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Analyzing DNA Topology and Transcription in Salmonella enterica serovar Typhimurium During Dichotomous Growth

Betty M. Booker
University of Alabama at Birmingham

ANALYZING DNA TOPOLOGY AND TRANSCRIPTION *IN SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM DURING DICHOTOMOUS GROWTH

by

Betty M. Booker

N. Patrick Higgins, CHAIR
David Briles
Natalia Kedishivili
Kiril Popov
Janet Yother

A DISSERTATION

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ANALYZING DNA TOPOLOGY AND TRANSCRIPTION *IN SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM DURING DICHOTOMOUS GROWTH

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BIOCHEMISTRY & MOLECULAR GENETICS

ABSTRACT

The bacterial chromosome is dynamic. The principle goal of my research is to understand how DNA topology is altered by transcription in *Salmonella enterica* serovar Typhimurium LT2. γδ-resolution requires two direct repeat Res sites to pair a plectonemic synapse. Previous work from our lab showed that the zones of high transcription inhibited γδ-resolution. Using phage λ recombineering methods, we have developed *Salmonella* strains to study ribosomal RNA operons, which are the most highly transcribed genes in bacteria. We propose a molecular model for how supercoiling generated by high levels of transcription modifies chromosome structure.

Keywords: DNA supercoiling, topoisomerase, gyrase, transcription, recombination, chromosome dynamics

DEDICATION

I would like to dedicate this dissertation to my friends and family who have constantly encouraged and supported me in all of my personal and academic endeavors. I am a direct product of all those I have encountered. I was blessed with such a giving family who surrounded me with faith and love. My Mother and three Aunts have all played a key part in my career. A special dedication is for my Aunt, Gail Myles of Jackson, Mississippi. This is for your endless support and early sacrifices that have afforded me this opportunity. You gave a huge part of your young adult life to prepare me for mines. Thank you for always providing me with another task to accomplish and seeing in me what I did not see in myself. I cannot imagine my life without your support and guidance. I must also dedicate my dissertation to my late grandmothers, Betty Lou Toles and Mae Catherine Booker, women of poise, courage, and humility. To my late maternal uncle, Larry Dell Toles, who always protected me and provided small unforgettable moments in the family. I have felt all of your spirits resting upon my soul since you departed; I hope I make you smile. And lastly, I dedicate this work to many children who may not have any other reason to dream except to escape their current circumstances. Keep on dreaming because dreams become thoughts and thoughts become actions.

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I would like to first acknowledge Dr. Patrick Higgins, for his effortless patience, guidance, and understanding throughout my graduate career. He teaches all of his students how to critically think beyond experiments and results in order to make sound analyses. I am honored that Dr. Higgins saw potential in me and allowed me to join his lab.

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LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
bp	base pairs
DNA	deoxyribonucleic acid
dif	deletion-induced filamentation
DSB	double strand break
Frt	Flp recombination target sites
γδ	gamma-delta
gn	gentamycin
H-NS	heat-stable nucleoid-structuring
IHF	integration host factor
kb	kilobase
kDa	kilodalton
kn	kanamycin
LB	Luria Bertani Broth
ml	milliliter
nt	nucleotides
Res	resolvase site
RNAP	RNA polymerase
rRNA	ribosomal RNA
TIF	transcription impact factor
W/C	Watson/Crick turns

INTRODUCTION

The three domains of life, bacteria, archaea, and eukaryotes, rely on multiple mechanisms to compact DNA into chromosomes (Holmquist and Ashley 2006; Aguilera and Gomez-Gonzalez 2008). Genomes of the bacteria are condensed 1000x's relative to the DNA's persistence length inside the cell (Reyes-Lamothe, Wang et al. 2008). Yet, this organization allows for efficient procession of all DNA processes. Elucidation of the mechanisms that control the organization of DNA as it undergoes transcription, replication, and to final segregation in two sister cells is a major goal in chromosome dynamics research.

THE BACTERIAL NUCLEOID

A major subject in chromosome dynamics focuses on DNA topology during growth. The chromosome of *Escherichi coli* is negatively supercoiled and separated by domains (Stonington and Pettijohn 1971; Worcel and Burgi 1972; Worcel and Burgi 1974). Upon the disruption of the cell wall and cytoplasmic membrane, DNA expands to reveal supercoiled domains extended from a central amorphous mass (Fig. 1). Inaugural studies by several labs support the supercoiled domain model but the identity of the proteins responsible remained undefined (Ryder and Smith 1974; Hecht and Pettijohn 1976; Drlica, Burgi et al. 1978). In this work, we analyze chromosome organization in bacteria by measuring the pattern of DNA supercoiling changes caused by RNA synthesis.

THE ACTION OF TOPOISOMERASES IN CHROMOSOME ORGANIZATION

Three topoisomerases regulate DNA supercoiling in bacteria. DNA supercoiling is the primary force that compacts bacterial chromosomes. Topoisomerases introduce (gyrase) or relax (topA or topoIV) negative DNA supercoils. Topoisomerases are conserved in organisms from prokaryotes to mammals (Schmid and Sawitzke 1993) as all organisms must resolve topological entanglement in the chromosome during growth (Cozzarelli 1980; Weitao, Nordstrom et al. 2000; Skarstad, Torheim et al. 2001; Maxwell, Costenaro et al. 2005). Viral particles have also been shown to rely on topology for packaging, transcription, and replication (Wong and Hsu 1989; Wong and Hsu 1990). Topoisomerases control DNA topology by supercoiling, relaxing, and decatenating strands during replication and transcription. In a recent review, Espeli and Marians outlined three topological problems the cell faces during growth: (1) positive supercoils generated during DNA replication, (2) topological equilibrium and (3) resolving linkages between sister chromosomes during segregation (Espeli and Marians 2004). Another conflict the authors failed to mention is the effect of transcription on topology. To overcome topological problems, *E. coli* and *Salmonella* have three essential (Giaever, Snyder et al. 1988; Steck, Franco et al. 1993) topoisomerases that regulate DNA topology.

Supercoiling occurs when the Watson and Crick strands wrap around each other at a higher (positive supercoils) or lower (negative supercoils) frequency than that found in normal B-DNA (10.5 bp/turn). Topoisomerases introduce or remove positive and negative DNA supercoils (Wang 1971; Gellert, Mizuuchi et al. 1976; Gellert, O'Dea et al. 1976; Marians, Ikeda et al. 1977). Negative supercoiled DNA is the natural state of the bacterial chromosome in vivo (Botchan, Wang et al. 1973; Higgins 1999). Two types of

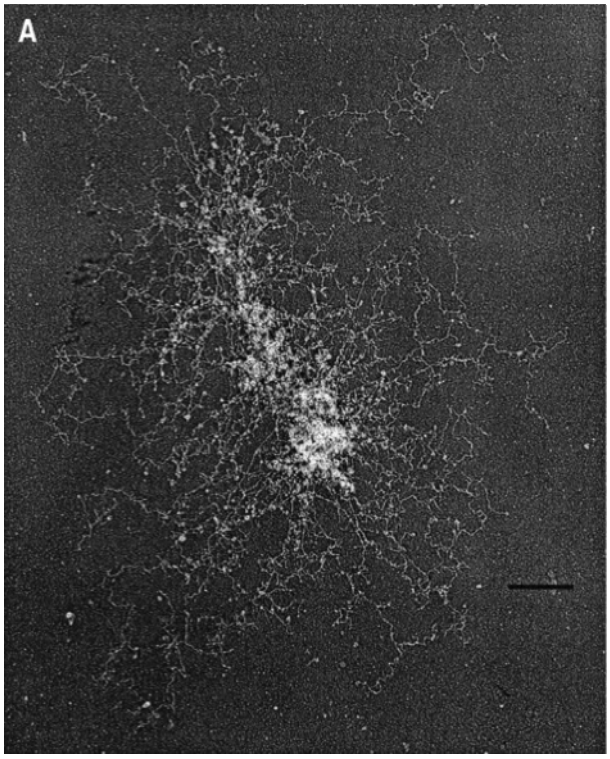

Figure 1. The isolated bacterial chromosome. Upon disruption of the cell wall and cytoplasmic membrane, DNA expands to reveal supercoiled domains extended from a central core region. It was suggested that RNA molecules (Pettijohn & Hecht, 1974) and DNA-binding proteins were present in the core and responsible for stabilizing the nuceloid's organization (Pettijohn, 1996). In the figure above, *E. coli* chromosomes were gently isolated by sucrose gradient sedimentation and spread onto carbon-coated electron microscopy grids. A representative chromosome is shown, photographed at 12,000× magnification. Bar, 500 nm. From "Topological domain structure of the *Escherichia coli* chromosome" by Postow et al, 2004, *Gen. Devel.,* p. 1766-79. Reprinted with permission.

supercoiling are found on the chromosome: Plectonemic (diffusible or unconstrained) and toroidal (constrained) (Bliska and Cozzarelli 1987; Willenbrock and Ussery 2004; Deng, Stein et al. 2005) (Fig. 2). Half of the chromosome is constrained and the other half is unconstrained, unlike eukaryotes where DNA is fully constrained by toroidal wrapping around nucleosomes. There are three bacterial topoisomerases that contribute to equilibrium of DNA supercoiling.

Type I topoisomerases (Topo I and Topo III) break and join one strand at a time by relaxing negative supercoils (Dekker, Rybenkov et al. 2002) independent of ATP. Topo IA relaxes negative supercoils to limit formation of unusual DNA structures that form in highly supercoiled DNA, like Z-DNA, H-form DNA, and R-loops. In the absence of Topo I, plasmids can become hyper-negatively supercoiled (Giaever, Snyder et al. 1988; Albert, Spirito et al. 1996; Zechiedrich, Khodursky et al. 2000). Topo III (a non-essential topoisomerase) can relax negative supercoils and decatenate circular DNA molecules (Hiasa and Marians 1994; Champoux 2001). Type II topoisomerases (Topo IV and Gyrase) break both strands simultaneously by changing supercoils in steps of two supercoils per reaction (Cozzarelli 1980; Paoletti 1993). Gyrase introduces negative supercoils in an ATP-dependent manner. The enzyme is vital for homeostatic superhelicity of the chromosome (Gellert, Mizuuchi et al. 1976; Snoep, van der Weijden et al. 2002). Negative supercoiling is required for DNA synthesis and for separating strands at cell division (Nollmann, Crisona et al. 2007). Gyrase is synthesized upon DNA relaxation (Menzel and Gellert 1983) and binds relaxed DNA at a higher preference than supercoiled DNA (Higgins and Cozzarelli 1982). The core heterotetramer cleavage/reunion complex is made up of two dimers of GyrB and the GyrA (Wang 1974;

Higgins, Peebles et al. 1978; Holmes and Cozzarelli 2000). The ATPase activity resides in GyrB. Gyrase changes the linking number of DNA topoisomers in steps of two by breaking both strands and passing a segment of DNA through a double strand break (DSB) (Brown and Cozzarelli 1979; Hardy, Crisona et al. 2004; Belotserkovskii, Arimondo et al. 2006) (Fig. 3). Approximately 1,000 molecules of gyrase bound to chromosomal DNA can produce 100,000 negative supercoils per minute (Higgins, Deng et al. 2005). Topo IV was identified as a protein required for efficient chromosome segregation in *E. coli* (Kato, Nishimura et al. 1990). Topo IV is a decatenase that is essential during replication and chromosome segregation (Kato, Suzuki et al. 1992; Zechiedrich and Cozzarelli 1995). Topo IV is an ATP-dependent enzyme that alters supercoiling in steps of two. Like gyrase it has two subunits, ParC and ParE, that form heteromeric tetramers (Kato et al., 1992). Unlike gyrase, Topo IV can only relax negative and positive supercoils (Higgins and Vologodskii 2004; Crisona and Cozzarelli 2006). Topo IV disentangles knots or pre-catenanes during replication. Without Topo IV, catenanes and knotted sister chromosomes result, which are lethal impediments to cell division (Zechiedrich, Khodursky et al. 1997).

