gyrB652* defect in *E. coli* came from the electroporation of two PCR products into recombination-competent cells with a *mutS* allele. A single C-A transversion in a 2.8 kb PCR product (Fig. 1*C*) caused at least a 1000-fold reduction in transduction efficiency of a Kan^R gene linked to the *gyrB* locus. Moreover, the *Salmonella gyrB652* allele was dominant as a merodiploid. Modest expression of the WT *Salmonella* GyrB was toxic in WT *E. coli*, and the GyrB652 mutation was 100 times more toxic. A pGem plasmid bearing *Salmonella gyrB652* gene cloned behind a T7 promoter was selectively eliminated from 99.99% of viable cells that did not contain T7 RNA polymerase (Fig. 2). Cells that spontaneously lost these plasmids overgrew cells that contain the plasmid. Although we don't know precisely how leaky the pRC03 GyrB652 expression was, it did not add enough protein to change the western blot band intensity of WT *E. coli* GyrB (data not shown). Two to three-fold over-expression of *E. coli* gyrase subunits does not hinder *E. coli*'s growth rate (Jensen *et al.*, 1999; Snoep *et al.*, 2002), so *Salmonella* GyrB protein is exceptionally toxic for *E. coli.*

Species-specific control of supercoil density. WT strains of *E. coli* growing exponentially in LB medium maintained a significantly higher supercoiled density than *Salmonella* (σ = -0.069 vs. -0.059 respectively in Fig. 4). The analysis with plasmid pRW478 showed that *E. coli* generated more torsional strain than *Salmonella. In vitro*, at

S_U = - 0.025, half of the pRW478 molecules adopt a left-handed conformation. Most of the plasmid molecules isolated from *E. coli* were hyper-supercoiled, indicating that the mean *in vivo* S_U was > - 0.025. The fraction of hyper-supercoiled plasmid in *Salmonella* was less than half, indicating the mean S_U was < - 0.025 (Fig. 5). These facts suggest a supercoil explanation for differential compaction and phenotypic differences of *topA*, *mukB*, and *seqA* null mutations in otherwise WT strains of *E. coli* and *Salmonella.* (Holmes and Cozzarelli, 2000; Sawitzke and Austin, 2000).

Supercoil density (σ) is regulated by the combined activities of gyrase and topoisomerase I as well as topoisomerase IV (Menzel and Gellert, 1983; Zechiedrich *et al.*, 2000). However, several-fold expression changes in gyrase or topoisomerase I produce a very small change in supercoil density and no significant alteration in growth rate (Jensen *et al.*, 1999; Snoep *et al.*, 2002). Our results suggest that one factor controlling the mean supercoil density is the GyrB subunit. Between *E. coli* and *Salmonella* there are 31 substitutions (3.9%) among the 804 amino acid residues of GyrB protein and 78 changes (8.9%) in 878 amino acids of GyrA. Internal metabolic flux changes the cellular phosphorylation state and ATP/ADP ratio, which modulates gyrase supercoiling activity (Hsieh *et al.*, 1991; Jensen *et al.*, 1999; Snoep *et al.*, 2002; Westerhoff *et al.*, 1988). However, these changes are modest relative to the15% difference shown in (Fig. 4 & 5). A significant fraction of the 15% difference in mean σ under identical growth conditions is likely caused by substitutions in GyrB. Previous work indicated that *E. coli* had higher supercoiling levels than three other gram-negative organisms (Jaworski *et al.*, 1991), so *E. coli* may have unusually high supercoiling among gram-negative organisms.

Species-specific phenotypes of chromosome dynamics proteins. Significant differences exist between *E. coli* and *Salmonella* in the phenotype of topologically sensitive genes and gene systems including *topA*, *mukB*, *seqA*, and bacteriophage Mu. The difference in WT supercoiling levels provides an explanation for each of these observations. For *topA* null mutations, *E. coli's* high torsional strain causes formation of transcription-driven inter-molecular triplexes or R-loops (Drolet *et al.*, 2003; Hraiky *et al.*, 2000). R-loops impede transcription of highly transcribed genes and stall replication forks (Higgins and Vologodskii, 2004). Because *Salmonella* maintains a 15% lower level of σ and a lower S_U, R-loop formation would be a less severe problem.

A SeqA null mutation in *E. coli* is more critical at fast growth rates than in *Salmonella* (Fig. 6). In both *E. coli* and *Salmonella*, SeqA dampens DnaA-dependent replisome assembly at the *oriC*. One critical step in the initiation of replication is unwinding of A/T rich sequences adjacent to DnaA binding sites at the origin (Baker *et al.*, 1986; Funnell *et al.*, 1986, 1987). Re-initiation at high frequency in the highly supercoiled *E. coli* chromosome causes cell filamentation and cell death due to replication fork failure (Grigorian *et al.*, 2003; Simmons *et al.*, 2004). The ambient supercoil level of *Salmonella* appears to lower initiation frequency to acceptable levels.

The exception that proves the rule is *mukB*, which shows a more severe defect in *Salmonella* than in *E. coli* (Fig. 7). *E. coli* strains with a deletion of *mukB* plate with equal efficiency on minimal and LB medium at 30°C. *Salmonella mukB* deletions plate with near 100% viability on minimal medium but were 6 orders of magnitude worse on LB medium. Again, a supercoil difference explains this discrepancy. The *E. coli* genome is sufficiently condensed by negative supercoiling that MukB is not required for

segregation at growth rates that include LB medium at 30°C. Sawitzke demonstrated that low levels of the GyrB inhibitor novobiocin increased dependence on MukB whereas the introduction of a *topA* mutation, which increased supercoil density, decreased MukB dependence and made it possible for *E. coli* to plate on LB up to 42°C (Sawitzke and Austin, 2000). *Salmonella's* low supercoil level makes it heavily reliant on MukBEF for condensation and efficient segregation in rich medium (see (Holmes and Cozzarelli, 2000).

Why do related enterics maintain different supercoil densities? We suggest that selective pressure for a different supercoiling level comes from prophages in the *Salmonella* genome. *E. coli* K12 harbors no plaque forming prophage while *S.* Typhimurium has 4 (Fig. 9); they are Gifsy-1, Gifsy-2, Fels-1, and Fels-2 (McClelland *et al.*, 2001). Gifsy-1 and Gifsy-2 harbor genes that contribute to pathogenicity in infected mice (Figueroa-Bossi and Bossi, 1999), which may explain the selective pressure to preserve these elements. Phage development changes with the growth conditions of the host. For lytic phages like T4, adsorption rate and burst size decrease an order of magnitude under poor growth conditions while the eclipse time increases (Hadas *et al.*, 1997). For lysogenic phage like Mu and λ, the lytic/lysogeny decision changes with physiology. Neither λ nor Mu produce significant single cell bursts in stationary phase, and gyrase modulates the lytic/lysogeny outcome in both viruses. λ excision is reduced from some chromosomal locations in *himB* (*gyrB*) mutants (Miller *et al.*, 1979; Miller *et al.*, 1981). The Mu burst size and plaque size is reduced in *E. coli gyrB* mutants (Yoshida *et al.*, 1982) and the lysis/lysogeny fate is linked to negative supercoiling (Higgins *et al.*, 1989; Krause and Higgins, 1986). Supercoiling is so critical for Mu that a

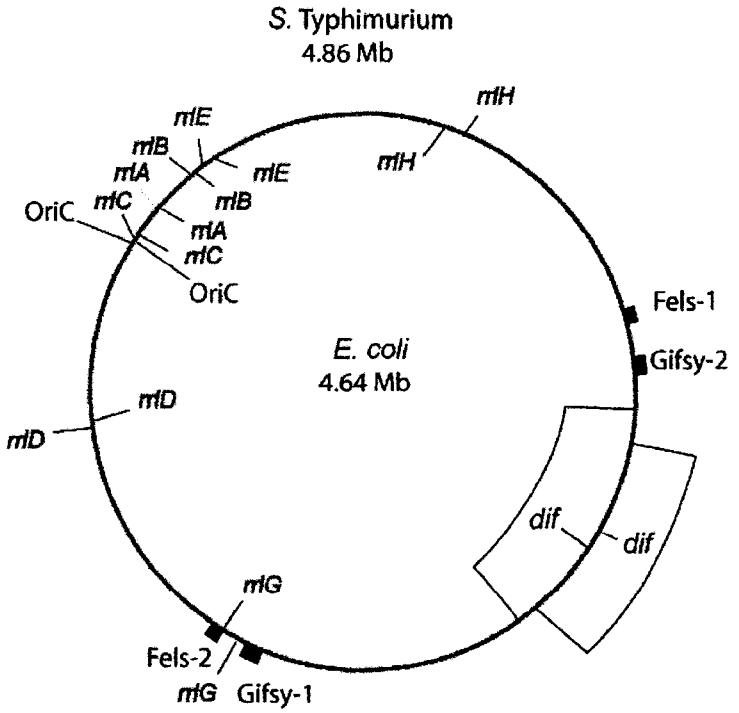

Figure 9. Map of *E. coli* (inner circle) and *S.* Typhimurium (outer circle) showing the positions of the seven 23S ribosomal RNA operons (*rrlA-H*) and 4 plaque-forming prophage in *Salmonella* (black arcs).

unique gyrase binding site at the virus center contributes to lytic development (Oram *et al.*, 2006; Pato *et al.*, 1990; Pato and Karlock, 1994; Pato *et al.*, 1995). Compared to *E. coli, Salmonella* has a longer eclipse and a dramatically increased survival of phage killing following Mu induction (Fig. 8). This we predicted, knowing that *Salmonella* has 15% lower mean superhelix density than *E. coli.*

The value of the *E. coli – Salmonella* comparison. The separation of *E. coli* and *Salmonella* from a common ancestor occurred about 200 my before present (Lawrence, 2005), which is about the time of separation of lineages for mouse and human. Both bacteria retain approximately 65% homologous genes and a conserved genetic map, but 35% of the genes in both organisms were introduced through horizontal transfer (Lawrence *et al.*, 1992; Lawrence and Roth, 1996; Lawrence and Roth, 1995). What sets the supercoiling level in *E. coli* and *Salmonella*? The mean σ varies with growth rate and the ATP/ADP ratio (Cook *et al.*, 1989; Drlica, 1992; Goldstein and Drlica, 1984; Higgins *et al.*, 1988). However, the difference in σ under optimal growth conditions is large and the simplest hypothesis is that supercoiling level is controlled by gyrase.

How many cellular functions might be influenced by different homeostatic supercoiling levels? Recent work shows that Mu transposition hotspots are different in *Salmonella* than in *E. coli,* even among the conserved homologous gene set (Manna *et al.*, Submitted). Because unusual DNA structures create high affinity targets for transposition (Yanagihara and Mizuuchi, 2002), transposon distribution patterns could be linked to supercoil level. One gene that influences both supercoiling and transposon targeting is H-NS. H-NS strengthens Mu lysogeny (Falconi *et al.*, 1991), modulates the target specificity of transposons (Swingle *et al.*, 2004), and is implicated in complex

supercoil behavior (Owen-Hughes *et al.*, 1992). Interestingly, *hns* mutants have a more severe phenotype in *Salmonella* than in *E. coli* (Navarre *et al.*, 2006).

All 4 *Salmonella* prophage have expression and gene amplification effects that can be measured after prophage induction using microarray technology (Frye *et al.*, 2005). The presence of Gifsy-1, Gifsy-2 and Fels-2 prophage make the *lexA* gene of *Salmonella* essential for survival under conditions where it is dispensable in *E. coli* (Bunny *et al.*, 2002). Also, regulation of the stationary phase RpoS sigma subunit is markedly different in *E. coli* and *Salmonella* (Tu *et al.*, 2006). Considering the growing list of differences between these two species, it would be interesting to learn how *Salmonella* fitness, growth rate, and transposition profiles would be altered by deletion of the 4 prophage by swapping the normal *Salmonella* genes with *E. coli gyrA* and *gyrB* sequences.

MATERIALS AND METHODS

Media. All cells were grown in LB broth (Higgins *et al.*, 1996). Antibiotics were added to the media at concentrations of 50 μg/ml for kanamycin and ampicillin, 20 μg/ml for chloramphenicol, 15 μg/ml for tetracycline, 10 μg/ml for gentamicin, and 5 μg/ml for nalidixic acid.

Plasmids and Strains. All plasmids and bacterial strains in this work are listed in Tables 1 and 2. Plasmids pRW478 is derived from pBR322 by the introduction of a $(CG)_{28}$ repeat into the *EcoRI* site (Jaworski *et al.*, 1991).

Chromosomal Modification. Chromosome modifications were made with the λ RED recombination system (Yu *et al.*, 2000). Synthetic oligonucleotides were from IDT

(Coralville, IA) and the sequences are given in Table 3. PCR products used for recombineering were made using a 1:1 mixture of *Taq* polymerase (Sigma) and *Taq* extender additive (Stratagene).

Plasmid Maintenance Assay. The ability of cells to maintain plasmids was measured by serial dilutions of culture both selectively and non-selectively. Fresh LB media containing 50 μg/ml ampicillin (and IPTG when indicated) was inoculated with fresh overnight culture 1:100. For log phase measurements the culture was allowed to grow for 240 min. at 30°C. For stationary phase measurements the culture was allowed to grow 8 h or more at 30°C. A portion of each culture was serially diluted 1:10 in a 96-well micro-titer plate. Next, 3 μl of each dilution was spotted onto LB plates both with and without 50 μg/ml ampicillin and incubated overnight in a 30°C incubator.