DOMAIN BARRIERS

A domain of supercoiling is defined by a segment of DNA bound by something that blocks free rotation (a barrier) (Pettijohn and Hecht 1974). Domains provide barriers against DNA relaxation, and are presumed to aid in compaction and confinement of local processes (Postow, Hardy et al. 2004) (Fig. 2). *E. coli* and *Salmonella* chromosomes have roughly 400-450 barrier domains that are mostly stochastic (Deng, Stein et al. 2004). New barriers appeared during transcription and disappeared when transcription was

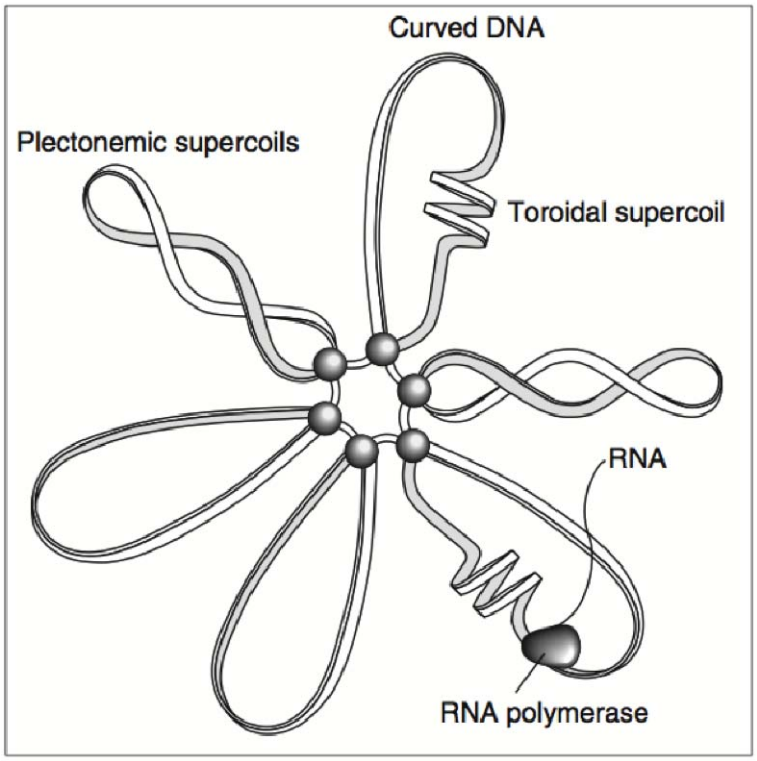

Figure 2. Supercoiling on the bacterial chromosome. Supercoiling occurs when the two Watson and Crick strands wrap around each other at a higher (positive supercoils) or lower (negative supercoils) frequency than normal. Two types of supercoiling are found on the bacterial chromosome: Plectonemic (diffusible, unconstrained) and toroidal (constrained). Toroidal supercoils are formed by wrapping DNA around proteins (not shown). DNA processes, such as transcription, can alter DNA supercoiling, as shown above. From "Chromatin architecture and gene expression in Escherichia coli" by H. Willenbrock & D.W. Ussery, 2004, *Genome Biol,* p. 252-268. Reprinted with permission.

repressed in the *tetA* module and bacteriophage *Mu* early promoter (Scheirer and Higgins 2001; Deng, Stein et al. 2004). Although specific domain barrier proteins (domainins) have not been proven, a few candidates are Fis, H-NS, IHF, gyrase, RNA polymerases, FtsK, MreB and MukB (Marenduzzo, Faro-Trindade et al. 2007; Dorman 2009; Higgins, Booker et al. 2010). Identification of specific domainins is necessary to entirely comprehend the bacterial chromosome's organization. For this reason, we analyzed the topology of regions of high transcription in *Salmonella.*

PROTEINS ASSOCIATED WITH CHROMOSOME STRUCTURE

The simplest bacterial chromosome is condensed 1000 times the actual cell size (Krawiec and Riley 1990; Marenduzzo, Faro-Trindade et al. 2007). Condensation occurs with the help of supercoiling and proteins that associate with DNA and alter DNA conformation by bending or wrapping DNA on their surface.

E. coli and *Salmonella* have the MukBEF protein complex that binds DNA and is suggested to promote condensation. Mutants of MukB have de-condensed nucleoids, aberrant chromosome partitioning, and elongated cells (Sawitzke and Austin 2000; Weitao, Dasgupta et al. 2000; Case, Chang et al. 2004). MukBEF is proposed to act like a eukaryotic condensin which compacts DNA and thickens fibers (Chen, Zinchenko et al. 2008; Woo, Lim et al. 2009).The nucleoid-associated proteins (NAPs) have low molecular weight (kDa) and bend and bind DNA in both a non-specific and sequence-specific manner (Higgins, Booker et al. 2010). Studies by various labs showed that mutants in NAPs display a loss in chromosome organization and viability. But none of

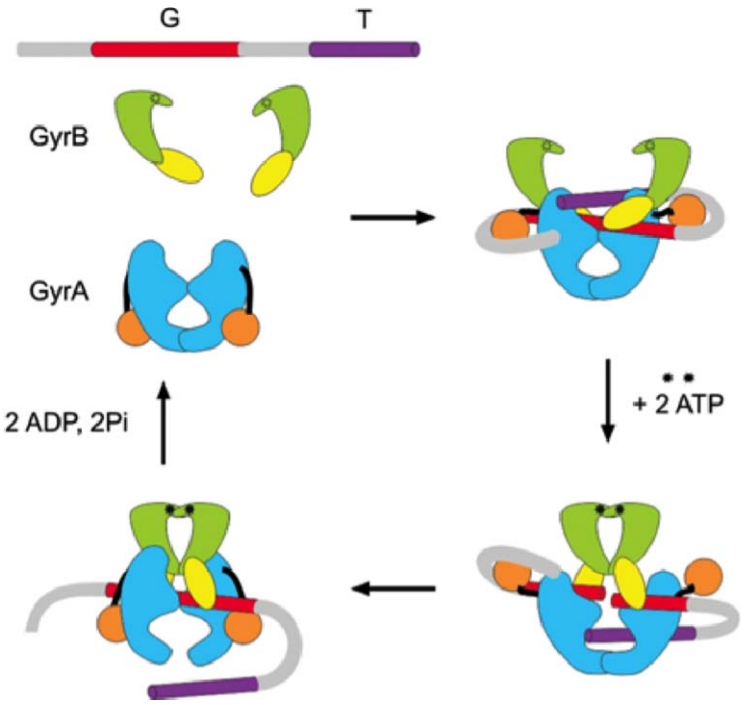

Figure 3. The action of gyrase on DNA. Gyrase, a type II topoisomerase, introduces negative supercoils in an ATP-dependent manner. It is a tetramer comprised of GyrA and GyrB subunits. Gyrase changes the linking number of DNA topoisomers in steps of two through a breakage mechanism of double strand breaks and passes the front segment of DNA through a DSB in the back segment It then catalyzes the ATP dependent passage of one duplex through another after resealing the break. From "Coupling ATP hydrolysis to DNA strand passage in type IIA DNA topoisomerases" by Maxwell, A et al, 2005, *Biochem. Soc. Trans*., p. 1460-1464. Reprinted with permission.

these proteins have proven to be essential (Hillyard, Edlund et al. 1990; McGovern, Higgins et al. 1994). Some NAPs are involved in gene regulation (Johnson, Johnson et al. 2005). H-NS binds foreign DNA and silences transcription of the DNA segment (Pflum 2006). IHF has also been reported to affect transcription of the phage Mu promoters, which promote lysogenic versus lytic development depending on the DNA supercoiling (Higgins, Collier et al. 1989).

A BIOCHEMICAL ASSAY TO ANALYZE CHROMOSOME MOVEMENT

The *Tn*3/γδ-resolvase assay is a transposon-based system developed to explore chromosome movement within a known DNA segment (Higgins, Yang et al. 1996). The resolution assay has yielded extensive data related to chromosome structure and movement. The transposon γδ is closely related to the *Tn*3 element, both sharing similar transposition requirements (Arthur and Sherratt 1979; Reed 1981; Reed 1981). The only protein required for either's (γδ or *Tn*3) resolution is the resolvase, making it a useful system to study genetics (Fig. 4).

γδ-resolvase, a 21,000 dalton protein, serves as a repressor and activator of site-specific recombination. The resolvase protein interacts with two direct repeat Res sites to catalyze a site-specific recombination event resulting in two independent molecules (Falvey, Hatfull et al. 1988). The two newly formed products each carry one Res site from the original substrate. The reaction depends on three factors: negatively supercoiled DNA, resolvase, and two directly repeated Res sites (Bliska and Cozzarelli 1987; Salvo and Grindley 1988; Higgins, Yang et al. 1996). The two Res sites must be plectonemically tangled with three superhelical nodes to form a synaptosome (Bliska,

Benjamin et al. 1991). This assay has proven beneficial in studying the behavior of specific chromosomal segments in *E.coli* and *Salmonella*. Using a subset of resolvases with shortened half-lives, the domains were proposed to follow a stochastic pattern averaging 10 kb (Deng, Stein et al. 2004; Stein, Deng et al. 2005). Here, I use the system to study the highly transcribed regions of the *Salmonella* chromosome.

CHROMOSOME ORGANIZATION IN REPLICATION

To reproduce, all organisms must separate the newly replicated strands, which entails eliminating all DNA crossings between sister chromosomes. Topo IV works during the final stages of replication to untangle the sister chromosomes promoting chromosome segregation (Zechiedrich, Khodursky et al. 1997; Draper and Gober 2002).

Replication in *Salmonella* starts at one origin of replication (*oriC*) with assembly of two replication forks that proceed in a bidirectional fashion (Kaguni, Fuller et al. 1982). Replication ends near the *dif* site and the newly replicated strands are segregated from one another (Bates and Kleckner 2005). DNA supercoiling helps to unwind the origin during replication initiation and it can stimulate initiation of RNA transcription (Botchan, Wang et al. 1973) (Fig. 5). Replication generates two topological domains: negative supercoils increase behind the fork and positive supercoils increase (or a loss of negative supercoils) ahead of the advancing replication fork (Postow, Crisona et al. 2001). The positive supercoils are generated by helicases and must be dissipated before replication can proceed. Both type II topoisomerases are important in the processivity and proficiency of replication (Strunnikov, Larionov et al. 1993; Hiasa and Marians 1994; Zechiedrich, Khodursky et al. 1997). Findings from our lab provide evidence that

mutants in either of the type II topoisomerases modify chromosome structure (Staczek and Higgins 1998; Pang, Chen et al. 2005).

XerCD SITE-SPECIFIC RECOMBINATION

The terminus region is where the two replisomes meet to end replication and finish segregation. Two distinct problems that segregating sister chromosomes face are dimers and pre-catenanes. Pre-catenanes are generated by the unwinding of the Watson and Crick plectonemic helix. Pre-catenanes turn into catenanes when replication completes both circles. Pre-catenanes are unlinked by topo IV before segregation (Grainge, Bregu et al. 2007). Gyrase provides a substrate for topo IV during decatenation of the two daughter strands (Pogliano, Pogliano et al. 2003). Chromosomal dimers arise due to an odd number of recombination events (Blakely, Colloms et al. 1991; Tecklenburg, Naumer et al. 1995). Dimer resolution occurs at resolvase sites called *dif* (deletion-induced filamentation) in the terminus region. Mutants in *dif* do not resolve dimeric chromosomes (Kuempel, Henson et al. 1991) and halt proper segregation. The XerCD recombinase promotes recombination between the two *dif* sites (Aussel, Barre et al. 2002; Ip, Bregu et al. 2003) to resolve chromosomal dimers into monomers (Lesterlin, Barre et al. 2004; Grainge, Bregu et al. 2007).