Supercoil density measurements. Plasmid supercoiling was determined by the band counting method using agarose gels containing chloroquine (Shure and Vinograd, 1976). DNA was prepared from log phase cells grown at 30°C. At indicated times cells were collected and concentrated by centrifugation. Plasmid DNA was extracted with the Promega Wizard Plus Midiprep DNA purification system and eluted into sterile water. DNA was further purified with phenol, phenol-chloroform, and chloroform extraction and concentrated with isobutanol. Ether was used to remove trace isobutanol. Purified plasmid DNA was separated in 25 cm 1.0% agarose gels containing chloroquine. For 1-dimensional gels, 1-2 μg of DNA was loaded into a gel containing 2.0 μM chloroquine and electrophoresis was carried out at room temperature at 2V/cm for 46 hours in 0.5x TBE also containing 2.0 μM chloroquine. For 2-dimensional gels, a topoisomer set from the first dimension was cut out, soaked in 0.5x TBE with 20 μM chloroquine for four

hours, turned 90°, and recast in a new 25 cm 1.0% agarose gel containing 20 μM chloroquine. Electrophoresis in the 2nd dimension was done at room temperature at 2V/cm for 46 hours in 0.5x TBE containing 20 μM chloroquine. After electrophoresis, gels were soaked in deionized water for 30 minutes each for 4 changes and stained 60 minutes with ethidium bromide. DNA was visualized with UV light and captured on BioRad Zeta Probe Membrane using vacuum transfer. Southern Blot analysis was done with probes made using Invitrogen's Random Primed DNA Labeling System with a pBR322 template. The membranes were exposed on a Molecular Dynamics Phosphor Screen and quantitated with BioRad Gel Doc Software.

TABLE 1. Strains used in this work.

Strain	Name	From	Genotype	Plasmid
NH0405	SL4213	*S.* Tm.	*hsdL6 hsdSA29*(r_{LT}^- m_{LT}^+ r_s^- m_s^+) *metA22 metE551 ilv-452 trpB2 xyl-404 rpsL120 galE496* Fels2$^-$ *nml*	
NH0572	JTT1	*E. coli*	*pyrF gal25 nirA strA195*	
NH0742	LT2	*S.* Tm.	*musA* Mu *cts62* pAp1	
NH0974	W3110	*E. coli*		
NH1013	N99	*E. coli*	*galE*	
NH1162	MC4100	*E. coli*	Mu *cts62* pAp1	
NH2002	LT2	*S.* Tm.	WT	
NH2678	LT2	NH2002	*gyrB652 zib-6794*::Tn*10d*Tc	
NH3379	LT2	*S.* Tm.	*seqA1::(swp) frt-Tc-frt*	
NH3381	LT2	*S.* Tm	*mukB1::(swp) frt-Tc-frt*	
NH3423	W3110	*E. coli*	λcI_{857} (Δ*cro-bioA*) *thi nad*$^+$ Δ*lac169*	
NH3612	W3110	*E. coli*	WT	pSim5
NH3702	LT2	NH2002	WT	pSim5
NH3703	W3110	NH0974	*gyrB*-<frt-Kan-frt>	pSim5
NH3704	W3110	NH0974	*mutS*::Tn*10d*Tc	pSim5
NH3705	N99	NH1013	*galE*	pAG111
NH3706	W3110	NH0974	λcI_{857} (Δ*cro-bioA*) *thi nad*$^+$ Δ*lac169*	pRC03
NH3707	W3110	NH0974	λcI_{857} (Δ*cro-bioA*) *thi nad*$^+$ Δ*lac169*	pRC05
NH3708	N99	NH1013	*galE*	pTTQ18
NH3709	N99	NH1013	*galE*	pZT382
NH3710	SL4213	NH0405	see above	pAG111
NH3711	LT2	NH2002	WT	pRC03
NH3712	LT2	NH2002	WT	pRC05
NH3713	SL4213	NH0405	see above	pRW478
NH3715	SL4213	NH0405	see above	pBR322
NH3716	JTT1	NH0572	*pyrF gal25 nirA strA195*	pRW478
NH3718	N99	NH1013	*galE*	pBR322
CC4001	MM294	*E. coli*	F$^-$ *supE44 hsdR endoA1 pro thiA ΔseqA::tet*	
CC4208	MG1655	*E. coli*	*ΔmukB:: (swp) kan* λ$^-$ F$^-$ *rph-1*	

TABLE 2. Plasmids used in this work.

Plasmid	Backbone	Insert	Reference
pSim5	pSC101	(ts *oriC*) *imm*λ cI_{857}-RED$^+$ *rex*<>CAT	(Datta *et al.*, 2006)
pTTQ18	pUC18		(Stark, 1987)
pZT382	pTTQ18	P_{tac} Tn*10 tnp* (G163-D)	Roth Lab
pAG111	pTTQ18	*E. coli gyrB*	(Hallett *et al.*, 1990)
pRC03	pGem-T	*S.* Typhimurium *gyrB652*	(Pang *et al.*, 2005)
pRC05	pGem-T	*S.* Typhimurium *gyrB*	(Pang *et al.*, 2005)
pBR322			(Sutcliffe, 1979)
pRW478	pBR322	$(dC\text{-}dG)_{28}$	(Jaworski *et al.*, 1991)

TABLE 3. Oligonucleotides

Name	Sequence	Length
Oligo #1	AAGGGGACTCCGCGGGCGGCTCTGCGAAGCAGGGGAGTAACCGCAAG AACCAGGCGATTCTGCCGCTGAA	70
Oligo #2	ACTTTGTACAGCGGCGGCTGAG	20
Oligo #3	GCGAGCGTGCCTGATGCGCTACGCTTATCAGGCCTACGATGTAGGCTG GAGCTGCTTCG	59
Oligo #4	TAGCTTCTTGCCGGATGCGGCGTGAACGCCTTACATATGAATATCCTC CTTAG	53
Oligo #5	TGTAAAGCGTTACGTGTTGA	20
Oligo #6	CCGAACTGTACCTGGTGGAAGGGGACTCCGCGGGCGGCTCTGCGAAG CAGGGGAGTAACCGCAAGAACCAGGCG	74
Oligo #7	CGCGCGTGAAATGACCCGCCGT	20

In oligo's #1 and #6, the underlined A indicates the C-A transversion that creates the *gyrB652* mutation. In oligo's #3 and #4, the underlined sequences indicate the homology for amplifying the Kan cassette.

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DIFFERENTIAL CHROMOSOMAL AND PLASMID SUPERCOIL LEVELS AND FLUCTUATION IN ESCHERICHIA COLI AND SALMONELLA ENTERICA

by

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ABSTRACT

Escherichia coli and *Salmonella enterica* serovar Typhimurium are highly conserved gram-negative bacteria that share a high degree of DNA and amino acid identity. Even though the majority of their defined physiological processes are homologous, there are several differences among genes involved in genome maintenance. Previously, using plasmid DNA isolated from wild type strains of each species, we showed that in mid-log phase, *E. coli* contains about 15% more unconstrained negative supercoiling than *Salmonella.* We believe this observation explains many of the underlying differences between the species. In the present study, we show a similar difference in supercoil-sensitive reporter expression with chromosomal DNA. We also expand these experiments to the entire logarithmic growth phase and see that both species undergo nearly identical chromosomal supercoil fluctuations throughout the cell cycle. We show that *E. coli* plasmid DNA also experiences this fluctuation pattern, however, plasmid DNA in *Salmonella* remains constant over the entire time course indicating a difference in plasmid DNA maintenance between the species.

INTRODUCTION

All organisms store their DNA in a supercoiled state. There are several reasons to maintain torsional strain in the genome. First, supercoiling causes the DNA to wind upon itself to assist in the condensation process. This helps accomplish the monumental task of compacting the genome over 1000-fold to fit into its given space (Holmes and Cozzarelli, 2000). Supercoiling also allows DNA a way to store energy since the underwound helix is a thermodynamically unfavorable conformation. This energy promotes proper strand separation in processes such as the initiation and elongation of both replication (Ryan, 1976) and transcription (Sanzey, 1979; Smith *et al.*, 1978). In prokaryotes, about half of the supercoils are in an unconstrained state (Pettijohn and Pfenninger, 1980) and form an interwound, plectonemic structure. This form allows for rapid interaction between distant sites via either a "slithering" (Marko, 2001) or "branch migration" movement (Kellenberger, 1990; Kolsto, 1997; Niki *et al.*, 2000; Staczek and Higgins, 1998) that assists the cell in many processes such as site-specific recombination (Gellert and Nash, 1987; Hayes and Boehmer, 1978; Holloman and Radding, 1976; Mizuuchi and Nash, 1976), cooperative repressor binding (Schleif, 1992), gene regulation (Bellomy and Record, 1990), and transposition (Craigie and Mizuuchi, 1986; Shapiro, 1979). Many genes are also regulated by the level of supercoiling (Borowiec and Gralla, 1985; Pruss and Drlica, 1989; Rudd and Menzel, 1987). Among these are the genes that code for DNA gyrase and topoisomerase I (Menzel and Gellert, 1983). In *E. coli*, it was recently found that over 7% of the genes are directly and substantially affected by supercoil levels (Peter *et al.*, 2004).

Because of the great importance of supercoiling, cells employ regulated systems to keep the supercoil level at an optimum. Topoisomerase mutant analysis has shown that for proper cell growth, supercoiling must be maintained within 15% of the medial value (Drlica, 1992). Increases in negative supercoiling beyond this range can cause the formation of cruciforms, Z-DNA, H-DNA, and R-loops, all of which can block the replication and transcription machinery (Higgins and Vologodskii, 2004). Conversely, a decrease in supercoiling beyond this amount results in improper DNA condensation that leads to defects in genome segregation, septum formation, and cell division. In prokaryotes, the level of supercoiling is maintained primarily by the actions of topoisomerase I and DNA gyrase (Menzel and Gellert, 1983). Topoisomerase I relaxes DNA and prevents hyper-supercoiling. DNA gyrase actively introduces negative supercoils and prevents relaxation.

Escherichia coli and *Salmonella enterica* serovar Typhimurium diverged evolutionarily about 200 million years ago and have an 80% sequence identity in 65% of their genes (Lawrence, 2005; McClelland *et al.*, 2001). This correlates into a 90% amino acid identity with much of the gene organization preserved among the two. Many genetic and biochemical processes have been well defined in both and have been found to be nearly identical. However, the literature shows several differences in the species that have not been fully understood. Many of these differences focus on the cells ability to properly maintain the genome such as: (1) *Salmonella* can easily tolerate a Δ*topA* mutation but *E. coli* cannot without a compensatory mutation in the DNA gyrase genes (DiNardo *et al.*, 1982; Pruss *et al.*, 1982), (2) in rich media *E. coli* strains can grow adequately with a Δ*mukB* mutation while *Salmonella* strains do not (Champion and

Higgins, Submitted; Nordstrom and Dasgupta, 2001), (3) *E. coli* cannot tolerate the inefficient *gyrB652* mutation as can *Salmonella* (Champion and Higgins, Submitted; Pang *et al.*, 2005), (4) a Δ*seqA* mutant grown in rich media has normal growth in *Salmonella* but has growth defects in *E. coli* (Champion and Higgins, Submitted; Lu *et al.*, 1994), and (5) bacteriophage Mu causes cell lysis faster and more thoroughly in *E. coli* than in *Salmonella* (Champion and Higgins, Submitted). Previously, using plasmid DNA, we showed that the unifying explanation for these differences is an elevated amount of negative supercoiling in *E. coli* in mid-log phase cells. This difference was in the form of unconstrained supercoils and was nearly 15% higher in *E. coli*. Here, using supercoil-sensitive reporter cassettes, we show that the difference we have seen in plasmid DNA is also present in mid-log phase chromosomal DNA. We expand these experiments to the entire logarithmic growth phase and find very similar chromosomal supercoil fluctuations in both species. Plasmid DNA in *E. coli* also showed this growth phase-dependent fluctuation, however, plasmid DNA in *Salmonella* remained constant over the entire time course. These observations suggest a difference in the way plasmid DNA is bound in each species.

RESULTS

Creating Supercoil-Sensitive Chromosomal Modules. Previously we established a supercoil difference between the closely related gram-negative bacterium *Escherichia coli* and *Salmonella typhimurium*. To estimate the supercoiling levels in these experiments, we monitored the topology of a pBR322 plasmid in wild type cells. Through these experiments we established that in mid-log phase cells (Klett $70/OD_{600}$ 0.4) *E. coli* maintains its DNA at about $\sigma = -0.069$ which is 14.5% higher than the $\sigma = -0.059$ value we found with *Salmonella*. This supercoiling difference explains the inability of *E. coli* to obtain the inefficient *gyrB652* allele characterized by Pang *et al.* (Pang *et al.*, 2005). This observation also explains differences between the two species in regards to Δ*mukB*, Δ*seqA* and Δ*topA* mutations as well as differences in lysis by bacteriophage Mu. Because the previous experiments were done using plasmid DNA, we wanted to confirm our findings using a chromosomal supercoil indicator since chromosomal DNA is susceptible to processes that may affect plasmid DNA differently (e.g. transcription, replication, etc.) or not at all (e.g. domain formation, dichotomous replication, etc.).