TRANSCRIPTION

RNA polymerase (RNAP) is the enzyme responsible for all transcription in a bacterial cell. In prokaryotic species, RNAP has five subunits; αI and αII, β, β', and ω. The σ

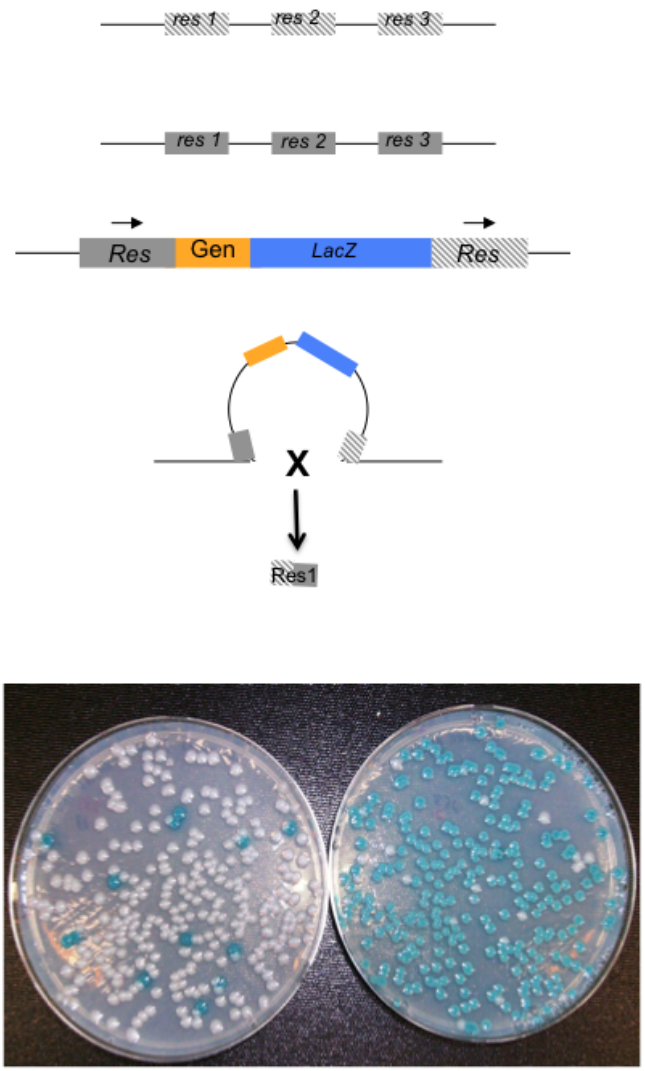

Figure 4. γδ-Resolution assays to measure DNA movement. A Res site includes three subsites labeled res-I, res-II and res-III (shown at top). The recombination event occurs at res-I. Resolution requires a dimer of resolvase to bind at each subsite (adapted from Staczek & Higgins 1998). γδ-Resolvase enzyme and 114bp Res sites can be introduced (via Red Recombineering) into the chromosome at specific locations. This assay is dependent on two res sites, negative supercoiling and the resolvase enzyme. If two Res sites form a plectonemic synapse, a recombination event can occur therefore deleting the intervening sequence. Shown above are γδ-Resolution indicator plates (induced-left, uninduced-right) using Chloramphenicol and 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal). Deletion frequency was scored by loss of color under induction.

subunit is responsible for finding the promoter and making initial contact with the gene sequence. σ70 is the major factor responsible for the bulk of transcription activity in the cell. If σ factors are not available, initiation becomes erratic and essential proteins are not made at proper critical levels (Artsimovitch 2008). Transcription elongation is processive and RNAP elongates until termination. The elongation rates for *E.coli* growing at 30°C averages 40-45 nucleotides per second (Gotta, Miller et al. 1991).

THE EFFECTS OF TRANSCRIPTION ON CHROMOSOME STRUCTURE

Transcription can be modulated by chromosome supercoiling, which influences initiation and elongation (Menzel and Gellert 1987; Richardson, Higgins et al. 1988; Straney, Krah et al. 1994; Spirito and Bossi 1996). Microarray studies demonstrate that 7% of *E.coli*'s genes respond to changes in supercoiling. Of the 400 supercoil sensitive genes, 100 increase and 300 decrease when supercoiling is lowered (Peter, Arsuaga et al. 2004). Transcription also affects DNA supercoiling. The twin domain model predicts the propagation of positive supercoils ahead of the RNAP translocating machinery and negative supercoils behind it. As DNA's helical turns are pushed ahead of the advancing transcription bubble, the DNA ahead of the bubble becomes wound (promoting positive supercoils) and the DNA behind the bubble becomes unwound (Liu and Wang 1987). Transcription causes circular DNA templates to undergo positive linking changes when gyrase is impaired and negative linking changes when topoisomerase I is impaired (Albert, Spirito et al. 1996). Negative supercoiling is a better template for most transcription as it is easier to denature than positive supercoiling. Other findings demonstrate transcription can be repressed or activated by negative supercoiling (Higgins, Collier et al. 1989; Higgins 1999; Chen and Wu 2003; Lim, Lewis et al. 2003).

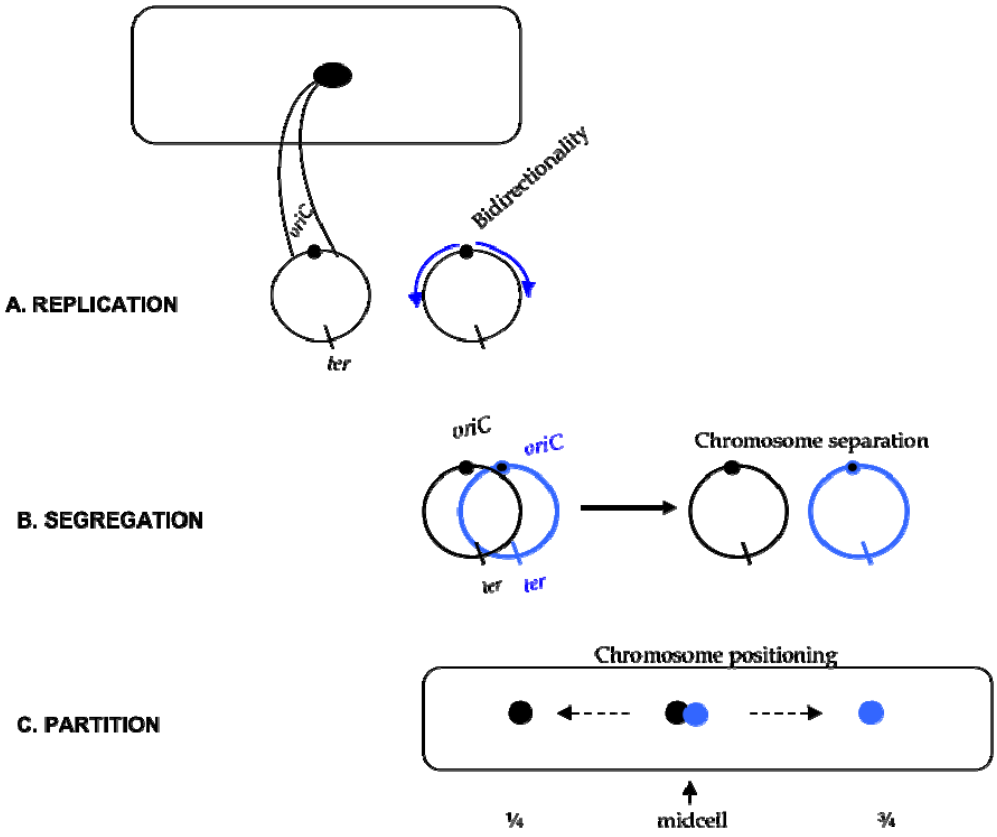

Figure 5**.** Chromosome replication involves several processes that ensure efficiency and fidelity of genetic material. (A) Replication proceeds in a bidirectional manner from *oriC* until it reaches termination sites (*ter*), results in two identical chromosomes. Many enzymes are responsible for replication processions: ligases, helicases, topoisomerases, and polymerases. (B) Segregation occurs along with replication; this process uses Topo IV and XerCD to decatenate dimers as replication ends. Segregation successfully separates the two newly replicated sister chromosomes and prepares for the next step. (C) Chromosome partitioning is responsible for positioning of the two sister chromosomes post replication but prior to cell division. This process ensures each cell gets one chromosome (blue or black circles).

The appearance of new domain barriers is also detected on chromosomal segments during transcription in vivo. Deng et al observed an inverse relationship between resolution and transcription activity (Deng, Stein et al. 2004). I have analyzed how topology is modified in and around the genes with the highest transcription frequency.

THE RIBOSOMAL RNA OPERONS IN BACTERIA

Salmonella Typhimurium LT2 has 4,733 genes and 7 rRNA operons (Mueller, Oebbecke et al. 1977; Nomura 1999; McClelland, Sanderson et al. 2001). Although, accounting for less than 1% of encoded genes, rRNA can make up more than 70% of cellular RNA. A cell can have over 70,000 ribosomes, which requires the synthesis of large amounts of rRNA. To meet this requirement, *Salmonella* has 7 copies of the rRNA genes located around the genome (Mueller, Oebbecke et al. 1977). The rRNA genes have strong promoters that can have up to 150 RNA polymerases transcribing each rRNA operon. With this activity, they are the most active genes (Schneider, Ross et al. 2003). The amount of rRNA activity depends on the need for protein synthesis. During amino acid starvation, rRNA and tRNA transcription is inhibited, thus halting ribosomal synthesis (Paul, Ross et al. 2004; Ferullo and Lovett 2008).

Previous studies have analyzed how transcription, in small plasmids, affects regions upstream and downstream without observing the actual site of barrier formation. My work provides evidence of transcription-induced domain barriers. The effort of this thesis is to understand molecular forces of topological constraint in highly transcribed regions on the chromosome.

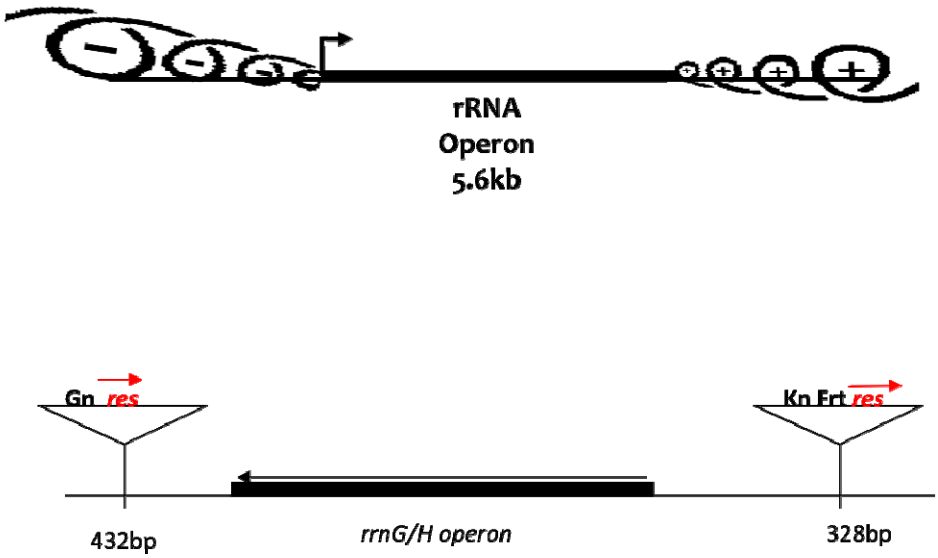

Figure 6. Supercoil diffusion in the rRNA operons. The twin domain model suggests a propagation of negative supercoils upstream and positive supercoils downstream of the transcribing polymerase (Liu & Wang, 1988).Utilizing recombineering methods, we insert *Res-Res* modules to measure supercoil diffusion across, upstream, and downstream of the most highly transcribed genes in bacteria (Yu *et al.*, 2000, Datsenko & Wanner, 2000).