To measure chromosomal supercoil levels, we fused the supercoil-sensitive promoter for the *gyrB* (P*gyrB*) gene to the *lacZYA* operon. This created a cassette where *lacZYA* expression is controlled by the negative supercoil level of the cell. Doing standard β-galactosidase assays on strains containing this element served as an indicator of chromosomal supercoil status. To create these elements we replaced the native *lacZ* promoter with the *gyrB* promoter sequence using a two-step lambda RED strategy (Fig. 1) (Yu *et al.*, 2000). We began with *E. coli* strain NH3734 that contains the entire

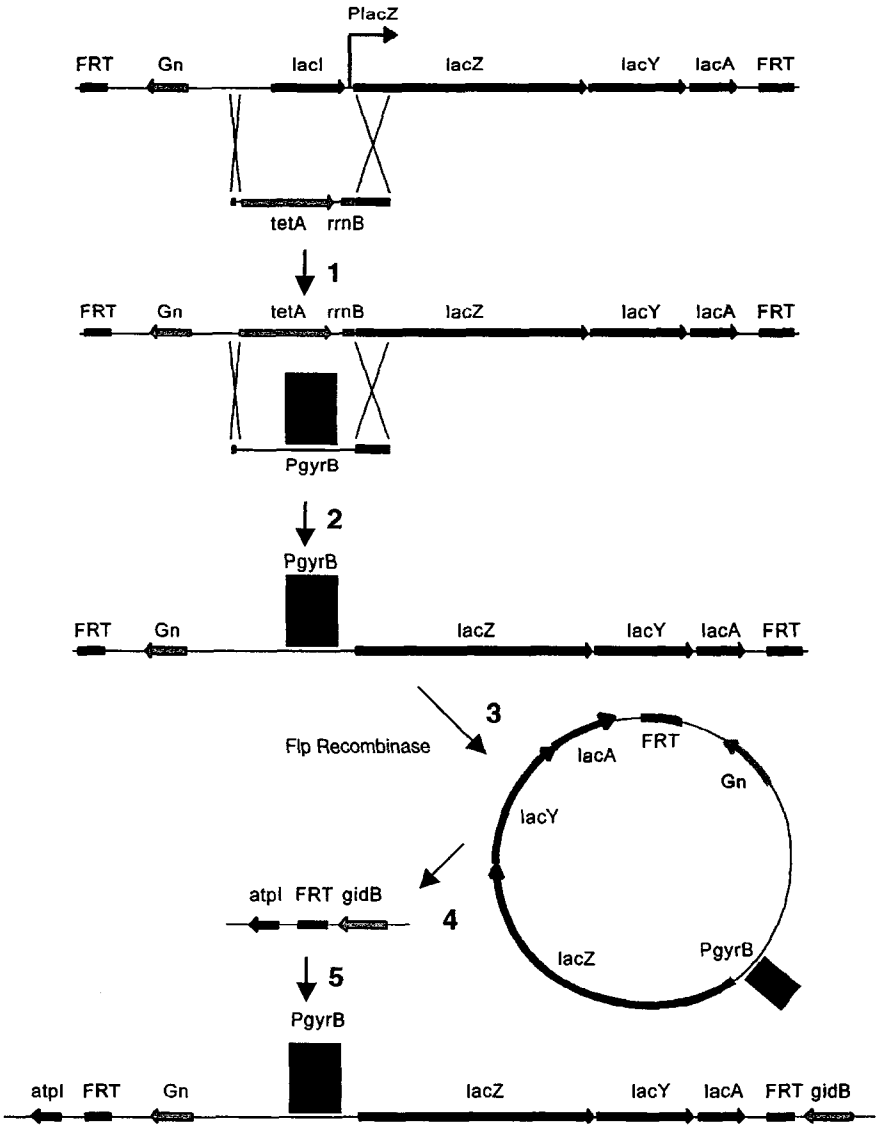

Figure 1. Promoter Swap and FLP-in Strategy. We changed promoters via a 2-step lambda RED strategy on a chromosomal locus that contained the *lacIZYA* operon and a gentamicin resistance cassette flanked by FRT sites. (1) The *lacI*-P*lacZ* region was replaced by a PCR product containing the *tetA* gene and a *rrnB* terminator (2) The *tetA-rrnB* region was then replaced by a PCR product containing the *gyrB* promoter (3) Expressing FLP recombinase from the pJB-FLP plasmid caused a recombination between the FRT sites and the formation of a DNA circle containing the P*gyrB-lacZYA* locus and the gentamicin resistance gene (4) Introducing the DNA circle into a strain with a single chromosomal FRT site located between the *atpI* and *gidB* genes and (5) expressing the FLP recombinase causes the circle to be integrated into the chromosomal locus.

lacIZYA operon, at its native locus, adjacent to a gentamicin resistance gene. The entire module is flanked by two FRT recombination sites that will aid in genomic insertion later. First we replaced the *lacI*-P*lacZ* region with a PCR product (LacI:TetA-*rrnB*-forward x LacI:TetA-*rrnB*-reverse) (Table 4) containing the Tn*10* tetracycline resistance gene (*tetA*) followed by the strong transcription terminator *rrnB*. We deleted *lacI* to avoid complications due to repressor binding. This first step created strain NH3735 that was tetracycline resistant and had a *lacZYA* operon with no promoter. We were able to select for this recombination using LB plates with tetracycline and confirm the change by their white growth on media containing 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal). In the second step, we replaced the *tetA* gene and *rrnB* terminator with the 11-182 base pair region upstream of the *gyrB* start codon (P*gyrB-lacZ*-forward x LacRev-02-II) to create strain NH3736 (Hardy and Cozzarelli, 2005). We were able to isolate recombinant strains by screening for pale blue colonies on LB plates with X-Gal. We then confirmed these strains by their sensitivity to tetracycline and later by PCR. Alternatively, we could select for blue tetracycline resistant colonies on Bochner plates containing X-Gal grown at 42°C (Bochner *et al.*, 1980).

After forming the P*gyrB-lacZYA* construct we tested its sensitivity to supercoiling levels by doing β-galactosidase assays (Miller, 1972) in the presence of the DNA gyrase inhibitor coumermycin A1. Increasing amounts of coumermycin A1 will inhibit DNA gyrase in the cell in a dosage-dependent manner and cause a decrease in the negative supercoil level. If the P*gyrB-lacZYA* locus is supercoil-sensitive, it will result in elevated β-galactosidase activity measured in Miller Units (Miller, 1972). We diluted overnight cultures of NH3736 1:100 in 5 ml of fresh LB media with 10 μg/ml gentamicin and grew

at 37°C for 2 hours. Next we added various amounts of coumermycin A1 and took 1 ml samples every 15 minutes. We then analyzed all samples as described previously (Simons *et al.*, 1987) and plotted the results as β-galactosidase activity against the OD_{600} of each sample (Fig. 2*A*). The slopes of each curve were determined and indicated the rate of β-galactosidase expression (Miller and Simons, 1993). Compared to the slope of cells that received no coumermycin A1, it was clear that the β-galactosidase expression was elevated with increasing amounts of DNA gyrase inhibitor. The 5 μg/ml coumermycin A1 sample gave a 1.74-fold increase and the 10 μg/ml sample gave a 3.04-fold increase. At 20 μg/ml coumermycin A1 the P*gyrB-lacZYA* construct gave in excess of 5-fold more β-galactosidase expression than the control strain (Fig. 2*B*). These results confirmed that our construct was sensing and reporting on the supercoil level of the cells. We next wanted to move our construct into equivalent positions in the *E. coli* and *Salmonella* genome using the FLP-FRT system described previously (Pang *et al.*, 2005). To do this we took advantage of the FRT sites that flanked the P*gyrB-lacZYA* cassette and gentamicin resistance gene (Fig. 1). By expressing FLP recombinase in strain NH3736, we could stimulate recombination between the FRT sites and create a DNA circle that contained our P*gyrB-lacZYA* cassette, the gentamicin resistance gene, and a single FRT site. We then isolated these circles and introduced them into strains NH3738 and NH3739, which contained a single chromosomal FRT site at a highly homologous region at minute 84 between the *atpI* and *gidB* genes. Expressing FLP recombinase in strains that contained the DNA circle and a chromosomal FRT site caused the P*gyrB-*

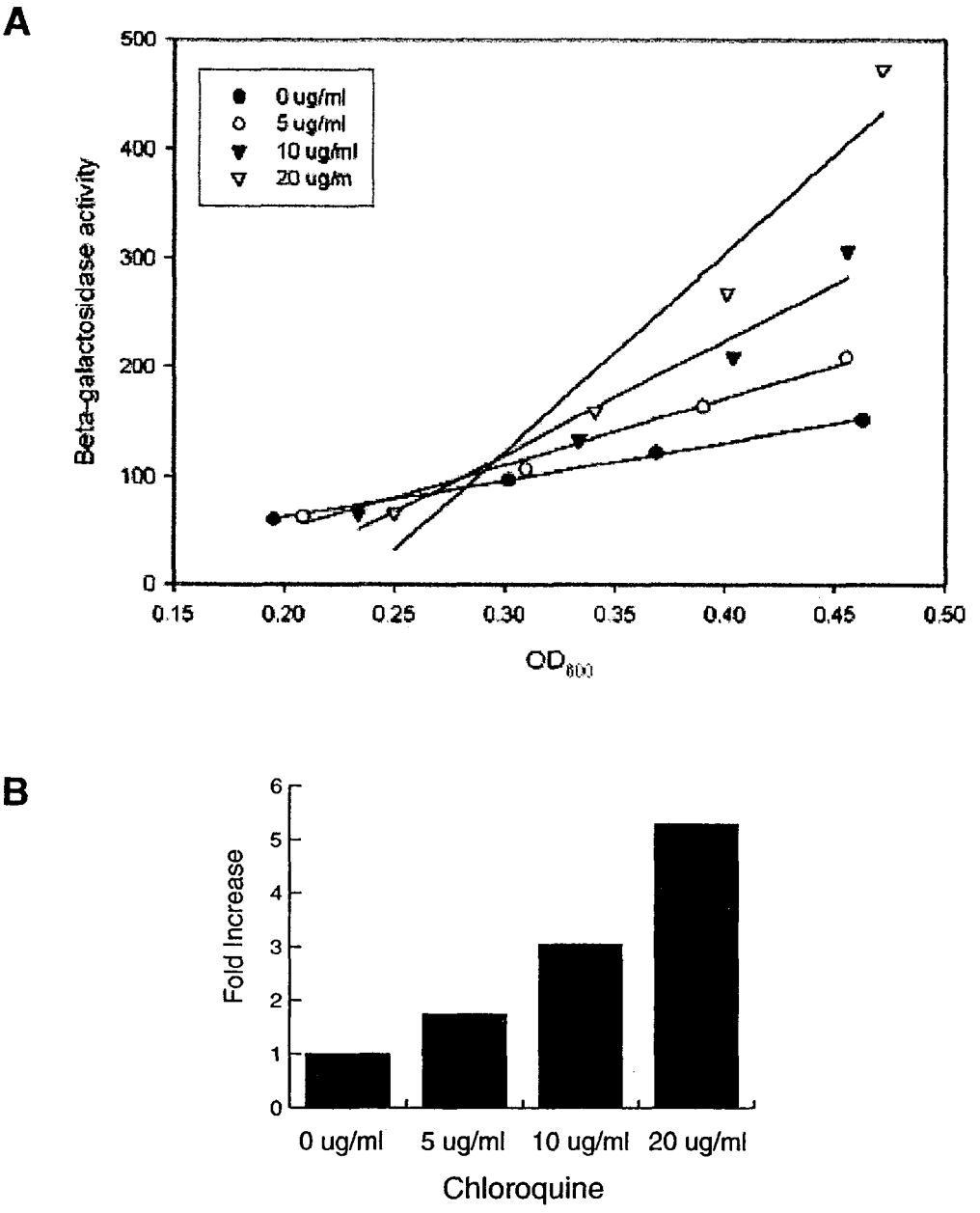

Figure 2. Testing Supercoil-Sensitivity of P*gyrB-lacZYA* Constructs. Constructs containing the P*gyrB-lacZYA* module were grown to mid-log phase and the indicated amount of the DNA gyrase inhibitor coumermycin A1 was added. Incubation was continued and samples were taken every 15 minutes. β-galactosidase assays were performed with each sample. (A) Results were graphed as β-galactosidase activity versus OD_{600} of the culture. Linear regression was used to fit a trend line to each data set. (B) The slope of each line compared against the assay with no coumermycin A1 indicated the fold increase with increasing amounts of drug.

lacZYA-gentamicin circle to recombine into the chromosome at the FRT site creating strain NH3740 in *E. coli* and NH3741 in *Salmonella*.

We used the above strategy to introduce two other types of cassettes into the same chromosomal loci. Strain WM283 contains a P*gyrA-lacZYA* fusion cassette that was used to estimate differential domain supercoiling in *E. coli* previously (Miller and Simons, 1993). Replacing the P*gyrB* PCR in the above strategy with the 378 base pair region upstream of the *lacZ* start site of strain WM283 produced the P*gyrA-lacZYA* strains NH3742 in *E. coli* and NH3743 in *Salmonella*. These strains, like the P*gyrB-lacZYA* strains, react by increasing expression with decreasing levels of negative supercoiling. The other type of cassette was made by using the FLP-mediated circle from strain NH3734, which contained the wild type *lacIZYA* operon, to create a P*lacZ-lacIZYA* module that we inserted into the same chromosomal loci to create strains NH3746 in *E. coli* and NH3747 in *Salmonella*. This construct is controlled by the native *lacZ* promoter and activated by elevated amounts of negative supercoiling, thus reacting in the opposite manner as the P*gyrA-lacZYA* and P*gyrB-lacZYA* constructs.

***E. coli* and *Salmonella* have Chromosomal Supercoil Differences in Mid-Log Phase Cells.** To determine supercoil difference via β-galactosidase expression, we diluted overnight cultures of strains with the above constructs 1:100 in 5 ml LB with 10 μg/ml gentamicin and grew at 37°C until the cultures reached Klett 70/OD_{600} 0.4. This part of the growth phase coincides with the harvest time used to isolate plasmid DNA previously (Champion and Higgins, Submitted). Since the P*lacZ-lacIZYA* construct contained the LacI repressor, 0.1 mM IPTG was added to these samples 10 minutes prior to collection to saturate LacI and allow unrepressed expression of *lacZYA*. After

incubating the strains on ice to stop growth, we performed routine β-galactosidase assays (Miller, 1972). The *E. coli* P*lacZ-lacIZYA* construct produced about 2500 Miller Units, which is 30% more expression than the 1700 Miller Units from the *Salmonella* strain (Table 1). Since production from the *lacZ* promoter is induced with increased negative supercoiling (Borowiec and Gralla, 1987; Miller and Simons, 1993; Sanzey, 1979; Sternglanz *et al.*, 1981), these results indicate a higher level of negative supercoiling in *E. coli*. However, because the supercoil-sensitivity of P*lacZ* is minimal (Peter *et al.*, 2004), a quantitative difference between the strains is probably not reliable. The more reliable comparison is represented with the P*gyrB-lacZYA* constructs where the overall expression numbers were in the 300 Miller Unit range. The vastly different amount of β-galactosidase activity verifies a change in the promoter region based on findings that the promoter strength of P*gyrB* is much less than P*lacZ* (Bernstein *et al.*, 2002; Wei *et al.*, 2001). In addition, the *gyrB* promoter is extremely sensitive to the supercoil level in the cell, making it a better candidate for a quantitative comparison (Peter *et al.*, 2004).

Promoter	*E. coli*	*Salmonella*	% Difference
P*lacZ*	2574 ± 227	1758 ± 312	31.7
P*gyrB*	306 ± 3	339 ± 7	9.7
P*gyrA*	1517 ± 117	1809 ± 58	16.1

TABLE 1. β-galactosidase expression in OD_{600} 0.4 culture. Strains were diluted 1:100 in fresh LB with 10 μg/ml gentamicin and grown at 37°C until the culture reached an OD_{600} of 0.4. Several assays were done with each strain. The average with the standard deviation are shown. The percent difference between the species is also shown.

Salmonella strains with this construct repeatedly gave higher expression levels than the same construct in *E. coli.* The average amount of this difference was nearly 10% (Table 1). Since P*gyrB* is inhibited by elevated supercoiling, this indicates that *E. coli* in mid-log phase cells contains more negative supercoiling than *Salmonella.* The P*gyrA-lacZYA* constructs, whose expression is controlled by the less supercoil-sensitive *gyrA* promoter (Peter *et al.*, 2004), gave Miller Unit values in the 1500-1800 range. Again, the finding that the P*gyrA* construct gave an intermediate β-galactosidase expression between P*lacZ* and P*gyrB* is in accordance with promoter strength data (Bernstein *et al.*, 2002; Wei *et al.*, 2001). As with the P*gyrB-lacZYA* results, *Salmonella* with this cassette consistently gave higher β-galactosidase expression than *E. coli* (Table 1). Data from these reporters showed that *E. coli* had 16.1% more expression than *Salmonella.* To summarize, all three supercoil-sensitive constructs, as well as our previous plasmid gel experiments show that in mid-log phase cells *E. coli* contains more negative supercoiling than *Salmonella.* However, the results with the β-galactosidase experiments do not correlate to actual negative supercoil levels and thus only indicate a similarity in chromosomal DNA to plasmid DNA. To assign supercoil density values to these results will require the production of a standard curve to correlate Miller Unit values from specific promoters to plasmid sigma values.

***E. coli* and *Salmonella* Plasmid DNA have Differential Supercoiling Patterns as a Result of Growth Phase.** The supercoil differences we have seen between *E. coli* and *Salmonella* using plasmid supercoiling and chromosomal reporters have all been done using mid-log phase cells (Klett 70/OD_{600} 0.4). It has previously been reported that *E. coli* cells exhibit a relaxation when moving toward stationary phase (Dorman *et al.*,

1988; Reyes-Dominguez *et al.*, 2003; Schneider *et al.*, 1999; Tse-Dinh and Beran, 1988) and an increase in supercoiling when a stationary phase culture is moved into fresh media (Reyes-Dominguez *et al.*, 2003). Therefore, we wanted to establish plasmid supercoil levels for both species throughout the cell cycle to see if *E. coli* remained more negatively supercoiled throughout. To do this, we isolated plasmid DNA from wild type strains of *E. coli* and *Salmonella* containing pBR322 (NH3718 and NH3715) (Champion and Higgins, Submitted) and performed 1-dimensional chloroquine gel electrophoresis. From an overnight culture, we diluted the strains 1:100 in fresh LB containing 50 μg/ml ampicillin and allowed the cells to grow at 37°C. At various time points we took samples, prepared plasmid DNA (Materials & Methods), and performed electrophoresis (Fig. 3*A*). After electrophoresis we quantitated each band in the topoisomer distribution and determined supercoil density via the band-counting method (Shure and Vinograd, 1976). Our results showed a dramatic supercoil fluctuation pattern with *E. coli* DNA and only slight changes with *Salmonella*. *E. coli* DNA at both the 45 and 90 minute time points was approximately $\sigma = -0.055$ which is slightly relaxed. Over the next 45 minutes, *E. coli* makes a dramatic shift toward the very negatively supercoiled value of $\sigma = -0.06814$. During the last 75 minutes, as well as into stationary phase (Data Not Shown), the plasmid population becomes more relaxed to a medial level of $\sigma = -0.06212$, very similar to the *Salmonella* value of $\sigma = -0.06170$ at this time point. The *Salmonella* plasmid showed almost no fluctuation throughout the cell cycle giving a maximum supercoil value of $\sigma = -0.06188$ at 45 minutes and a minimum of $\sigma = -0.06067$ at 135 minutes. This represents a less than 2% supercoil fluctuation over the entire time course compared to a nearly 20% fluctuation in *E. coli*. These results are represented

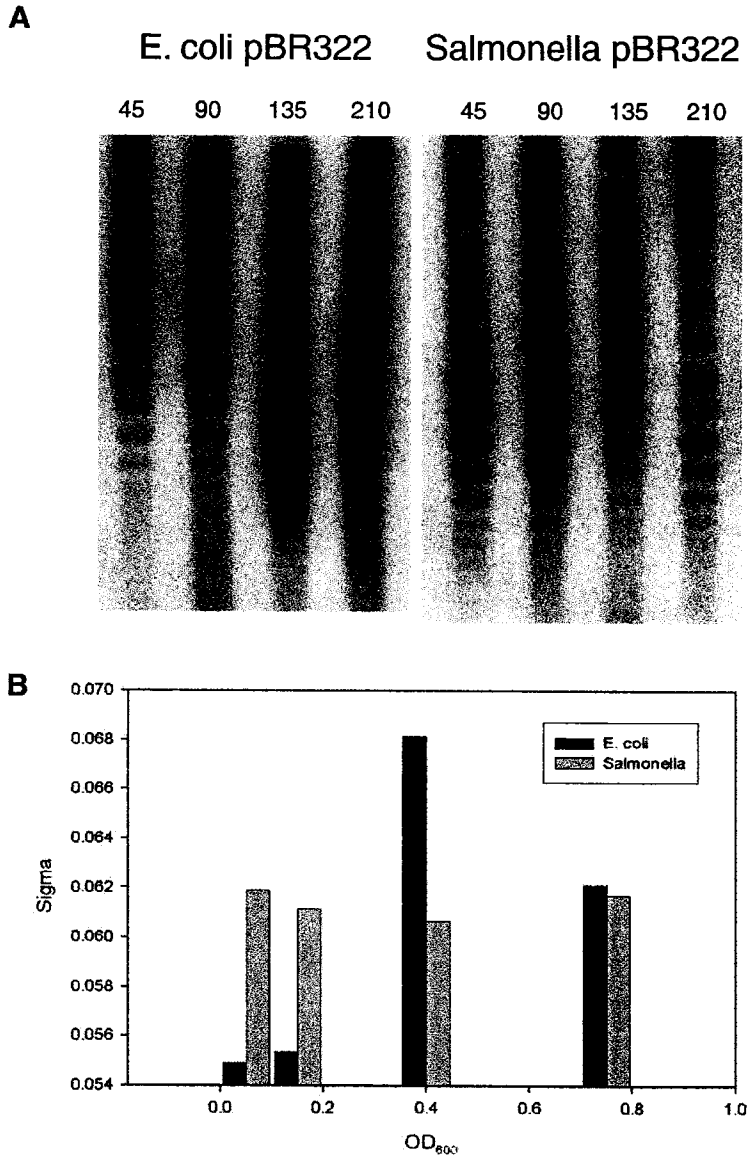

Figure 3. Chloroquine Gel Time Course Experiment. Overnight cultures of wild type *E. coli* and *Salmonella* with pBR322 were diluted 1:100 into fresh LB containing 50 μg/ml ampicillin and grown at 37°C. (A) Samples were taken at the indicated time points and plasmid DNA was isolated and run on a 1.5 μM chloroquine gel as described previously. (B) The sigma of each sample was determined and plotted against the OD_{600} of the culture.

graphically in Figure 3*B* where the absolute value of sigma at each time point is plotted against the OD_{600} of the culture when plasmid DNA was isolated. Of note in the graph is that the point of maximal difference between the two species is at an OD_{600} of about 0.4 where *E. coli* is 11% more negative than *Salmonella*. This correlates well to Klett 70 where we isolated DNA for our previous chloroquine gel experiments (Champion and Higgins, Submitted) and did β-galactosidase assays (Table 1). This pattern of supercoiling in *E. coli* is in agreement with previous reports that showed a decrease in negative supercoiling when *E. coli* cells approach stationary phase (Dorman *et al.*, 1988; Reyes-Dominguez *et al.*, 2003; Schneider *et al.*, 1999; Tse-Dinh and Beran, 1988) and an increase in negative supercoiling when stationary phase cells are inoculated into fresh media (Reyes-Dominguez *et al.*, 2003). Also of note is that with the exception of the time period when cells are coming out of stationary phase, *E. coli* is more negatively supercoiled than *Salmonella*, which is in agreement with our earlier results and our observation that the *gyrB652* mutation is not viable in *E. coli* (Champion and Higgins, Submitted). This may be due to a need for a higher level of supercoiling in *E. coli* that the sluggish *gyrB652* enzyme cannot provide.

***E. coli* and *Salmonella* have Similar Growth Phase Dependent Chromosomal Supercoil Fluctuations.** The changes in *E. coli* plasmid DNA throughout the cell cycle, as well as the lack of changes in *Salmonella* plasmid DNA, prompted us to investigate if the same phenomena occur in chromosomal DNA. To do this we diluted strains NH3740 and NH3741, which contain the P*gyrB-lacZYA* element, 1:100 in fresh LB containing 10 μg/ml gentamicin and grew at 37°C. We isolated samples at time points that correlated

with the OD_{600} levels of the plasmid DNA time course experiments. The results showed that both species have a very similar supercoil fluctuation over the cell cycle that correlated very well with the *E. coli* plasmid data (Fig. 4). The first sample in both species showed a high level of β-galactosidase activity with *E. coli* having a slightly lower level of supercoiling than *Salmonella*, which is in agreement with plasmid data from Figure 3. The next sample showed a dramatic increase in negative supercoiling that coincides with the increase in the *E. coli* plasmid experiments. This time point has an OD_{600} of about 0.4 which is equivalent to the mid-log phase time point we used for chromosomal DNA comparisons earlier (Table 1) as well as previously reported plasmid DNA results (Champion and Higgins, Submitted). Here, the difference in the two was 9.6% with *E. coli* being more negatively supercoiled. This is in almost perfect agreement with the 9.7% difference given in Table 1 for this time point. The next time point shows a slight relaxation in both species as they approach stationary phase. Again, even though the results with the chromosomal DNA are not as dramatic, they correlate very well with the *E. coli* plasmid time course experiments and previous observations about supercoil changes throughout the cell cycle (Reyes-Dominguez *et al.*, 2003).

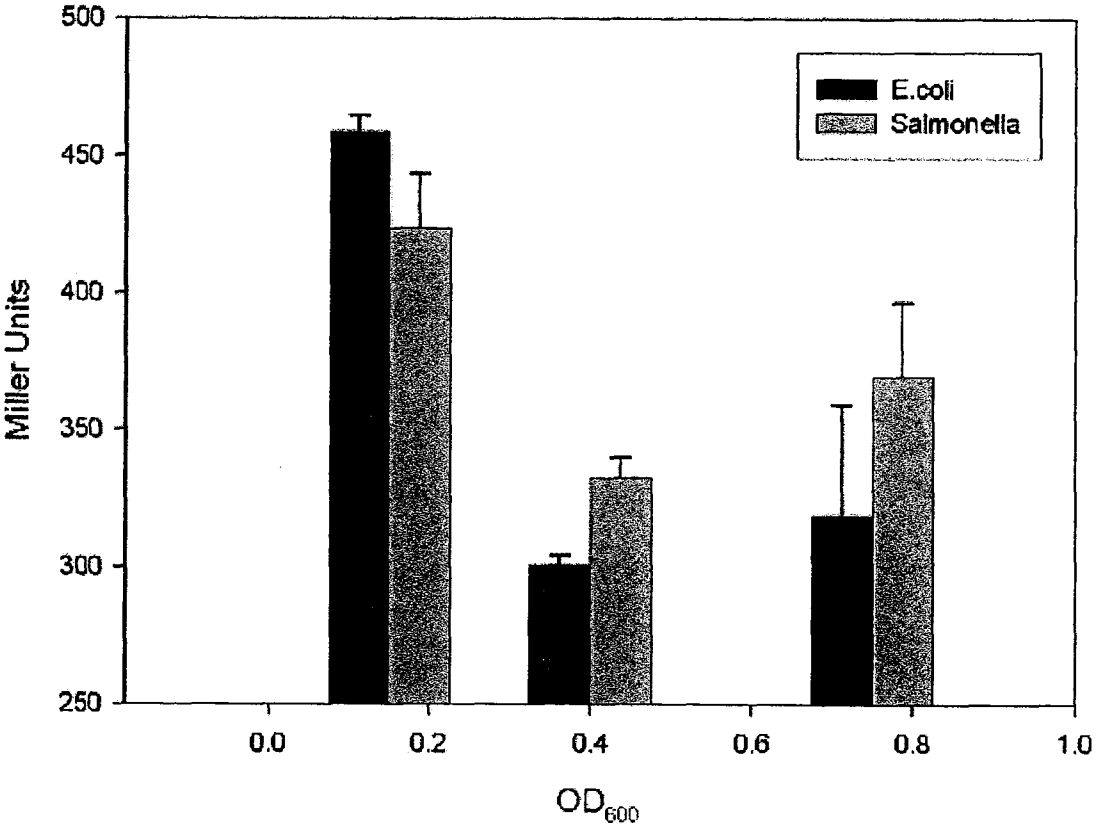

Figure 4. P*gyrB-lacZYA* Construct Time Course Experiment. Overnight cultures of strains containing the P*gyrB-lacZYA* reporter cassette were diluted 1:100 into fresh LB containing 10 μg/ml gentamicin and grown at 37°C. Samples were taken at various time points and routine β-galactosidase assays were performed. The resulting Miller Units were plotted against the OD_{600} of the culture.