DNA TOPOLOGY OF HIGHLY TRANSCRIBED OPERONS IN *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM

by

BETTY M. BOOKER, SHUANG DENG, AND N. PATRICK HIGGINS

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Format adapted for dissertation

ABSTRACT

Transcription-induced barriers to chromosomal supercoil diffusion can be measured using Tn*3*/γδ resolution in vivo. We used resolution to measure DNA topology upstream, downstream, and across highly transcribed operons in *Salmonella*. Inhibition of resolution was observed whenever highly transcribed genes or operons were positioned between two *Res* sites. The barriers to supercoil diffusion across each region reflected the level of transcription. Ribosomal RNA operons had the highest transcription rates, and experiments at the *rrnG* and *rrnH* operons showed that transcription of these operons resulted in the largest inhibitory effects. Resolution of intervals containing these segments were nearly 2 orders of magnitude lower than measured at comparable length intervals in low transcription zones of the chromosome. However, transcription caused only modest effects in the "twin domain" regions. Upstream of the *rrnG* operon, resolution efficiency was slightly higher than expected (150%), while downstream resolution efficiency was 56% lower than expected, indicating an increase and decrease of $\sigma = -0.007$ respectively at both regions. A model is discussed that accounts for high levels of constrained supercoiling along the transcription track with modest effects on diffusible supercoiling immediately adjacent to the rare highly transcribed zones of living cells.

INTRODUCTION

Escherichia coli and *Salmonella enterica* serovar Typhimurium chromosomes are organized into about 400 independent supercoiled domains in vivo (Postow, Hardy et al. 2004; Deng, Stein et al. 2005). Most topological barriers to supercoil diffusion in both *E. coli* and *Salmonella* are stochastically located along the DNA sequence (Higgins, Yang et al. 1996; Staczek and Higgins 1998; Postow, Hardy et al. 2004; Higgins, Deng et al. 2005; Stein, Deng et al. 2005). Therefore, each cell has a unique local chromosome structure with an average domain size that varies around 10 kb. Identifying specific structures, boundaries, or supercoiling organizational motifs is a major goal for building a working model of the dynamic prokaryotic nucleoid.

During dichotomous growth in rich medium, approximately 2000 RNA polymerases transcribe > 4000 genes at varying levels and frequencies while multiple replication forks move from the replication origin to the terminus at a rate that is more than 10-fold faster than transcription. How do cells form compact nucleoids that allow both complete and partial genome replicas to move into daughter cell compartments at cell division while DNA replication and transcription occur unabated? What is the DNA topology of the most highly transcribed DNA?

Transcription causes DNA to rotate about its axis and generates an increase in negative (-) supercoils upstream and a decrease in (-) supercoils downstream of transcribed genes (Liu and Wang 1987; Wu, Shyy et al. 1988). Most biochemical studies of supercoiling effects associated with transcription have been carried out using small

multi-copy plasmids, which are single domain molecules (Higgins and Vologodskii 2004). Very little is known about transcription dynamics in the 400 domain bacterial chromosome.

Site-specific recombination assays based on the Tn*3*/γδ resolvase can be used to study supercoil diffusion in the *Salmonella* chromosome *in vivo* (Higgins, Yang et al. 1996). The resolution reaction requires the precise movement of duplex supercoiled strands to form a synaptic crossing of three nodes that promote recombination (Fig. S1) (Stark, Sherratt et al. 1989; Benjamin, Abola et al. 1996). Scheirer discovered that gene expression from the early promoter of bacteriophage Mu created a barrier to Tn*3* resolution near the site of transcription in the chromosome of *Salmonella* (Scheirer and Higgins 2001). Deng *et al*. later showed that transcription from a Tn*10*-derived TetA promoter also created barriers to site-specific recombination in the chromosome (Deng, Stein et al. 2004).

Here, the effects of transcription are mapped at 6 regions of the *Salmonella* chromosome that span a full range of gene expression levels. Site-specific resolution was impaired whenever efficiently transcribed genes were flanked by directly repeated Res sites. The magnitude of resolution inhibition increased with transcription efficiency, ranging from very small or un-detectable effects at poorly transcribed genes to 100-fold effects at operons with the highest transcription level, the ribosomal RNA operons. However, supercoiling effects upstream of the promoter and downstream of a terminator were small. These results lead to model wherein RNA polymerase locks in high levels of

constrained supercoiling that dramatically blocks resolution and dominates DNA polymer dynamics in the rare highly transcribed zones of the bacterial chromosome.

RESULTS

Measuring Resolution Inhibition Over a Full Range of Chromosomal Transcription Levels. Previous work from this lab uncovered four rules that connect transcription to supercoil behavior in the 400 domain bacterial chromosome (Deng, Stein et al. 2004; Deng, Stein et al. 2005). A synthetic operon in which the Tn*10* repressor (TetR) regulates expression of different genes fused to the TetA reading frame revealed four properties of transcription-generated domain behavior. First, induction of gene expression from a TetA promoter caused the appearance of new barriers that block resolution in the region near the induced transcript. Second, termination of transcription reversed the effect and the region-specific barriers disappeared. Both the appearance and the disappearance of barriers took place within 10-15 min. of induction or repression. Third, transcription of different types of expressed proteins caused similar effects. A small cytoplasmic protein (kanamycin transacetylase), a large cytoplasmic protein (β-galactosidase), or a medium size membrane protein (TetA tetracycline pump) gave similar results. Fourth, the level of TetR expression controlled the strength of resolution interference. By combining the level of a *lacZ* transcript with the magnitude of resolution inhibition, we could predict transcription effects at chromosomal regions where mRNA levels were mapped as steady state RNA/DNA ratios. However, these experiments only covered a partial range of expression levels (Deng, Stein et al. 2004).

To test the characteristics of domain behavior in natural chromosome contexts, we introduced Res sites into five regions that include the regions with the highest

transcription activity (Table I). Previous studies showed that resolution can be measured between regions placed 100 kb apart, but that stochastic 10 kb domains are predominant in both *E. coli* and *Salmonella* (Postow, Hardy et al. 2004; Stein, Deng et al. 2005). Therefore, all test intervals lacked essential genes and were designed to be close to a 10 kb interval (Table 1).

Selectable markers linked to 114 bp Res sites (pink boxes in Fig. 1) were introduced using the phage λ *red* recombineering methods (Datsenko and Wanner 2000; Yu, Ellis et al. 2000). Then, a plasmid was introduced that produced either the WT Tn*3* Res (Higgins, Yang et al. 1996) or the 30 min. time-restricted γδ-Res-SsrA-L9D enzymes (Stein, Deng et al. 2005). Both enzymes were expressed under the regulatory control of p_L promoter and the lambda cI^{857} repressor. To measure recombination efficiency, cells were grown in LB at 30°, shifted to 42° for 10 min. to induce resolvase expression, and then returned to 30° to allow segregation of recombinant chromosomes (Stein, Deng et al. 2005). Colonies arising on non-selective agar medium were patched onto drug selection plates to determine the frequency of recombination. Each data point represents the average ±1 standard deviation of at least three independent experiments in which ≥ 200 colonies were counted for drug sensitivity.

RNA/DNA = ≤ 1 The first interval tested was a low transcription zone. NH4057 contains two Res sites that span a *Salmonella* segment from *yliJ* to *ybjC* (Fig.1). Microarray experiments showed that all genes in this sector are poorly transcribed with

steady state RNA/DNA ratios ≤ 1. Many cells have no RNA for each of the genes in this cluster during steady state growth.

Previous experiments using the WT Tn*3* resolvase to delete markers spaced from 5 kb to 100 kb apart showed a first order decay curve (Fig. S2). Interpolation of this curve predicts that a 14 kb bp segment should have a deletion efficiency of 70% in mid-log growth (Higgins, Yang et al. 1996; Staczek and Higgins 1998). The deletion interval in NH3500 resulted in a deletion efficiency of 65% (Fig. 1, Table 2). To compare results from different regions with slightly different size intervals, we define a term Transcription Impact Factor (TIF) to be the value obtained from the first order decay curve in previous work divided (Fig. S2) by the observed recombination efficiency. The segment spanned by Res sites in *Salmonella* strain NH3500 is close to the expected value TIF = 1.

RNA/DNA = 10 A more active operon is present in NH4058 (Fig. 1, 2nd line). Gn-Res and Kn-Res modules flank an interval of 8,724 bp that includes the ATP operon. The ATP operon encodes a family of membrane-associated subunits that are transcribed from a single promoter. The process of co-transcriptional translation and insertion into the membrane (transertion) causes the RNA polymerases within this operon to be restricted from rotation around the DNA because transertion anchors transcription machinery to the inner membrane (Lynch and Wang 1993). Genes in the ATP operon show steady state RNA/DNA ratios near 10. For a 9 KB interval we expect a resolution efficiency of 95% after expression of the WT resolvase (Fig. S2). The observed

resolution efficiency was 22%, which yields a TIF of 4.5. Thus, the ATP operon shows reduced recombination compared to the efficiency measured for a low transcription zone.

RNA/DNA = >600 The most highly transcribed genes in enteric bacteria are the ribosomal RNA operons (Schneider, Ross et al. 2003). Electron microscopic analyses show that for *E. coli* cells growing exponentially in LB, each ribosomal RNA operon is densely packed with around 150 transcribing RNA polymerase molecules. A "Christmas tree" appearance on EM spreads reflects individual transcripts that are being actively assembled into ribosomes (French and Miller 1989; French 1992). Approximately 70% of all active RNA polymerases are engaged in transcribing the seven *rrn* operons during rapid dichotomous growth (Dennis and Bremer 1974; Dennis, Ehrenberg et al. 2009). After adjusting for the stability of the ribosomal RNA product, each *rrn* operon generates a steady state RNA/DNA synthesis ratio over 600, which is > 60-fold higher than the most highly expressed protein-encoding gene in *Salmonella*.

Strain NH4029 has a Kn-Res module upstream and a Gn-Res module located downstream of the *rrnG* operon (Fig. 1, line 3). This is the *rrn* operon farthest from the origin of replication in replichore II. The Res modules form a 13,030 bp interval that is deleted and lost from cells following recombination. The expected recombination frequency for a 13 KB interval is 88% (Fig. S2). However, the measured recombination efficiency across the *rrnG* locus was ≤ 1%, giving a TIF ≥ 88.

This striking result shows that one of the seven highest transcribed regions in *Salmonella* had the highest TIF, which is consistent with the previous observation that

effects on chromosome structure increase with higher transcription efficiency. To confirm this result, Res modules were introduced up and down stream of the *rrnH* operon in NH4030 (Table I). The *rrnH* operon is the ribosomal RNA cluster farthest from the origin in replichore I. The Gn-Res and Kn-Res modules flanking the rrnH operon form a 10,647 bp deletion (see below). Experiments with NH4030 gave results similar to NH4029. The expected deletion efficiency is 97% but measured recombination was <1% giving a TIF of 97 (Table 2). Thus, recombination experiments in two of the 7 ribosomal RNA operons showed that both have recombination efficiencies that are 100-fold lower than those found for similar sized domains in low transcription zones of the genome.