DISCUSSION

In the present study, we have confirmed previous findings that *E. coli* cells in mid-log phase contain more negative supercoiling than *Salmonella*. In our previous experiments we used pBR322 DNA and chloroquine gel technology. Because of possible differences that could arise in chromosomal DNA as compared to plasmid DNA, we wanted to compare chromosomal supercoil levels. We did this by creating supercoil sensitive reporter cassettes that we inserted into a homologous locus in wild type strains of both species. For the supercoil-sensitive promoter we chose the control region of the *gyrA* and *gyrB* genes of DNA gyrase. We chose these because they have been well characterized and used previously in fusion cassette experiments (Menzel and Gellert, 1987; Miller and Simons, 1993; Neumann and Quinones, 1997). We focused on the data using the *gyrB* promoter based on the very high level of supercoil sensitivity that it has shown (Peter *et al.*, 2004). Next, we fused each promoter to the *lacZYA* operon in place of the native *lacZ* promoter and the *lacI* repressor gene such that β-galactosidase expression would be controlled by the amount of negative supercoiling in the cell (Fig. 1). We tested the construct sensitivity to the DNA gyrase inhibitor coumermycin A1 (Fig. 2) and moved the construct into the genome using a FLP-FRT strategy described previously (Fig. 1) (Pang *et al.*, 2005).

Strains used in our previous experiments were collected at an OD_{600} of about 0.4 (or Klett 70) when cells are in the mid-log phase of growth. We collected strains with our chromosomal reporter cassettes at this OD_{600} and performed standard β-galactosidase assays (Table 1). With the P*gyrB-lacZYA* construct we measured 306 Miller Units from *E. coli* and 339 from *Salmonella*. This represents a 9.7% increase of expression in *E. coli*

over *Salmonella*. Using the P*gyrA-lacZYA* construct, which is less supercoil-sensitive, gave 1517 Miller Units in *E. coli* and 1809 in *Salmonella*, which equals a 16.1% greater expression level in *E. coli*. In our previous plasmid data, we measured *E. coli* $\sigma = -0.069$ which is 14.5% higher than the $\sigma = -0.059$ we measured in *Salmonella*. As a confirmation of this trend, the P*lacZ-lacIZYA* construct gave much higher Miller Unit values in *E. coli* than it did in *Salmonella*. Since the *lacZ* promoter is activated by negative supercoiling, these results also indicate that *E. coli* cells contain more negative supercoiling than *Salmonella*. As mentioned previously, the β-galactosidase results do not necessarily correlate to absolute supercoil density but give a fair approximation. Experiments to more precisely correlate β-galactosidase results to supercoil density values are currently in progress.

Previous reports detailing supercoil changes as cells enter and exit stationary phase (Dorman *et al.*, 1988; Reyes-Dominguez *et al.*, 2003; Schneider *et al.*, 1999; Tse-Dinh and Beran, 1988) prompted us to investigate whether *E. coli* maintained its DNA at a higher supercoil density at all stages of the cell cycle. To do this, we inoculated our P*gyrB-lacZYA* reporter cassette strains into fresh media and took samples at several time points to cover log phase growth. The data from both species gave a nearly identical supercoil pattern (Fig. 4). At the earliest time point, both were slightly relaxed and *Salmonella* appears to be slightly more supercoiled than *E. coli*. As the cell cycle progressed, both species underwent a significant increase in supercoiling as the cells approached OD_{600} of 0.4. Here the *E. coli* sample gave 301 Miller Units and the *Salmonella* gave 333. This gives a difference of 9.6% and is nearly identical to both the raw Miller Unit numbers (306 for *E. coli* and 339 for *Salmonella*) and difference (9.7%)

that we saw from the experiments in Table 1. As the strains approached stationary phase, both species became slightly more relaxed with *E. coli* maintaining its elevated amount of supercoiling over *Salmonella*. This supercoil fluctuation pattern is in agreement with previous observations that as *E. coli* cells approach stationary phase, the amount of supercoiling decreases slightly (Dorman *et al.*, 1988; Reyes-Dominguez *et al.*, 2003; Schneider *et al.*, 1999; Tse-Dinh and Beran, 1988), and when stationary phase cells are inoculated into fresh media, they undergo an increase in negative supercoiling (Reyes-Dominguez *et al.*, 2003). Our results confirm these observations for both *E. coli* and *Salmonella* chromosomal DNA.

We also did similar experiments with the wild type pBR322 strains NH3718 and NH3715 using 1-dimensional chloroquine gels to look at plasmid DNA supercoiling over the time course (Fig. 3). The *E. coli* results were very similar to the β-galactosidase results with the chromosomal reporters. As cells begin to move into log phase, they are slightly relaxed but then become much more supercoiled as the cells reach a time point near OD_{600} of 0.4. At this time point, *E. coli* pBR322 was $\sigma = -0.06814$ which is very close to the –0.069 value reported previously (Champion and Higgins, Submitted). From here, the *E. coli* plasmid DNA becomes more relaxed as it moves toward stationary phase. This fluctuation pattern is almost identical to the above results with the β-galactosidase assays. However, the *Salmonella* plasmid DNA showed almost no fluctuation. As cells entered log phase, they were measured at $\sigma = -0.06188$ and became slightly more relaxed over the time course. The supercoil density fluctuation over the entire experiment was less than 2%. Of note is the observation that for most of the cell cycle *E. coli* remains more supercoiled than *Salmonella*, which is in agreement with the

inability of *E. coli* to obtain the *gyrB652* mutation. A comparison of the OD_{600} growth points with all experiments highlights this increased superhelicity in mid-log phase cells (Table 2). The plasmid time course shows an *E. coli* σ of -0.06814 at this growth point, which is 11% higher than the -0.06067 value from *Salmonella*. This is in close agreement to the 14.5% difference between the σ values of -0.069 and -0.059 from previous experiments (Champion and Higgins, Submitted). This is also in close agreement with chromosomal supercoiling data from this time point. Constructs with P*gyrB-lacZYA* gave Miller Units of 306 and 339 for the single time point (Table 1) and 301 and 333 for the time course (Fig. 4). These show a 9.7% and 9.6% difference. The P*gyrA-lacZYA* results gave 1517 and 1809 Miller Units, which represent a 16.1% difference (Table 1). Taken together (Table 2), all of these results confirm the hypothesis

Experiment		EC σ	ST σ	EC β-gal	ST β-gal	%Δ
2D Chloroquine Gel	REF[1]	-0.069	-0.059			14.5
1D Chloroquine Gel	Fig.3	-0.068	-0.060			11.0
P*gyrB* β-gal	Tab.1			306	339	9.7
	Fig.4			301	333	9.6
P*gyrA* β-gal	Tab.1			1517	1809	16.1

1 – Champion & Higgins, Submitted

TABLE 2. Summary of OD_{600} 0.4 data. Results from this report or previous work in our lab are summarized for the OD_{600} 0.4 time point. Chloroquine gel data from Fig. 3 and Champion & Higgins (2006) are given as well as β-galactosidase assay results from the P*gyrB-lacZYA* OD_{600} 0.4 experiments (Table 1), the OD_{600} 0.4 time point of the P*gyrB-lacZYA* time course (Fig. 4), and the P*gyrA-lacZYA* OD_{600} 0.4 experiments (Table 1.). The percent difference between the species in each set of experiments is given.

that in mid-log phase cells, *E. coli* contains more negative supercoiling than *Salmonella*. The plasmid results suggest the amount of this difference is about 15%.

The difference in supercoiling in mid-log phase cells may explain several differences between *E. coli* and *Salmonella* in respect to genome maintenance. A Δ*mukB* mutation in *Salmonella* is several orders of magnitude more growth compromised in rich media than is *E. coli* (Champion and Higgins, Submitted). Increases in negative supercoiling can partially suppress the *muk* phenotype and explains this observation (Sawitzke and Austin, 2000). Conversely, *E. coli* strains with a Δ*seqA* mutation grown in rich media have a much more severe growth penalty than do these mutants in *Salmonella* (Champion and Higgins, Submitted). Since the opening of DNA in the *oriC* region to allow replication initiation is dependent on negative supercoiling, the need for SeqA to prevent over- initiation is more critical in *E. coli* than in *Salmonella* and also supports our results. Observations with bacteriophage Mu are also explained by a difference in supercoiling. It has been shown that Mu lyses *E. coli* faster than *Salmonella* (Champion and Higgins, Submitted). Since increases in negative supercoiling have been shown to increase lysis by up to 40 fold (Higgins *et al.*, 1989), this fits well with our model. Finally, a supercoil difference may also explain the long-standing observation that a Δ*topA* mutation requires compensatory mutations in *E. coli* but not in *Salmonella* (DiNardo *et al.*, 1982; Pruss *et al.*, 1982; Zumstein and Wang, 1986). *E. coli* already contains a higher supercoil set-point and cannot tolerate further negative supercoiling resulting from a loss of topoisomerase I activity. *Salmonella* contains a lower level of negative supercoiling and, because of this, can tolerate increases in negative supercoiling. It has also been observed that *Shigella flexneri* can tolerate a Δ*topA* mutation without

compensatory mutation much like *Salmonella* (Bhriain and Dorman, 1993). Along these lines, in an assay for Z-DNA forming potential in CG repeats, it has been shown that *Morganella*, *Klebsiella*, and *Enterbacter* contain less torsional strain than *E. coli* (Jaworski *et al.*, 1991). These data suggests that *E. coli* contains a higher supercoil set-point than many other gram-negative bacterium.

The finding of differential supercoil fluctuation in plasmid DNA is also of interest. Why does the *Salmonella* plasmid DNA not show a fluctuation pattern like plasmid DNA in *E. coli*? The observation that plasmid DNA has supercoil fluctuation differences, but chromosomal DNA does not, suggests that there is a difference in the way plasmid DNA is bound. Fis is a non-specific DNA binding protein that is identical in *E. coli* and *Salmonella* (Osuna *et al.*, 1995). Both Fis and *fis* mRNA are present at low levels in stationary phase, but become very abundant upon nutrient up-shift, coinciding with the increase in negative supercoiling (Osuna *et al.*, 1995; Schneider *et al.*, 1997). It has been shown that Fis in stationary phase binds and stabilizes moderately supercoiled plasmid DNA and delays the actions of both DNA gyrase and topoisomerase I. In addition, it has been shown that Fis inhibits transcription of both subunits of DNA gyrase. Because of these activities, Fis allows easier recovery from stationary phase by preventing deleterious unwinding of the DNA due to the fast increase in negative supercoiling (Schneider *et al.*, 1997). It has been reported that in mid-log phase cells, *Salmonella* contains more Fis molecules than *E. coli*. It also appears that *Salmonella* Fis is not depleted in stationary phase to the extent it is in *E. coli*. In addition, because of differences in the promoter regions, *fis* auto-regulation is 4-fold less efficient in *Salmonella* (Osuna *et al.*, 1995). Increased levels of Fis in *Salmonella* could bind and

stabilize moderately supercoiled plasmid DNA, shield the plasmid from topoisomerases, and prevent the fluctuations we see in *E. coli* where Fis is not as prevalent. The chromosomal markers in both species are probably not affected because of other chromosomal DNA binding proteins, domain barriers, and replication and transcriptional forces acting on the chromosome or by a higher affinity of Fis for plasmid DNA.

MATERIALS AND METHODS

Media. All cells were grown in LB media prepared as usual. Antibiotics were added to the media at concentrations of 50 μg/ml for ampicillin, 15 μg/ml for tetracycline, and 10 μg/ml for gentamicin. Bochner plates were made as described previously (Bochner *et al.*, 1980).

Beta Galactosidase Assays. Levels of β-galactosidase were determined with cells grown for given lengths of time at 37°C. After sufficient growth, cultures were incubated on ice for at least 20 minutes to stop growth. 100 μl of the culture was added to 900 μl Z-Buffer containing beta-mercapto ethanol, SDS, and chloroform. Samples were then vortexed for 30 seconds and incubated in a 30°C shaking water bath for 2 minutes. 200 μl of 4 mg/ml ONPG was then added to each sample and incubated in a 30°C shaking water bath until a yellow color appeared. At that time, 500 μl of 1M Sodium Carbonate was added to the assay to stop the reaction. The reaction was measured at 420λ and 550λ, the culture was measured at 600λ, and Miller Units were determined (Miller, 1972).

Chromosomal Modifications. LacZYA fusion constructs were made in a two-part experiment using the lambda RED recombination system (Fig. 1) (Yu *et al.*, 2000). First,

the *lacI* gene and the *lacZ* promoter were replaced with the *tetA* tetracycline resistance cassette followed by a *rrnB* transcription terminator to prevent read-through. Tetracycline resistant colonies were screened for the loss of *lacZYA* production on plates containing 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal). In the second step, replacement of the tetracycline-*rrnB* cassette with a PCR product containing the promoter of interest was either screened on LB plates containing X-Gal or selected using Bochner plates. Tetracycline sensitive constructs with the new promoter driving *lacZYA* production were verified by reaction to coumermycin A1 and by PCR.

Inserting the *lacZ*-fusion constructs into the *E. coli* and *Salmonella* chromosome was accomplished using FLP mediated recombination (Pang *et al.*, 2005). Kanamycin resistant plasmids containing a gentamicin resistance cassette and either the wild type P*lacZ-lacIZYA* operon or a modified P*gyrA/B-lacZYA* construct flanked by FRT sites were introduced into *lac*$^-$ strains containing pJBFlp and a chromosomal FRT site. These cells were grown to mid log phase at 30°C and then shifted to 42°C for 60 minutes to induce Flp recombinase expression from pJBFlp. Cells were then returned to 30°C and grown overnight. Phage P1 lysates were grown on the overnight cultures and transductions were done with these lysates on a wild type *lac*$^-$ strain. Gentamicin resistant colonies were selected and screened for kanamycin sensitivity and β-galactosidase production on X-Gal plates.

Plasmid Supercoil density measurements. Plasmid supercoiling was determined by the band counting method using agarose gels containing chloroquine (Shure and Vinograd, 1976). DNA was prepared from log phase cells grown at 37°C. At indicated times cells were collected and concentrated by centrifugation. Plasmid DNA was extracted with the

Promega Wizard Plus Midiprep DNA purification system and eluted into sterile water. DNA was then further purified with phenol, phenol:chloroform, and chloroform extraction and concentrated with isobutanol. Ether was used to remove trace isobutanol. 1-2 μg of purified plasmid DNA was separated in a 25 cm 1.0% agarose gels containing 1.5 μM chloroquine at 2V/cm for 46 hours in 0.5x TBE also containing 1.5 μM chloroquine. After electrophoresis, gels were soaked in deionized water for 30 minutes each for 4 changes and stained 60 minutes with ethidium bromide. DNA was visualized with UV light and captured on BioRad ZetaProbe Membrane using vacuum transfer. Southern Blot analysis was done with probes made using Invitrogen's Random Primed DNA Labeling System with a pBR322 template. The membranes were exposed on a Molecular Dynamics Phosphor Screen and quantitated with BioRad Gel Doc Software.

TABLE 3. Strains used in this work.

Strain	Name	From	Genotype	Plasmid
NH0405	SL4213	*S.* Tm.	*hsdL6 hsdSA29*(r_{LT}^- m_{LT}^+ r_s^- m_s^+) *metA22 metE551 ilv-452 trpB2 xyl-404 rpsL120 galE496* Fels2$^-$ *nml*	
NH0402	MC4100	*E. coli*	F$^-$ *araD139 Δ(argF-lac) U169 rpsL150 RelA1 flbB5301 deoC1 ptsF25 rbsR*	
NH0974	W3110	*E. coli*	λcI_{857} (*Δcro-bioA*) *thi nad*$^+$ *Δlac169*	
NH1013	N99	*E. coli*	*galE*	
NH2002	LT2	*S.* Tm.	WT	
NH2854	W3110	*E. coli*	PgyrB-bla PgyrB-lacZ	
NH3715	SL4213	NH0405	see above	pBR322
NH3718	N99	NH1013	*galE*	pBR322
NH3734	N99	NH1013	*cynX*<FRT *res lacIZYA gen res* FRT>*mhpR*	pSim5
NH3735	N99	NH3734	*lacI-PlacZ* (swp) *TetA-rrnB*	pSim5
NH3736	N99	NH3735	*TetA-rrnB* (swp) *PgyrB*	pJBFlp
NH3738	W3110	NH0974	*atpI*<FRT>*gidB*	pJBFlp
NH3739	LT2	NH2002	*atpI*<FRT>*gidB*	pCP20
NH3740	MC4100	NH0402	*atpI*<FRT *res PgyrB-lacZYA gen res* FRT>*gidB*	
NH3741	LT2	NH2002	*atpI*<FRT *res PgyrB-lacZYA gen res* FRT>*gidB*	
NH3742	MC4100	NH0402	*atpI*<FRT *res PgyrA-lacZYA gen res* FRT>*gidB*	
NH3743	LT2	NH2002	*atpI*<FRT *res PgyrA-lacZYA gen res* FRT>*gidB*	
NH3746	MC4100	NH0402	*atpI*<FRT *res PlacZ-lacIZYA gen res* FRT>*gidB*	
NH3747	LT2	NH2002	*atpI*<FRT *res PlacZ-lacIZYA gen res* FRT>*gidB*	
WM283	DR459	*E. coli*	*ins100 pGyrA-lacZ*$^+$ F'*WM1*	

TABLE 4. Oligonucleotides

Name	Sequence	Length
LacI:TetA-*rrnB*-forward	ttggctgtctgaatctggtgtatatggcgagcgcaatgacCATTAA TTCCTAATTTTTGTTGAC	64
LacI:TetA-*rrnB*-reverse	gttgtaaaacgacggccagtgaatccgtaatcatggtcatCTCAGG AGAGCGTTCACCGA	60
P*gyrB*-*lacZ*-forward	ttggctgtctgaatctggtgtatatggcgagcgcaatgacTGCCTC TGAGCTTGATGATG	60
LacRev-02-II	CCGAGTTAACGCCATCAAAA	20

The upper-case sequences indicate the homology for amplifying the TetA-*rrnB* or P*gyrB* cassettes. The lower-case sequences indicate the targeting homology to place the insert upstream of the *lacZYA* operon.

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DISCUSSION

Every organism on earth maintains its DNA in a negatively supercoiled conformation. In bacteria, an underwound helix promotes proper condensation of the nucleoid, replication initiation, RNA polymerase elongation, gene expression, and the ability to monitor the growth environment and adapt to changes in nutrient availability, growth phase, osmolarity, and oxygen levels. The ubiquitous impact of supercoiling on cell viability highlights the importance of understanding its regulation and association with other cellular processes.

The contour length of prokaryotic genomes is 1000 fold longer than the cellular space. Negative supercoiling forces DNA to wind upon itself and promotes the binding of proteins that further condense the molecule. The prokaryotic condensin homolog MukB contributes to higher order structures. Proper condensation is even more amazing when you consider the extensive organization that must be maintained to allow the cell easy access to all genomic segments. Also, many processes such as replication and transcription counteract the condensation process by their need to separate the DNA strands. Therefore, bacteria must compact their genomes with minimal topological stress to prevent interfering with replication and transcription (Holmes and Cozzarelli, 2000; Trun and Marko, 1998).

Negative supercoiling also provides energy needed for separating the DNA strands during processes like the initiation and elongation of replication and transcription. This requirement is highlighted in the DnaA-dependent pathway of replication initiation.

The *oriC* region of the chromosome contains 4 unique sequences that bind the DnaA protein. Upon binding, the proteins wrap the DNA and form a complex that causes a nearby AT-rich patch to become unwound. This allows access for the DnaB helicase to start formation of a replisome. Proper supercoiling is required for this strand separation (Messer *et al.*, 2001) as shown by the inability of certain *gyrB* mutants to replicate (Filutowicz, 1980; Filutowicz and Jonczyk, 1981; Orr *et al.*, 1979). Conversely, certain *topA* mutations contain a high enough level of supercoiling to initiate replication in the absence of the DnaA protein (Louarn *et al.*, 1984).

In *E. coli,* 7% of all genes are directly affected by supercoiling in mid-log phase cells grown in rich media (Peter *et al.*, 2004). In addition, several reports indicate that cells grown in poor growth conditions adapt to new metabolic conditions through supercoiling (Balke and Gralla, 1987; Dorman *et al.*, 1988; Hsieh *et al.*, 1991; McClellan *et al.*, 1990). For example, cells grown in low salt conditions, and then shifted to higher salt conditions, increase torsional strain that stimulates the expression of *proU.* ProU is a glycine betaine transporter that restores proper osmolarity to the cell (Higgins *et al.*, 1988). Aside from gene expression, global supercoil levels must be properly maintained to prevent deleterious increases or decreases in torsional strain. Hyper-negative supercoiling causes formation of alternative DNA structures including cruciform extrusions, left-handed Z-DNA, R-loops, and H-DNA (Higgins and Vologodskii, 2004). All of these structures can act as blockades to transcription and replication as well as providing a substrate for recombinational proteins. Conversely, a 15% loss in supercoiling can cause improper DNA condensation and lead to defects in segregation and cell division (Drlica, 1992). Maintaining a proper supercoil level is achieved by the

enzymes topoisomerase I and topoisomerase IV, which relax supercoils, and DNA gyrase, which actively pumps supercoils into the genome (Menzel and Gellert, 1983; Zechiedrich *et al.*, 2000). This is best shown by early *par* mutants, which formed anucleate and guillotined cells as a result of DNA gyrase mutations (Hirota *et al.*, 1968; Kato *et al.*, 1989).

The purpose of this dissertation was to study supercoil levels in both plasmid and chromosomal DNA in closely related gram-negative bacteria. *Escherichia coli* and *Salmonella enterica* serovar Typhimurium share an 80% sequence identity and a 90% amino acid identity in 65% of their genes (McClelland *et al.*, 2001). Most established cellular processes appear to be similar and map position of all conserved genes is generally preserved. However, unexplained differences persist in highly homologous systems. One longstanding observation is that a *topA* mutant cannot survive in *E. coli* without a compensatory mutation to relieve the increased torsional stress (DiNardo *et al.*, 1982; Pruss *et al.*, 1982; Zumstein and Wang, 1986). In contrast, a *topA* mutant survives without compensatory mutations in *Salmonella* (Richardson *et al.*, 1984) as well as in the closely related *Shigella flexneri* (Bhriain and Dorman, 1993). In a related observation, it was shown that the torsional strain, measured via Z-DNA forming potential, was significantly higher in *E. coli* than in *Klebsiella*, *Morganella*, and *Enterobacter* (Jaworski *et al.*, 1991).

To investigate supercoil dynamics, we began with the *gyrB652* mutation. This C-A transversion was discovered in *Salmonella* where its complex phenotype included temperature sensitivity at 42°C, induction of the SOS response, "*rec*-less" degradation of DNA at temperatures above 37°C, and synthetic lethality when combined with a *recA* or

recB/C mutation (Gari *et al.*, 1996). Pang *et al.* showed that the *gyrB652* mutation formed a sluggish DNA gyrase enzyme with a low catalytic efficiency, k_{cat}. The temperature sensitive phenotype resulted from the inability of the defective DNA gyrase to resolve the topological problems associated with DNA replication at high temperatures (Pang *et al.*, 2005). To move the *gyrB652* mutation into *E. coli,* we employed lambda RED recombineering methods. We were unable to isolate the mutation regardless of recombination homology length. To eliminate the possibility of differential phenotypes, we linked the mutation to a kanamycin resistance cassette. Interestingly, *E. coli* strains that became kanamycin resistant through recombination all had a wild type DNA gyrase sequence. The strong bias in *E. coli* against *gyrB652* was confirmed in recombineering experiments with *mutS*::Tn*10* strains that were unable to perform mismatch repair. In these strains, there was a 1000-fold reduction in recombination efficiency in donor DNA containing the C-A transversion over donor DNA containing only WT sequence. These results strongly suggest that *gyrB652* is lethal in *E. coli.*

We next investigated the effect of *Salmonella* DNA gyrase expressed in *E. coli* using plasmids with either WT *gyrB* or *gyrB652 Salmonella* subunits. *E. coli* strains with the normal complement of topoisomerases were able to maintain a plasmid expressing extremely low levels of *Salmonella gyrB652* under selection, but grew slowly compared to controls strains that contained empty vector. However, when selection was removed 99.99% of the cells lost the plasmid indicating a high *gyrB652*-associated penalty. Somewhat surprising was the observation that the same plasmid system expressing WT *Salmonella gyrB* experienced a 99% plasmid loss without selection. These are in stark contrast to the >99% retention of the same vector containing no insert. These results

show that *Salmonella* GyrB652, as well as WT *Salmonella* GyrB, is toxic in *E. coli* even in the presence of WT *E. coli* DNA gyrase and explain our inability to introduce the mutation into the chromosome.

Next we did the reciprocal experiment to see if *E. coli* GyrB was toxic to *Salmonella*. A plasmid that contained the WT *E. coli gyrB* gene under the control of a *lac*-inducible promoter was introduced into WT strains of each species and monitored for plasmid loss at various inducer levels. In *E. coli*, high expression destabilized the plasmid, which means that *E. coli* is sensitive to overproduction of its own WT GyrB. In contrast, *E. coli gyrB* expression in *Salmonella* had no harmful effects up to 0.1 mM of inducer. At the 1 mM concentration of inducer, there was only a slight (10-fold) reduction in plasmid maintenance. Taken together, these results indicate that *E. coli* is hyper-sensitive to both the efficiency and concentration of DNA gyrase in the cell. In contrast, *Salmonella* tolerates inefficient DNA gyrase as well as high concentrations of *E. coli* DNA GyrB. These observations, as well as the differences in Δ*topA* mutants and Z-DNA forming potential discussed above, suggested an underlying reason for these results. We investigated normal negative supercoil levels as a unifying explanation.

To determine absolute supercoil levels for *E. coli* and *Salmonella* in mid-log phase cells, we used 2-dimensional chloroquine electrophoresis. Plasmid pBR322 was introduced into WT strains of each species and cultures grown to the mid-log phase Klett value of 70 (OD_{600} of 0.4) when plasmid DNA was isolated and purified. Electrophoretic topoisomer separations showed significant difference in mean supercoil density (σ). Plasmid DNA isolated from *E. coli* was 3-4 topoisomers more negative than *Salmonella*. Using the band-counting method (Shure and Vinograd, 1976) we established that *E. coli*

σ = -0.069 and *Salmonella* σ = -0.059. This 15% difference suggests why *E. coli* cells may not tolerate an inefficient *gyrB652* mutation that is unable to maintain a viable supercoil level. This is especially true if the GyrB protein is involved in setting the proper supercoil set-point for the cell.