Modulation of Transcription and Recombination at Ribosomal RNA Operons. It is clear that transcription efficiency is inversely correlated to recombination efficiency across 4 different locations that vary in gene expression over a 600-fold range. But mechanisms other than transcription might be involved in blocking resolution. For example, proteins that form cross-bridges between two sites on the chromosome should impede supercoil diffusion (Saldanha, Flanagan et al. 1987; Mercier, Petit et al. 2008). The ribosomal RNA operons could have special sites for the MukBEF condensin or other proteins that might isolate ribosomal RNA from the rest of the genome. To experimentally test whether transcription is a prime cause of effects on recombination, resolution was measured under several conditions that lower the transcription frequency of ribosomal RNA operons.

To confine measurements to a shorter time frame, we removed the plasmid expressing WT Res and introduced a plasmid that makes the γδ-Res-SsrA-L9D resolvase (Stein, Deng et al. 2005) to make NH4031 (Fig. 2). The WT Tn3-resolvase is a long-lived protein that persists for much longer than an hour after induction at concentrations sufficient to carry out recombination. The γδ-Res-SsrA-L9D protein contains a modified ClpXP degradation tag at the C-terminus. This enzyme is fully proficient for resolution but it has a 30 min. half-life and this restricts the assay period to one cell generation [(Stein, Deng et al. 2005) see Fig. S2]. NH4031 has the same chromosome structure as NH4029 (Fig. 1), but the first order decay curve in over the same 100 kb interval is different (Fig. S1 lower curve). The recombination efficiency of the γδ-Res-SsrA-L9D resolvase in log phase was measured. <1% of the cells underwent recombination, which is the same as that measured with the WT Tn*3* resolvase. For an interval of 13 kb in cells with the 30 min. resolvase, the expected resolution efficiency is 40% (Stein, Deng et al. 2005). Thus the TIF = ≥ 40, which compares with a TIF = 90 for the WT enzyme. The difference between the two numbers reflects division by a very small number (less than 1%). Note that the actual recombination efficiency was the same for both enzymes.

Transcription was temporarily interrupted by adding rifampicin (Rif) immediately after the 10 min. resolvase induction period. Rif was washed out of the cells 30 min. later and the efficiency of recombination that occurred during the transcription interruption was measured after cells resumed growth through several divisions to segregate chromosomes into daughter cells. Rif addition caused no detectable loss in colony

forming units, but the deletion efficiency at the *rrnG* operon locus soared to 95%. Thus, the TIF in the absence of inhibitor is 40, but after Rif addition TIF = 0.4. Interrupting transcription caused a 100-fold increase in resolution efficiency. Clearly, transcription blocks resolution in the *rrnG* operon in rapidly dividing cells.

A second way to change the ribosomal RNA transcription rate is to allow cells to enter stationary phase. Transcription of ribosomal RNA genes is coordinated with the need for protein synthesis, and when cells grow slowly on defined medium or when cells enter stationary phase, transcription of the ribosomal RNA genes is reduced by a process called growth rate regulation (Schneider, Ross et al. 2003). Cells grown into stationary phase by overnight incubation in LB were subjected to thermo-induction at 42° without addition of fresh medium. Cells were subsequently returned to 30°, diluted into fresh LB, and incubated at 30° to allow chromosome segregation. The recombination efficiency at the *rrnG* locus in stationary phase was 50%. Addition of Rif for 30 min. immediately after thermo-induction boosted the efficiency to 75% resolution. We cannot assign a TIF in stationary phase that is comparable to a TIF in exponential phase because the half-life of SsrA-tagged resolvase increases in stationary cultures of *Salmonella* (Stein and Higgins, unpublished observations). Nonetheless, in stationary phase the ribosomal RNA locus recombines at levels expected for a low transcription zone in rapidly growing cells.

The effect of transcription was also analyzed at the *rrnH* operon (Fig. 3). An efficiency of 45% recombination is expected for a 10 kb interval after expression of the 30 min γδ-Res-SsrA-L9D resolvase (Fig. S2). In log phase cells, the *rrnH* operon gave

the same resolution rate observed for the *rrnG* operon (<1% with a TIF of ≥ 45). Addition of Rif caused the resolution efficiency to rise to 27%. In stationary cells, the resolution efficiency of the *rrnH* operon was 14%, and it rose to 41% after Rif addition. Thus, in two ribosomal RNA operons, temporary blockage of transcription stimulated 30-100-fold increases in resolution frequency. Differences between the two operons at slow growth rates and after rifampicin addition may indicate that the *rrnH* operon is more highly transcribed than the *rrnG* operon at slower growth phases. Little is known about differences in expression between the 7 operons. The near identity of all 7 ribosomal RNA sequences makes telling them apart very difficult. Nonetheless, each operon contains different tRNA genes and they may well have different transcription profiles during sub-optimal growth.

In both of the highly transcribed ribosomal RNA operons, recombination falls to 1% using both the WT and 30 min. resolvase. The meaning of 1% recombination in unclear. Many bacterial cultures have a population around 1% that is not in exponential phase. Some of these cells may be repairing broken chromosomes generated during replication via the recABCD pathway. There are also genetic systems that take cells out of the cell cycle by expressing cell division inhibitors. For example, approximately 1% of *Escherichia coli* K12 cells are out of the growth cycle due to the *hip* operon. The *hip* operon provides a stochastic mechanism that allows drug-sensitive cultures to persist during bouts of drug treatment (Korch, Henderson et al. 2003). *Salmonella* does not have the *hip* operon but it has other elements, including four prophages, that may have

elements that cause a similar effect. Whatever accounts for the 1% recombination observed across actively transcribing ribosomal RNA operons, this represents the lowest recombination rate we have measured at all sites analyzed in *Salmonella*.

Supercoiling in the "Twin Domains" of ribosomal RNA DNA. In vivo transcription of plasmids with strong promoters generates negative supercoiling upstream and a loss of negative supercoiling (or perhaps even positive supercoiling) downstream of transcription termination (Liu and Wang 1987; Wu, Shyy et al. 1988). This is often called the twin domain effect of transcription (Higgins and Vologodskii 2004). To examine supercoiling in the twin domains of the *rrnG* operon, 34 bp Frt sites were introduced at positions that caused no disruption to normal gene expression (Fig. 4). Then, an 8.6 KB module (Pang, Chen et al. 2005) with a complete *lac* operon plus a selectable Gn gene flanked by two Res sites was "Flipped in" to each Frt site.

There are two advantages of this system. First, absent lactose or IPTG, the module has a very low transcription profile. Thus, recombination efficiency of this module is influenced only by transcription effects of the adjoining chromosomal segments. Second, resolution deletes the *lac*-Gn circle and leaves a Frt-Res-Frt scar (Fig. 4C.) Results can be scored on X-Gal medium by simply counting blue (non-recombinant) and white (recombinant) colonies.

In NH3868, the recombination module is situated beyond the terminator of the *clpB* gene, and upstream of the Fis binding site (or UP element) in the *rrnG* promoter (Fig. 4B). For an 8.6 kb interval, the 30 min. resolvase is predicted to delete the region at

a frequency of 50% (Fig. S2). The measured resolution frequency was 75%, which gives a TIF of 0.7. This 50% increase in recombination is consistent with a small increase in negative supercoiling upstream of the *rrnG* operon (see below).

Supercoiling effects downstream of the *rrnG* terminator were analyzed in NH4028. Here the recombination module was located beyond the *rrfG* terminator at the end of the rrnG operon and upstream of the probable promoter of the SMT2655 gene. SMT2655 is a hypothetical protein that lies immediately downstream of the *rrnG* operon. The measured recombination efficiency was 28%, which is 56% of the expected recombination efficiency that results in a TIF of 1.8. This result indicates a small but measurable decrease in downstream negative supercoiling, and is inconsistent with downstream positive supercoils, which would abolish resolution.

In summary, resolution efficiencies measured across different transcribed zones increased with higher transcription activity. Operons with steady state RNA/DNA ratios of 1, 10, and >600 showed TIF values of 1, 5, and >70 respectively. But in the upstream and downstream twin domain regions of the highly transcribed *rrnG* operon, recombination efficiency was increased or decreased by a small value relative to the efficiency of recombination observed in a low transcription domain. At the rrnG operon, >95% of the transcriptional impact on supercoil diffusion is confined to the transcribed track and this inhibition requires continuous transcription.

DISCUSSION

In this study we used Tn3/γδ resolution to measure supercoil dynamics of chromosomal DNA experiencing different levels of transcription. Negative supercoiling plays two roles in the resolution reaction (Fig. S1). First, supercoiling promotes the synapse of two 114 bp Res sites, forming an interwound, three noded braid of negatively supercoiled duplex DNA (Benjamin and Cozzarelli 1988; Stark and Boocock 1995). Without this synapse, recombination cannot occur (Fig. S1). Second, negative supercoiling drives the rotation of the protein-DNA complex to complete a round of phosphodiester bond exchanges between the 4 participating strands.

Diffusible Supercoiling Upstream and Downstream of Highly Transcribed Genes. Supercoil density, s, is defined as the ratio of the linking difference ΔLk of a supercoiled chromosome or plasmid divided by the linking number of a nicked or relaxed molecule Lk_0. In *Salmonella* and other Gram-negative organisms that have been analyzed, σ has two components. Constrained (σ_C), which is DNA topology associated with bound chromosomal proteins and R-loops, and diffusible (σ_D) supercoiling, which is generated by and maintained at defined levels by DNA gyrase (Higgins and Vologodskii 2004). In *E. coli* and *Salmonella*, supercoiling is partitioned equally between the two states (Pettijohn and Pfenninger 1980; Bliska and Cozzarelli 1987; Jaworski, Higgins et al. 1991; Higgins and Vologodskii 2004) (Fig. 6A). Only diffusible supercoiling promotes γδ recombination.

In the region upstream of *rrnG* transcription, resolution efficiency increased by 50% compared to the rate measured for a module in a low transcription zone (Fig. 4). The dependence on negative supercoiling has been established using defined substrates in vitro (Benjamin, Abola et al. 1996). Assuming that diffusible supercoiling is evenly distributed, the value would be $\sigma_D = -0.03$ in poorly transcribed regions. A 50% increase in recombination efficiency indicates an increase of - 0.007 to $\sigma_D = -0.037$ (Fig. 5, pink arrow). This increase in diffusible supercoiling would result in the upstream domain having a value of $\sigma = -0.067$ (Fig. 6 B and C).

In the region downstream of the transcription terminator of *rrnG*, a decrease in resolution of 56% was observed. This change in efficiency correlates with a decrease of supercoiling of 0.007 to $\sigma_D = -0.023$ (Fig. 5, green arrow). This means supercoiling downstream of the *rrnG* operon would drop to the range of $\sigma = -0.053$. Significantly, the domain is clearly not positively supercoiled. To our knowledge, this is the first quantitative estimate of supercoil density in a chromosome region immediately upstream and downstream of one of the strongest known bacterial promoters. However, these measurements are consistent with plasmid studies that show qualitative increases in negative supercoiling in the region upstream of less active moderate to strong promoters in plasmids in *E. coli* (Rahmouni and Wells 1992; Albert, Spirito et al. 1996). And they agree with effects reported for supercoil changes near the chromosomal leu-500 promoter mutation in *Salmonella* (Fang and Wu 1998; El Hanafi and Bossi 2000).