To verify these results, we measured chromosomal expression levels in both species with supercoil-sensitive reporter modules. Using lambda RED recombineering technology (Yu *et al.*, 2000) and a FLP-FRT recombination strategy (Pang *et al.*, 2005) reporter cassettes were introduced into similar chromosome locations in each species. The reporter cassettes consisted of the *lacZYA* operon driven by either the *gyrA* or *gyrB* promoters. Calibrations with coumermycin A1 confirmed the supercoil-dependent expression of the cassettes. Using routine β-galactosidase experiments as an indicator of supercoil level, we showed that, with both promoters, *E. coli* constrained more negative supercoiling than *Salmonella* at mid-log phase. Expression from the *gyrB* promoter, which is extremely sensitive to torsional strain (Peter *et al.*, 2004), gave a 9.7% difference between the species. Using the *gyrA* promoter, which is less sensitive (Peter *et al.*, 2004) gave a 16.1% difference. The results with the β-galactosidase experiments do not correlate to actual negative supercoil levels and thus only indicate a similarity in chromosomal DNA to plasmid DNA. To assign supercoil density values to these results will require the production of a standard curve to correlate Miller Unit values from specific promoters to plasmid sigma values. Regardless, three independent experiments show that in mid-log phase cells, *E. coli* is more negatively supercoiled than *Salmonella.* As a control in the above experiments, we used a module regulated by the WT *lacZ*

promoter, which is mildly supercoil-sensitive in the opposite direction than the *gyrA* and *gyrB* promoters. The results with this reporter cassette confirmed the above findings.

In prokaryotes, supercoiling occurs in two distinct forms ($\Delta Lk = S_U + S_C$). Constrained supercoils are associated with transcribing RNA polymerases as well as DNA binding proteins such as HU, H-NS, Fis and IHF (Higgins and Vologodskii, 2004). Unconstrained supercoils exist as torsional strain and wrap onto themselves in a plectonemic structure. We next wanted to determine if the supercoil difference we were seeing between the species was a result of increased torsional strain (unconstrained supercoils) or differences in DNA binding proteins (constrained supercoils). To do this we repeated the above chloroquine gel experiments using pRW478 instead pBR322. This plasmid has a 56 bp CG repeat that adopts a left-handed Z-DNA structure at $S_U = -0.025$ (Jaworski *et al.*, 1991). When the "flip" to Z-DNA occurs, it absorbs torsional strain and the cell introduces additional negative supercoiling into the molecule. By monitoring hyper-supercoiled topoisomers on a 2-dimensional gel we determined that over 70% of the *E. coli* plasmid DNA was hyper-supercoiled compared to less than 40% in *Salmonella*. These results show that both supercoil density and torsional strain are higher in *E. coli* than in *Salmonella*.

Knowing the different species supercoil densities, we hypothesized that cellular processes with supercoil components would behave differently in response to the same deletion mutations. An example of this is the longstanding observation that *E. coli* and *Salmonella* respond differently to a Δ*topA* mutation (DiNardo *et al.*, 1982; Pruss *et al.*, 1982; Zumstein and Wang, 1986). This is explained with our findings. In *E. coli*, where supercoiling is elevated, a loss of topoisomerase I causes excessively high torsional strain

that causes the formation of RNA-DNA intermolecular triplexes, or R-loops (Drolet *et al.*, 2003; Hraiky *et al.*, 2000). These R-loops stall replication forks and RNA polymerases (Higgins and Vologodskii, 2004), become substrates for recombinational enzymes, and have been shown to cause serious growth problems (Drolet *et al.*, 1995). In *Salmonella*, where torsional strain is much lower, R-loop formation is not as prevalent and the *topA* deletion is allowed. In the *E. coli* Δ*topA* strain, it would be interesting to over-express RNaseH, an enzyme that will degrade the RNA in this hybrid structure, to see if it alleviates some of the phenotype. We investigated two other cellular processes with supercoil components, replication initiation and chromosome condensation, to look for phenotypes that could be explained by differential superhelical strain.

The opening of DNA in the *oriC* region is controlled by the DnaA protein and negative supercoiling. DnaA binds to special sequences in the origin and makes a complex that causes a nearby AT-rich segment to become unwound with sufficient negative supercoiling (Baker *et al.*, 1986; Bramhill and Kornberg, 1988; Funnell *et al.*, 1986). Over-initiation is prevented by the binding of a protein called SeqA to hemi-methylated GATC sites in *oriC* and preventing re-initiation until *dam* methylase is able to act upon the daughter strand and make the origin amenable to another round of replication (Brendler *et al.*, 1995; Brendler and Austin, 1999; Shakibai *et al.*, 1998). The role of negative supercoiling in this process is shown by the inability of certain *gyrB* mutations to initiate (Filutowicz, 1980; Filutowicz and Jonczyk, 1981; Orr *et al.*, 1979). Conversely, it has been shown that *dnaA* strains, which are initiation-defective, can be suppressed with *topA* mutations that cause excess negative supercoiling (Louarn *et al.*, 1984). Our results showed that, when grown on rich media, an *E. coli* Δ*seqA* mutant is

more severe than the same deletion in *Salmonella*. This is explained by the increased need for *E. coli* to negatively regulate initiation due to higher supercoil levels.

As mentioned previously, negative supercoiling is of the utmost importance in chromosome compaction. Another layer of condensation is provided by the prokaryotic condensin homolog, MukB, which has been studied extensively in *E. coli*. Strains with a deletion of *mukB* have diffuse nucleoids and severe growth defects due to problems in condensation, segregation, and cell division (Hiraga *et al.*, 1989). The *muk* phenotype can be suppressed by mutations in topoisomerase I which implies that the role of MukB in the cell can be partially compensated by increases in negative supercoiling (Sawitzke and Austin, 2000). This being the case, we found that *Salmonella*, with less negative supercoiling, is much more sensitive to a Δ*mukB* mutation than *E. coli*. Our results showed a dramatic 6 order of magnitude difference between the species when grown on rich media. This heavy reliance of *Salmonella* on MukB for proper condensation suggests a lower supercoil density.

In addition to affecting cellular processes involving DNA movement, differences in supercoil levels should have an effect on determining cell fate in processes such as bacteriophage infection. The lysis/lysogeny decision of bacteriophage Mu is determined by two convergent supercoil-sensitive promoters (Krause and Higgins, 1986). At high supercoil levels, the promoter controlling lytic functions is expressed at a higher rate and cell lysis occurs. This effect has been demonstrated *in vivo* with *gyrB* mutations that fail to enter the lytic phase (Ross *et al.*, 1986). It has also been shown directly, as increases in supercoil density can increase lysis by 40-fold (Higgins *et al.*, 1989). To compare the lysis/lysogeny decision in *E. coli* and *Salmonella*, we used temperature-inducible Mu

monolysogens to monitor cell lysis. The eclipse phase in *Salmonella* was about 10 minutes longer than in *E. coli*. In addition, there was a 7-log decrease in CFU in *E. coli* over *Salmonella* after lytic induction. These results indicate that in *Salmonella* the Mu lytic cycle is less potent than *E. coli*. As discussed later, this finding suggests a possible explanation as to why two closely related enterics have vastly different supercoil set-points.

Other aspects of Mu biology may also be affected by supercoiling. Mu transposes into a degenerative consensus sequence that occurs about every 55 bp (Manna *et al.*, 2005). Sites that show a high transposition rate are called "hot spots". Conversely, areas that are rarely the target of transposition are called "cold spots". The explanation for what causes a site to be a "hot spot" or "cold spot" may be dependent on DNA structure. This is shown by the observation that transcription has a clear negative impact on transposition (Manna *et al.*, 2004). It has also been shown that Mu has a strong target site preference for single base mismatches. The most likely explanation for this observation is that these sites create a unique, deformed structure that attracts the transposition machinery (Yanagihara and Mizuuchi, 2002). The idea that alternative DNA structures become transposition "hot spots" is of significance since supercoil levels attribute to their formation. Along these lines, recent work shows that the pattern of Mu transposition is different in *E. coli* and *Salmonella*. Most importantly, this is even true among highly homologous genes (Manna *et al.*, Submitted).

Taken in full, the evidence strongly argues for a difference in supercoil levels between *E. coli* and *Salmonella* in mid-log phase cells. However, several reports have shown that when *E. coli* cells begin to enter stationary phase, they undergo a relaxation of

their genomes (Dorman *et al.*, 1988; Reyes-Dominguez *et al.*, 2003; Schneider *et al.*, 1999; Tse-Dinh and Beran, 1988). On the other end of the growth cycle, it has been demonstrated that when stationary phase cells are diluted into fresh media, there is an increase in supercoil density (Reyes-Dominguez *et al.*, 2003). Considering these results, we wanted to establish whether negative supercoiling is greater in *E. coli* throughout all stages of log phase growth. To do this we used our WT strains with pBR322 plasmid. After inoculating a stationary phase culture into fresh media 1-dimensional chloroquine electrophoresis was carried out to determine plasmid supercoil density throughout the growth phase. Like our previous experiments, we found stark differences between the species. *E. coli* had a dramatic supercoil fluctuation that coincided with previous observations on entering and exiting log phase. Early log phase cells were slightly relaxed but became highly supercoiled in mid-log phase. After this point, the plasmid DNA once again became slightly relaxed as cells entered and persisted into stationary phase. *Salmonella* was quite different, showing almost no fluctuation from stationary phase through log and back. Early growth points showed a medial level of negative supercoiling and subsequent samples only showed a 2% fluctuation over the time course. These results suggest a difference in the way *Salmonella* maintains and supercoils plasmid DNA. Two observations about these differential supercoil cycles are especially pertinent. The maximal difference between the species is 11% and comes at the mid-log phase point (Klett 70) that coincides with our previous experiments. The reason for the slight variance in the amount of difference from experiment to experiment may be due to growth cycle fluctuations. The other important observation is that, with the exception of early log phase, *E. coli* DNA is more supercoiled than *Salmonella* at all stages of the cell

cycle. This fits well with our previous observations and our inability to isolate a *gyrB652* mutation in *E. coli*.

As with our mid-log measurements, plasmid fluctuation data was measured on the chromosome. Expression from P*gyrB-lacZYA* supercoil reporter cassettes were measured at various stages in the growth cycle. Results showed close agreement with *E. coli* plasmid DNA. The mid-log phase data point here shows a difference of 9.6%, which is in strong agreement with the 9.7% difference we found with the previous β-galactosidase experiments.

E. coli and *Salmonella* are separated by about 200 million years of evolution (Lawrence, 2005). They share extensive sequence and amino acid homology in 65% of their genes. In addition, the genetic maps are very similar. Why would two highly homologous organisms evolve such different levels of negative supercoiling? We suggest that the difference is to protect cells from lytic prophage. *Salmonella* contains 4 plaque-forming prophage (McClelland *et al.*, 2001) whose lytic/lysogeny pathway is partially regulated by negative supercoiling. *E. coli* contains no prophage capable of lytic growth and therefore can tolerate increased negative supercoil levels without risk of lysis. But why would a bacterium increase the level of negative supercoiling? A series of experiments showed that passaging 12 identical *E. coli* strains for 20,000 generations resulted in a 17% increase in supercoiling in stationary phase cells. Identical mutations in the *topA* ORF and the regulatory region of *fis* caused the increases in supercoiling and resulted in increased fitness as determined by competition experiments with the parental strain (Crozat *et al.*, 2005). Both of these sequences are preserved between *E. coli* and *Salmonella* but demonstrate how simple changes can cause drastic supercoil differences.

Of interest in these experiments is the observation of a 17% supercoil difference when a 15% change should be lethal (Drlica, 1992). This is probably due to the change coming in two steps. The *topA* mutation, which always came first, accounted for 12% of the supercoil difference and the *fis* mutation accounted for the other 5%. Since the resultant double mutant strain was viable it indicates that the cells became adapted to the *topA* change and were then able to tolerate the *fis* change. This example serves as proof that, not only can this type of "re-tuning" occur, but can happen in a relatively short time span. Also of note in these experiments was the observation that the double mutant strains, with increased negative supercoiling, were fitness enhanced and had a faster growth rate in rich media. This could be due to increased transcription from the ribosomal operons to provide the raw materials needed for faster growth. In keeping with this theory, they found an increased RNA:DNA ratio in the double mutant strains. Taken in full, these results suggest an increase in fitness with higher negative supercoiling. It could be possible that *E. coli* has become more negative through evolution to become more fit but *Salmonella* is unable to change due to the lytic prophage it harbors. It would be interesting to compare RNA:DNA ratios between the species to see if they correlate with the supercoil differences.

Previous experiments showed that *E. coli* also contains a higher level of torsional strain than *Klebsiella*, *Morganella*, and *Enterobacter* (Jaworski *et al.*, 1991). *Klebsiella* contains two known plaque-forming prophage known as FR2 and AP3 (Satta *et al.*, 1978) similar to the situation in *Salmonella*. Even though *Morganella* and *Enterobacter* are sensitive to many strains of phage (Gabrilovich *et al.*, 1998; Loessner *et al.*, 1993) it is not clear whether these species harbor plaque-forming prophage in their genomes. As

mentioned previously, *Shigella* can tolerate the *topA* deletion without compensatory mutation (Bhriain and Dorman, 1993), suggesting it has a supercoil level similar to *Salmonella*. Interestingly, *Shigella* is known to harbor 11 different prophage, even thought it is not clear if any of these have supercoil-controlled regulation (Nie *et al.*, 2006; Petrovskaya and Licheva, 1982). It would be interesting to see how *Salmonella* physiology responds to the deletion of all 4 prophage. For instance, an evolution experiment similar to the one mentioned above (Crozat *et al.*, 2005) using WT *Salmonella* versus *Salmonella* without prophage would give great insight into phage/host relations and the role of supercoiling in evolution. Also, what type of supercoil changes will these prophage tolerate before inducing lytic growth. From our experiments, we know high levels of *E. coli* GyrB have no effect on *Salmonella* physiology indicating that either supercoiling is not changing, or that any change is insufficient to cause prophage lysis or altered phenotype.