Our results also agree with qualitative experiments on the downstream effect of inducible promoters in *E. coli*. Moulin et al showed that a strong lambda pR promoter, regulating a copy of the *uidA* gene under control of the TS lambda $c1^{857}$ repressor, stimulated expression of a downstream *gyrA* promoter driving *lacZ* (Moulin, Rahmouni et al. 2005). Shift from 30° to 37° caused large increases in *uidA* expression and small increases in β-gal levels that varied from 15% to 40% of uninduced levels. These β-gal measurements indicate a small supercoil change. Note that torsional effects should be the same if one polymerase is moving alone or if 100 enzymes move along DNA together. It may also be the reason that there is no topological difference between a gene encoding an integral membrane protein that is transerted by coupled transcription and translation and a cytoplasmic protein like the Kan protein (Deng, Stein et al. 2004). Once ribosomes start translating any protein in a WT cell, the RNA polymerase might stop rotating around DNA.

RNA Polymerase is a Major Barrier to Supercoil Diffusion During Transcription.

Transcription across the ATP operon (Fig. 1) lowered resolution efficiency 4.5-fold. This decrease is equivalent to changing the effective diffusible supercoil density from $\sigma_D = -0.030$ in a low transcription zone to $\sigma_D = -0.016$ (Fig. 6B). This effect is much more dramatic than what is seen up- and downstream. And as transcription becomes more efficient culminating in the *rrnG* and *rrnH* operons, the recombination across the transcription zone falls to near zero > 1%. Can RNA polymerase alone account

for a 100-fold impact of transcription on resolution?

A number of abundant proteins contribute to constrained supercoiling (σ_C) *in vivo* (Johnson, Johnson et al. 2005; Pul and Wagner 2010). They include the small nucleoid associated proteins IHF, HU, H-NS, and FIS that contribute to transcriptional regulation (Browning, Grainger et al. 2010; Dorman 2010), the structural maintenance of chromosome (SMC) complex MukBEF, and RNA polymerase. In general, supercoiling dynamics are similar in the *E. coli* and *Salmonella* chromosome and in small circular plasmids introduced into cells (Miller and Simons 1993; Pavitt and Higgins 1993) (Fig. 6A.) However, for highly transcribed genes, the RNA polymerase constrained supercoiling level must becomes huge. Each enzyme unwinds the Watson-Crick strands to maintain an RNA-DNA hybrid at the active site as it moves along DNA. RNAP constrains the equivalent of -1.7 supercoils as DNA writhes (Gamper and Hearst 1982) through the enzyme. The DNA segment bound by polymerase is about 35 bp. Therefore, RNAP has a much higher constrained supercoiling value than even the eukaryotic nucleosome, which constrain -1.6 supercoils in 146 bp of DNA . (Hamiche, Carot et al. 1996; Hamiche and Richard-Foy 1998; Musgrave, Forterre et al. 2000).

With an *rrnG* transcript 5200 nt long and the DNA template in the Lk_0 state of 495 WC turns of B-form DNA (5200 bp/10.5 bp per turn) the 150 RNA polymerases transcribing each 7 *rrn* operon generate a cumulative constrained supercoiling value σ_C = - 0.50 (495 W/C turns / 150 RNPs x - 1.7)! This value is 15 times higher that the average constrained level due to all DNA-binding proteins (σ_C = - 0.03). It is also 8

times the average value of total σ = - 0.06 distributed over most of the genome, and it is 4 x the constrained supercoiling level present in eukaryotic nucleosomes (Hamiche, Carot et al. 1996; Hamiche and Richard-Foy 1998).

Transcription of the *rrn* operons is equivalent to putting DNA into 150 pieces of tiny PVC tubing. This can completely block plectonemic slithering and branching across transcribed genes (Fig. 6). It also provides a compelling explanation for why highly transcribed genes in both *E. coli* and *Salmonella* are shielded from insertions by transposable elements like bacteriophage Mu (Manna, Breier et al. 2004; Manna, Porwollik et al. 2007). It is hard to imagine any organization scheme near highly transcribed genes that would not block plectonemic interactions between opposite ends of a transcribed zone.

Why are Downstream Chromosomal "Positive Supercoiling" Effects Small?

The *in vivo* rate of RNA synthesis in *E. coli* growing at 30° is 25 – 40 nt/ sec for both the highly transcribed stable RNA operons (Ryals, Little et al. 1982; Gotta, Miller et al. 1991) and for protein encoding genes (Golding and Cox 2004). Measurements using single molecule techniques *in vitro* show lower rates of RNA polymerase movement of 0.5 to 11 nt/sec (Kasas, Thomson et al. 1997; Davenport, Wuite et al. 2000). At 40 nt/ sec, the supercoiling impact of transcriptions is 4 supercoils/sec, (- and + respectively).

With a Res site 328 bp beyond the transcription terminator in the rrnG operon, the effect of transcription on resolution was a TIF = 1.4). This value correlates with a drop in σ to around -0.052. Presumably, something ameliorates a greater decrease in negative

supercoiling, and the most likely factor is gyrase. The DNA footprint of a processively acting gyrase bound to the strong binding site in Mu is about 140 bp (Oram, Travers et al. 2006). Gyrase acting downstream of each *rrn* terminator is the simplest explanation for the lack of dramatic changes in supercoil levels downstream of the most active promoters. Which is also consistent with the gyrase binding properties at multiple *rep* sequences that occupy locations near the ends of many genes in the *E. coli* and *Salmonella* genomes (Stern, Ames et al. 1984; Yang and Ames 1988).

Topo I controls most of the negative supercoiling effect upstream, and single molecule studies indicate that TopA can relax DNA at a rate of 4 supercoils/sec (Corbett and Berger 2004). *In vitro*, gyrase can supercoil DNA at a sustained rate of 1 supercoil/sec for over an hour in a distributive supercoiling assay (Higgins, Peebles et al. 1978). Rates of 4 supercoils/sec have been measured in single molecule experiments during processive supercoiling bursts (Nollmann, Stone et al. 2007). Note that DNA rotates at the same rate with one RNA polymerase restricted for rotation as with 150 enzymes moving together. Thus, assuming no increased efficiency of gyrase supercoiling *in vivo* compared to *in vitro* assays, a single enzyme working near a transcription terminator could eliminate most of the downstream losses in negative supercoils topological at the strongest promoters.

Assuming that these kinetics hold for all genes transcribed at RNA/DNA levels from 10, which generates a TIF = 2 for with the γδ-SsrA-L9D resolvase, to the *rrnH* and *rrnG* operons which make RNA/DNA transcripts near 600, there would be 30-50 sites of

high transcription in the genome (see below) and only 30-50 gyrase molecules would be required to handle the positive supercoil problem posed by the most abundantly expressed genes. With 500-1000 molecules of gyrase in the average log phase cell, there is ample supercoiling power for the task. ChIP-chip studies analyzing genome-wide gyrase binding patterns during different stresses and under varying growth conditions shows that gyrase presence is highly correlated with the active transcription zones (Jeong, Ahn et al. 2004; Jeong, Xie et al. 2006).

Transcription Domains Have a Conserved Order In Two Bacteria. The genomes of both *E. coli* and *S. typhimurium* evolved from common ancestors millions of years ago. Dramatic changes in each genome include the frequent horizontal transfer of genes in both organisms. Presently, over 30% of all genes are species unique (Lawrence and Ochman 1997; Lawrence and Roth 1999; Lawrence 2005) and each organism has a completely different set of mobile DNA elements (Fig. 6A). From a perspective of autonomously mobile DNA, it is striking how different the chromosomes are in both number and type of elements. *E. coli* K12 contains a large cast of insertion sequence (IS) elements, which includes multiple copies of IS1, IS3, IS4, IS5. IS30, IS150, IS186, and IS911. *E. coli* has no inducible plaque-forming prophages. By contrast, *Salmonella* has none of the IS elements present in *E. coli*. It contains a single IS200 element, which is found at 6 locations in addition to four viable plaque-forming prophages, Gifsy-1, Gifsy-2, Fels-1 and Fels-2. Looking at this map the two organisms appear unrelated.

The opposite impression is seen in a map of highly transcribed genes (Fig. 6B). Whole genome arrays reporting the RNA/DNA ratio for cells growing exponentially in LB are published for *E. coli* (Bernstein, Khodursky et al. 2002) and S. *typhimurium* (Manna, Porwollik et al. 2007). When one analyzes the top 100 transcribed genes, they are 100% conserved under these growth conditions. Map positions for all genes/operons with steady state RNA/DNA ratios near 10 (TIF with 30 min. resolvase = ≥ 2) are shown in Fig. 6B. Three things are remarkable. First, highly transcribed genes are distributed throughout the genetic map in roughly 30 clusters, and all genes except *nmpC* have a conserved order in *E. coli* K12, with a 4.64 Mb genome, and *S. typhimurium*, with a 4.86 Mb genome. There are two differences in the high transcription gene order. One is a symmetrical inversion about the terminus of replication, but the gene order persists within the inversion.

Second, the only highly transcribed gene with a position change, *nmpC,* is an exception that proves the rule. In *E. coli*, *nmpC* (b0553) is listed as an outer membrane pseudo-gene that is truncated by an IS5 insertion in its C-terminal domain. It lies clockwise from the *rrnH* operon in replichore 2 and has a low transcription rate (RNA/DNA = 1) that runs opposite to the direction of DNA replication. In *Salmonella*, *nmpC* is listed as a highly transcribed outer membrane porin (RNA/DNA = 38) located within the inversion near the Dif site. Thus, the transformation to high transcription status appears to have necessitated a different location in the genome.

The important theme is that 30-50 efficient transcription islands, which represent about 5% of the sequence space, are dispersed in a sea of low transcription DNA accounting for 95% of the sequence space. This landscape of long segments of poorly transcribed genes interrupting highly transcribed gene islands has been noted for many bacteria (Wright, Kharchenko et al. 2007). The same pattern is also found in the well-studied eukaryotic genomes including Drosophila, yeast, and human cells (Furlong, Anderson et al. 2001; Kuznetsov, Knott et al. 2002; Spellman and Rubin 2002).

Dispersing Transcription Could Stabilize Chromosomes. We propose that transcription distribution is under strong selective force to maintain a conserved genetic backbone of the chromosome. Note that the gene order in Fig. 7 is not the only structure found in normal bacterial populations. Duplications are present in up to 2% of individual cells in exponentially growing populations (Andersson and Roth 1979; Anderson and Roth 1981). One large duplication/amplification becomes the dominant chromosome genotype when cells are grown on media containing a poor carbon source (Sonti and Roth 1989). Inversions in normal populations are detectable by PCR and new linkage arrangements are frequently generated by recombination between homologous sequences like the ribosomal RNA genes and mobile DNA sequences that are closely related and spread around the genome (Schmid and Roth 1983; Segall, Mahan et al. 1988; Mahan and Roth 1991). The order of genes in Fig. 7 has persisted for millions of year in the face of the myriad of genetic events including gene transposition and homologous and non-homologous recombination that can and do reorder the sequence.

We suggest that this structure represents the fastest replication order during dichotomous growth in rich medium. Dichotomous growth allows cells to double every 25 min even though the time required to complete a round of replication is 50 min. To double rapidly, dichotomously dividing cells inherit two complete sister chromosomes, and 2 or 4 partially replicated arcs of replicating chromosomes at each cell division. High transcription zones may function like traffic cops, punctuating high speed of DNA synthesis with pauses to promote untangling between domains in each replichore. Evidence for the importance of spacing between highly transcribed genes has been noted in yeast. Yeast have a segment of chromosome with about 150 copies of ribosomal RNA genes in tandem array. But less than a third of these are transcribed at any one time. If the copy number is decreased to 20, the chromosome is destabilized because when every gene is transcribed for many kb, the ability of important proteins like condensins and cohesins to function efficiently in mitosis deteriorate (Ide, Miyazaki et al. 2010). Spacing out high transcription regions with idle zones allows room for binding of proteins that function during the formation of a compact genome during the cell cycle.