Another intriguing question is deciding what controls the supercoil set-point in each species. From our experiments, it appears that the GyrB subunit could be at least partially responsible. If this is true, it further validates our inability to create the *gyrB652* mutation in *E. coli,* since this would not only create an inefficient enzyme but cause a drastic reprogramming of supercoiling. *E. coli* and *Salmonella* GyrB have a >96% amino acid identity, leaving only 28 residue differences between them. The majority of these differences reside in the C-terminal domain in close proximity to the *gyrB652* mutation. Among these differences are a few residues that are highly conserved in most enteric bacteria but differ in *E. coli*. It would be of great interest to individually or serially change these residues and monitor the resultant supercoil density.

Is it feasible for a few amino acids to account for such large differences in a topoisomerase? The *gyrB652* mutation is a single base transversion that lowers the Lk of pBR322 by 1.5 topoisomers relative to WT (Pang *et al.*, 2005; Staczek and Higgins, 1998). A spontaneous base pair substitution in *topA* has been shown to increase supercoiling by 12% and seemed to improve the fitness of the cell (Crozat *et al.*, 2005). In another example, *dnaA* and *parC*, which encode subunits of DNA gyrase and topoisomerase IV respectively, are highly homologous genes whose products have very different functions. A structural comparison of the C-terminal domains of these subunits may explain their differences. Both form a unique β-pinwheel structure (Corbett *et al.*, 2004; Hsieh *et al.*, 2004). However, GyrA contains a 7-residue, unstructured motif termed the "GyrA-box" that is the only distinguishing characteristic between these regions (Ward and Newton, 1997). The β-pinwheel structure of topoisomerase IV is in an open conformation that cannot wrap DNA and thus functions mainly as a decatenase. The same structure in DNA gyrase is held closed by this GyrA-box and allows DNA gyrase to wrap DNA and insert negative supercoils. Deletion or alanine-substitution of the GyrA-box abolishes this ability but does not affect the relaxation or decatenase activity (Kramlinger and Hiasa, 2006). Therefore, DNA gyrase without the GyrA-box is effectively converted into topoisomerase IV. All of these examples highlight situations where slight changes can cause drastic phenotypic differences in topoisomerases.

At first glance, some of our results appear contradictory to the role of GyrB in setting supercoil level. When expressing various *gyrB* constructs in the two species, we found that any change in expression levels or DNA gyrase efficiency in *E. coli* caused a severe penalty, in keeping with our hypothesis. However, we also made the observation

that highly over-expressing *E. coli* GyrB in *Salmonella* caused no apparent problems. If GyrB controls the supercoil set-point, and *E. coli* GyrB encodes a more negative supercoil value than *Salmonella*, this observation seems out of place. There are several plausible explanations as to how GyrB could be responsible for setting supercoil set-point and yet be seemingly inactive in *Salmonella*. The first could be a difference in DNA gyrase affinity for certain DNA. More precisely, DNA gyrase could be more active on the *E. coli* chromosome than the *Salmonella* chromosome. How could this be? An intermediate step in DNA gyrase activity is the cleavage of both strands and a transient binding to the enzyme. If cells are treated with the DNA gyrase inhibitor oxolinic acid followed by a detergent like SDS, the enzyme is removed and leaves a double strand break. Initial experiments found that oxolinic acid-induced cleavage caused a break about every 100 kb (Snyder and Drlica, 1979). This equaled about 45 cleavage sites per genome and was in near perfect agreement with domain estimates at the time (Sinden and Pettijohn, 1981). However, another group found that this same treatment caused cleavage sites about every 12-25 kb (Bejar and Bouche, 1984). These differences are probably from experimental variance and represent high and low affinity DNA gyrase binding sites. This is in agreement with others who have found DNA gyrase sites with different affinities (Condemine and Smith, 1990; Franco and Drlica, 1988). The best-known DNA gyrase-binding sites are the repetitive extrangenic palindromic (REP) sequences. These 35 bp sites are usually found in clusters outside of coding regions. It is predicted that there are about 500 REP sequences per chromosome, which would make up about 0.5% of the total DNA. The REP site sequence is highly conserved between *E. coli* and *Salmonella* and does not appear in eukaryotic chromosomes where there is no

DNA gyrase. Of great interest is the 35 bp inverted repeat structure of the REP sites (Stern *et al.*, 1984). Since secondary structures, like cruciforms, are highly dependent on supercoil levels, the amount of activity at REP sites may be a function of supercoiling. This would mean that the REP sites in *E. coli* are more amenable than the REP sites in *Salmonella* and explain why DNA gyrase seems to be more active in *E. coli*. This could be reminiscent of the observation that single base mismatches, which cause a deformed structure, attract the Mu transposition machinery (Yanagihara and Mizuuchi, 2002). In addition to the possible difference of REP sites caused by supercoiling levels, it has been shown that the location of REP sites is different in *E. coli* and *Salmonella*. Only on rare occasions were REP sites from one species found in equivalent locations in the other species (Gilson *et al.*, 1987; Yang and Ames, 1988).

Another possible explanation for differences in GyrB tolerance between the species may be due to the stability of hybrid enzymes. Since we only expressed one gyrase subunit, the interaction between subunits from different species may be poor or create inactive enzyme. This is especially plausible when considering that the C-terminal domain of GyrB, which has the area of most heterology, is responsible for interacting with GyrA (Gellert *et al.*, 1979). An example of this is shown by mutation of the Y5 residue of GyrB. When this tyrosine residue is substituted with a serine or phenylalanine, plasmid maintenance is severely decreased. The residue change probably causes poor interaction between the subunits and results in an inefficient enzyme. Since many plasmids have a supercoil-regulation component to their copy number control, defects in DNA gyrase efficiency can cause defects in plasmid maintenance (del-Solar and Epinosa, 2000). In addition, it was shown that these mutant forms competed with WT DNA

gyrase (Brino *et al.*, 1998). These results are similar to plasmid instability experiments done with TS DNA gyrase strains or WT DNA gyrase strains treated with novobiocin. In these strains, the plasmid instability penalty of a mutant DNA gyrase was measured to be as high as 99% (Wolfson *et al.*, 1982; Wolfson *et al.*, 1983). In the case of novobiocin-induced elimination, DNA gyrase alleles resistant to novobiocin showed no plasmid defect. Differences in hybrid enzymes and their competition with WT DNA gyrase could explain our differences. For example, it is possible that a hybrid *E. coli* GyrA/*Salmonella* GyrB enzyme competes with native *E. coli* DNA gyrase and causes a decrease in supercoiling. On the other hand, maybe hybrid *Salmonella* GyrA/*E. coli* GyrB is unstable and cannot compete with native *Salmonella* DNA gyrase. Along similar lines, a difference in affinity of WT DNA gyrase subunits from the same species could explain the inactivity of *E. coli* GyrB in *Salmonella*. If the subunits of DNA gyrase in *E. coli* are more loosely bound together, expressing a foreign GyrB may easily cause the formation of potentially defective hybrid enzymes. Conversely, if the subunits of DNA gyrase in *Salmonella* were tightly bound together, exogenous GyrB would not be able to form stable enzyme and be ineffective. Since the region of interaction between the subunits contains many differences, this is a plausible, although exclusively hypothetical, explanation.

Finally, another explanation for the difference in GyrB tolerance may be due to roles of GyrA and GyrB in the cell apart from supercoiling. As an example, in *Borellia burgdorferi*, a naturally occurring 34 kDa C-terminal GyrA fragment was found that had activity nearly identical to HU. It was even shown that this fragment, called Gac, could complement *hu* deletions in *E. coli* (Knight and Samuels, 1999). The same *E.*

coli fragment, though not naturally occurring, can also act as a binding protein (Reece and Maxwell, 1991b). If the GyrA subunit has a DNA-binding role separate from supercoiling in *E. coli* and/or *Salmonella*, expressing GyrB would cause the excess GyrA to be bound in enzyme form and be unavailable for this secondary function. Alternatively, if GyrB has another role in the cell, overproducing this subunit would not only cause changes in supercoiling via complete DNA gyrase enzyme, but also through the other activity of GyrB.

In addition to changing certain residues in one species to match the other, as mentioned above, it would be of great interest to do a "swap" between the species with respect to both DNA gyrase subunits. However, there is a chance that this may not be possible if each species is delicately tuned to a specific supercoil level. For instance, in *Salmonella* it may be necessary to remove the 4 prophage before a swap is possible. Also, differences in the regulation of other topoisomerases may have to be considered. Even being mindful of obvious requirements to allow a swap to occur, the differences caused by and affected by supercoiling may potentially be too vast to overcome. Perhaps an intermediate step may be required to allow the 15% supercoil difference to change in more than one step. Experiments such as these may provide a wealth of information about differences in supercoiling, topoisomerases, and gene expression.

The finding that there are significant supercoil differences between the species suggests that there must have been a "re-tuning" of supercoil-sensitive genes. A recent study in *E. coli* showed 7% of all genes are supercoil-sensitive in mid-log phase cells in rich media (Peter *et al.*, 2004). There are several cellular systems that work by sensing the environmental condition and effecting change through gene regulation via

supercoiling (Balke and Gralla, 1987; Dorman *et al.*, 1988; Higgins *et al.*, 1988; Hsieh *et al.*, 1991; McClellan *et al.*, 1990; Nicholson and Setlow, 1990). In addition, the genes encoding the topoisomerases themselves are controlled by a homeostatic feedback mechanism (Menzel and Gellert, 1983). Changes in supercoiling between two species would require compensation in the regulation of many of these systems. This seems like a monumental change. However, such change is not without precedent. For example, T3 and T7 are closely related coliphage whose RNA polymerases share over 80% identity. In spite of this homology, these species cannot recognize promoters specific for the other. In fact, T3 evolved from a T7 progenitor species and harbors a vestigal T7 promoter that is not recognized (Bailey *et al.*, 1983; Garcia *et al.*, 2003). How is such specificity accomplished by two closely related species? The answer is in a single locus in the promoter and the contact it makes with a single residue on the RNA polymerase. Experiments have shown that a T3 RNA polymerase can be converted to a T7-recognizing enzyme by the conversion of this single amino acid, and vice versa. Additionally, a similar change in the promoter sequence can also provide this type of specificity switch (Raskin *et al.*, 1992). This example shows how the divergence of closely related species altered specificity. A similar mechanism could also change regulation. As proof of this, it has been shown that a *topA* mutation, that increases supercoiling by 12% in stationary phase, can be accommodated and even result in a "fitter" organism (Crozat *et al.*, 2005). A thorough examination of promoter differences between the species may give insight as to how similar cells accommodate different torsional strain. However, certain supercoil-sensitive genes, including the ribosomal operons, may not alter their regulation and choose to exist with higher expression from

these genes. It has even been suggested that these types of expression changes could result in better growth (Cooper *et al.*, 2001; Crozat *et al.*, 2005; Vasi *et al.*, 1994). It would be of great interest to do a series of promoter swap experiments between the species to see if changes in expression mirror the changes we see in supercoil level.

Another question to address is why DNA goes through a supercoil cycle throughout the growth phase? This may reflect the need for cells to relax the genome as they exit log phase so that the stationary phase sigma factor, σ^S, can properly function (Reyes-Dominguez *et al.*, 2003). Once cells are diluted into fresh media, the supercoil density increases to allow transcription and replication. As cells move to late log phase, they again relax their genome in preparation for stationary phase. Interestingly, in *E. coli* a burst of DNA gyrase production in late log phase helps cells quickly reestablish supercoil levels upon nutrient availability (Reyes-Dominguez *et al.*, 2003). An explanation for why we do not see plasmid supercoil fluctuation in *Salmonella* may be associated with this fast increase in negative supercoiling upon nutrient up-shift. The Fis DNA-binding protein is identical in *E. coli* and *Salmonella* (Osuna *et al.*, 1995). In *E. coli*, it is present at very low levels in stationary phase but experiences a dramatic increase upon entrance into log phase. This increase coincides with the increase in negative supercoiling and may protect the DNA from deleterious unwinding due to very fast topological changes (Osuna *et al.*, 1995; Schneider *et al.*, 1997). This is a feasible explanation because it has been shown that, in stationary phase, Fis preferentially binds and stabilizes a moderately supercoiled plasmid population and delays the action of both topoisomerase I and DNA gyrase (Schneider *et al.*, 1997). In addition, Fis is more prevalent in *Salmonella* and its regulation is 4-fold less efficient because of differences in

the promoter regions (Osuna *et al.*, 1995). In *Salmonella*, the binding persists longer than in *E. coli* because of differences in Fis level and regulation (Osuna *et al.*, 1995) and/or decreased topoisomerase activity in *Salmonella*. It would be of interest to know if the storage of DNA gyrase that occurs in *E. coli* also happens in *Salmonella*. If not, it may explain why plasmids in *Salmonella* do not show a dramatic supercoil increase. The cycle difference is not seen with genomic DNA because of domain formation, other binding proteins, differential affinity, and replication and transcription differences.

In summary, there is a significant supercoil difference between *E. coli* and *Salmonella*. The difference exists as torsional strain and is present in plasmid DNA as well as in genomic reporters. The difference is maximal at mid-log phase where *E. coli* is about 15% more negative than *Salmonella*. Following the supercoil fluctuations throughout the cell cycle shows that *E. coli* is more negative at nearly all points. This supercoil difference is clearly shown by the inability of *E. coli* to acquire the inefficient *gyrB652* mutation. This difference also manifests itself in cellular systems that have a supercoil-control element to their regulation. As examples, replication initiation and chromosome condensation mutants act differently in the two species. Also, phage Mu lysis, cell survival, and transposition patterns (Manna *et al.*, Submitted) are different. These are in addition to the long-standing differences in topoisomerase I deletions in the literature (DiNardo *et al.*, 1982; Pruss *et al.*, 1982; Zumstein and Wang, 1986). We suggest that the GyrB subunit partially controls the supercoil set-point and that lower supercoil values protect against lytic prophage induction.

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