To have such a significant impact on chromosome structure, dichotomous growth would have to provide a significant selective advantage. One point where dichotomous growth appears to be a critical factor in the natural environment for *E. coli*, *S. typhimurium*, and *Pseudomonas* is the competition for adhesion sites during the establishment of a stable niche following primary infection (Freter, Brickner et al. 1983; Mathee, Narasimhan et al. 2008).

Finally, our results raise a number of important questions. First, since the average supercoiling level is higher in *E. coli* than in *Salmonella* (Champion and Higgins 2007), what is the supercoil density up- and downstream of a ribosomal RNA operon in *E. coli*? Presumably *E. coli* maintains higher averages up and downstream, but the constrained supercoiling level associated with RNA polymerase should be the same. Moreover, because the structure/function properties of RNA polymerase are highly conserved from bacteria to humans, constrained supercoiling in highly transcribed zones should also be significant to eukaryotic genomes.

Second, what happens to supercoil density in the twin domains in cells that harbor mutants of TopoI and gyrase? Do R-loops form behind the RNA polymerases in a hyper-supercoiled wake, or might the R-loops form directly at the enzyme active site like they do during the formation of R-loops that prime replication of ColE-1 related plasmids (Masukata and Tomizawa 1990)?

Third, it is often assumed that supercoil domains reflect loop structures in the genome. Most often these loops are believed to be stabilized by the abundant proteins, with H-NS and FIS the most popular candidates (Dame 2005; Skoko, Yoo et al. 2006). However, the most dramatic barrier to supercoil diffusion, the rrnG operon, shows no sign of having a looped structure. If RNA polymerase has affinity for some cellular component like MreB , even rather weak interactions could organize the chromosome by selective aggregation of the highly transcribed regions (Kruse, Blagoev et al. 2006; Woldringh and Nanninga 2006; Fan, Tuncay et al. 2007).

Lastly, the only *in vivo* looping mechanism that we know of that works to block resolution in *Salmonella* involves the MatP/*matS* system discovered by Mercier et al. (Mercier, Petit et al. 2008). In *Salmonella*, the protein MatP binds to a cluster of 14 13 bp *matS* sties which occupy a 590 kb region surrounding the *dif* site. Resolution is impaired across *matS* sites when the MatP protein is expressed in vivo, and this is consistent with stable looping mechanism (Booker, and Higgins, unpublished). But as yet we have not found evidence consistent with any of the other known abundant proteins contributing to the 10 kb domain behavior in the large poorly transcribed regions. There could be other systems like the MatP/*matS* system that are yet undiscovered. But an alternative explanation is that the 10 kb diffusion rule in enteric bacteria is caused by random DNA interlinks between supercoiled strands rather than by a specific protein crosslinking mechanism. Topological interlocks known to occur in vivo include knots, catenane links between sisters, and hemicatenanes. In support of this notion, gyrase appears to be the most crucial enzyme for chromosome segregation. It was discovered in searches designed to find mutants that fail to segregate both the F plasmid and the *E. coli* chromosomes (Hiraga, Niki et al. 1989; Hiraga 1992). Moreover, recent results in yeast demonstrated that hemicatenanes are involved in stabilizing DNA interactions under several conditions (Lopes, Cotta-Ramusino et al. 2003; Robinson, Blood et al. 2007).

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Table 1. Strains used

STRAIN	GENOTYPE	PLASMID	SIZE
NH3868	LT2 *clpB* ::<*FRT* Res- Gn *lacIZYA* Res FRT>	pJBRES *30'*	8.6 KB
NH4028	LT2 *STM2655* :: <*FRT* Res- Gn *lacIZYA* Res FRT>	pJBRES *30'*	8.6 KB
NH4029	LT2 *rluD* ::<Gn-Res > STM2662 :: <Kn-Res >	pJBRES*c*I	13 KB
NH4030	LT2 *metN* :: <Gn-Res > *STM0257*:: <Kn-Res >	pJBRES*c*I	11.7 KB
NH4031	LT2 *rluD*:: <Gn-Res > STM2662 :: <Kn-Res>	pJBRES *30'*	13 KB
NH4032	LT2 yeaD :: <Gn-Res > STM0257 :: <Kn-Res >	pJBRES *30'*	11.7 KB
NH4057	LT2 STM0860::<TetR-A Res > *madA*::<Kn-Res >	pJBRES*c*I	13.7 KB
NH4058	LT2 STM3863:: <Gn-Res> *atpI*:: <*Kn-res*>	pJBRES*c*I	8.7 KB

All strains were derived from *S. Typhimurium* LT2 and were constructed for this work. Chromosomal disruption within or after a designated gene is indicated by :: and the relevant markers inserted into the chromosome are listed between brackets < >**.** All chromosomal replacements were made using lambda Red recombineering techniques (Yu, Ellis et al. 2000) or the yeast Flp site specific system (Huang, Wood et al. 1997).

Table 2.
Resolution efficiency measurements in the Salmonella Chromosome

Strain #	Deletion Interval	Log phase % Res	**TIF**	Stat. phase % Res	RIF Log phase % Res	RIF Stat. phase %Res
3868	*clpB <Res* Gn-lac Res >	75 ± 6	**0.7**	86 ± 3	N.D.	N.D.
4028	STM2655<*Res* Gn-lac Res	28 ± 3	**1.8**	74 ± 13	N.D.	N.D.
4029	*yfiH-kgtP*	1 ± 0.1	**88**	37 ± 4	85 ± 8	90 ± 6
4030	*metN-STM0257*	< 1	**90**	30 ± 4	82 ± 4	85 ± 4
4031	*yfiH-kgtP*	1 ± 0.7	**40**	51 ± 11	97 ±0.5	76 ± 1
4032	*metN-STM0257*	< 1	**45**	14 ± 10	27 ± 11	41 ± 7
4057	*yliJ- ybjC*	65	**1.2**	N.D.	N.D.	N.D.
4058	*atpC-atpI*	22	**4.5**	N.D.	N.D.	N.D.

Recombination reactions were carried out with cells grown in LB medium grown at 30° as described in the Materials and Methods. The NH strain numbers are given in the first column and the interval specifics and sizes are listed in the second column. Recombination efficiencies were measured in triplicate with 1 standard deviation shown for all intervals analyzed. The Transcription Impact Factor (TIF) for log phase cell is shown in Column 4. It represents the recombination efficiency from the standard curve (Fig. S1)divided by the efficiency measured experimentally for each interval. Recombination was also measured in stationary phase (column 5) and after addition of rifampicin (Rif) in either log or stationary phase (columns 6 and 7). N.D. indicates not determined.

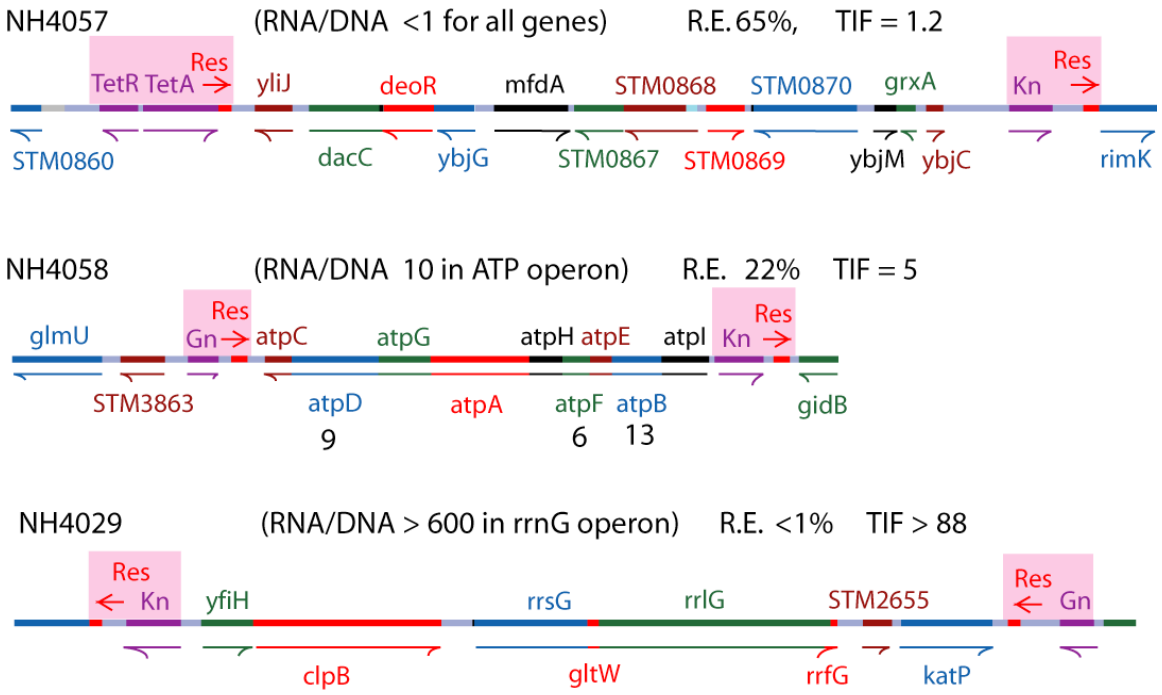

Fig. 1. Resolution frequencies in 3 chromosomal regions with different transcription activity. Regions with different transcription levels were tagged with modules carrying an antibiotic resistance gene and a 114 bp Res site (red arrow). The transcription level determined from microarray analysis is shown as a steady state ratio of RNA/DNA in the most highly expressed genes. The recombination efficiency (R.E.) is shown above each map along with the Transcription Impact Factor (TIF), which is described in the text.

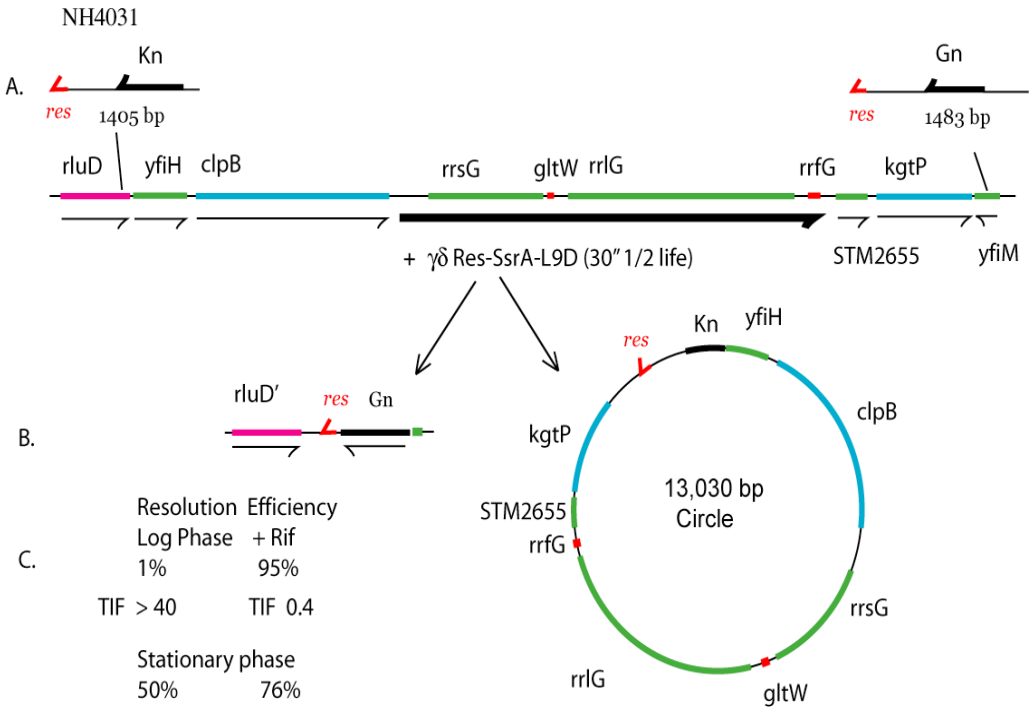

Fig. 2. Resolution efficiency measured across the ribosomal *rrnG* operon transcription domain. A. LT2 was modified by introducing a 1405 bp element containing a Kanamycin resistance gene and a Res site into the 3'end of the *rluD* gene upstream of the *rrsG* gene encoding 18S RNA of the bacterial ribosome. A 1483 bp element was then introduced into the *yfiM* gene downstream of the *rrnG* operon. B. Recombination efficiencies were measured by inducing γδ -Res-SsrA-L9D enzyme that produces a 30 min. enzyme. The reaction deletes the *rrnG* operon from the bacterial chromosome (left) as a 13,030 bp circle that is lost during subsequent growth. C. Recombination was monitored by loss of Kn resistance (see Table II). For log phase cells resolution was below 1%, but 30 min treatment with Rifampicin stimulated recombination 90-fold.

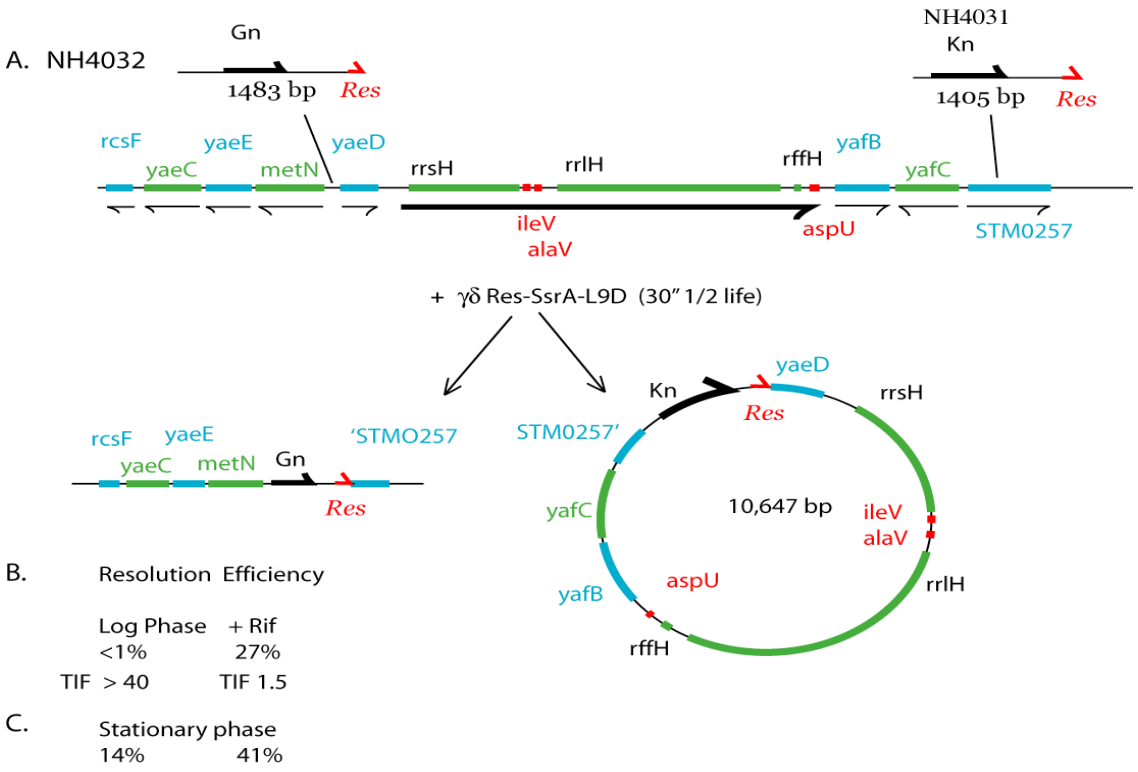

Fig. 3. Transcription domain of the ribosomal operon *rrnH*. A. LT2 was modified by introducing a Gn-Res element in the intergenic region between *metN* and *yaeD* genes upstream of the *rrsH* gene. A Kn-Res element was introduced into the STM0257 gene downstream of the *rrnH* operon. B. Recombination efficiency was measured by inducing expression of a 30 min. γδ resolvase. Resolution generates a deletion of the *rrnH* operon from the bacterial chromosome (left) and a 13,030 bp circular DNA that is lost during cell division (right). C. Recombination was monitored by loss of Kan resistance (see Table Y). For log phase cells resolution was low, but addition of Rif stimulated recombination 30-fold. In stationary cells, resolution efficiency increased from <1% to 14%, but rose to 40% recombination after Rif addition.

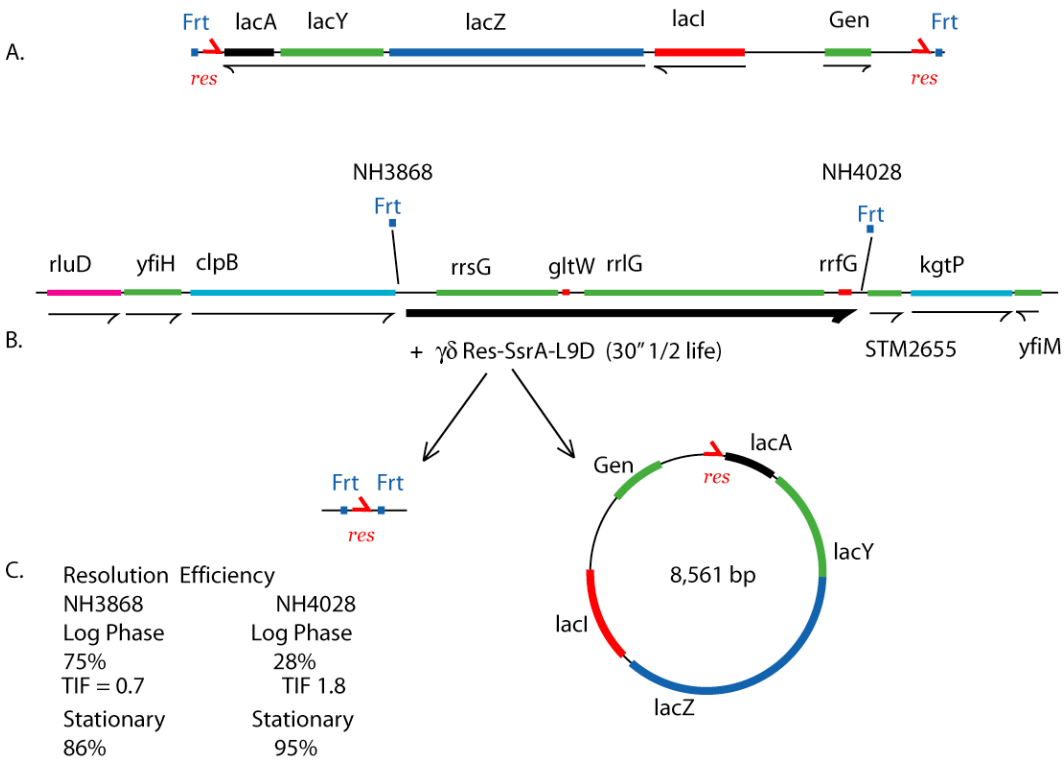

Fig. 4. Supercoil Diffusion Upstream and Downstream of the *Salmonella rrnG* RNA operon. A. Strains NH3868 and NH4028 were constructed by placing single Frt sites into the chromosome upstream and downstream of the *rrnG* operon. A module containing the complete *lacZ* operon, a Gn resistance gene, and flanking Res and Frt sites was inserted into the genome using Flp recombinases and selection for Gn resistance. B. Resolution efficiency was measured by inducing expression of the γδ Res-SsrA-L9D resolvase and scoring Lac^- colonies. C. The TIF upstream of the *rrnG* operon in log phase was 0.5. The TIF downstream with a Res site 268 bp beyond the *rrnG* terminator was 1.4.

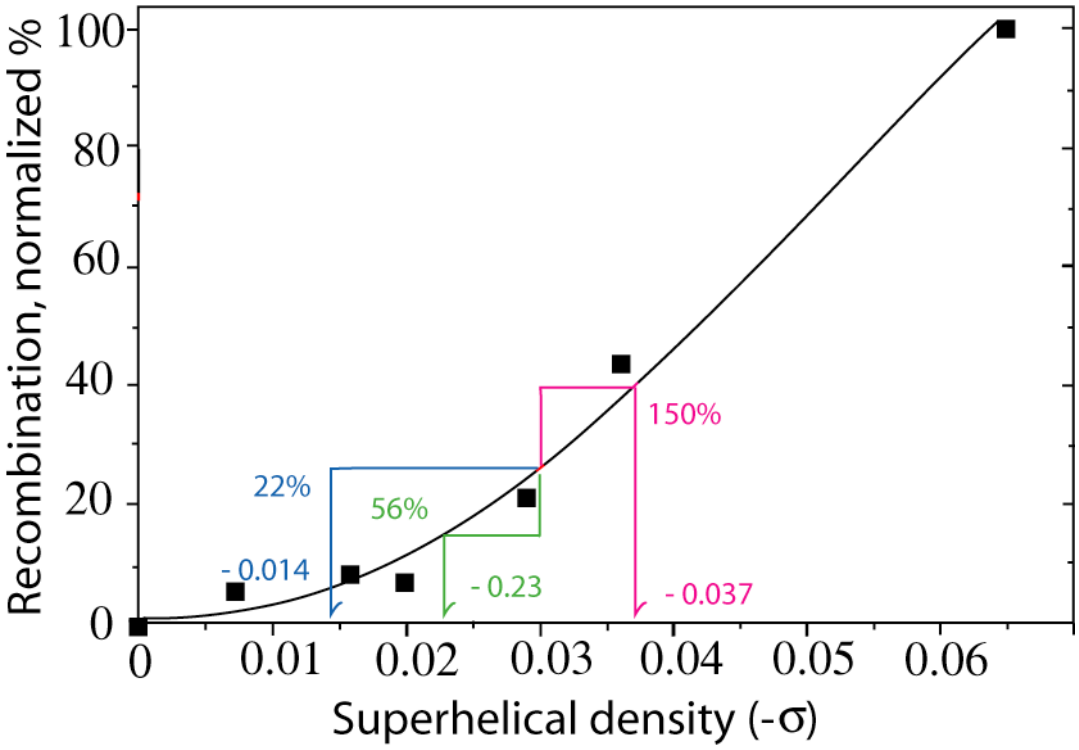

Fig. 5. Supercoil density dependence of site-specific recombination. The dependence of substrate superhelical density (σ) on the efficiency of recombination by Tn3 resolvase is plotted. Plasmids with different average superhelical densities were incubated with resolvase for 60 minutes at 37°C. The fraction of DNA that reacted was normalized to the highest value obtained (76% recombinant). Data from (Benjamin, Abola et al. 1996). Changes in diffusible supercoiling caused by transcription are interpreted for: 1) A 50% resolution increase upstream of *rrnG* promoter σ = - 0.037 (magenta), 2) a 56% resolution decrease downstream of the rrnG terminator σ = - 0.023 (green), and 3) a resolution decrease to 22% the expected frequency across the ATP operon σ = - 0.014 (blue).

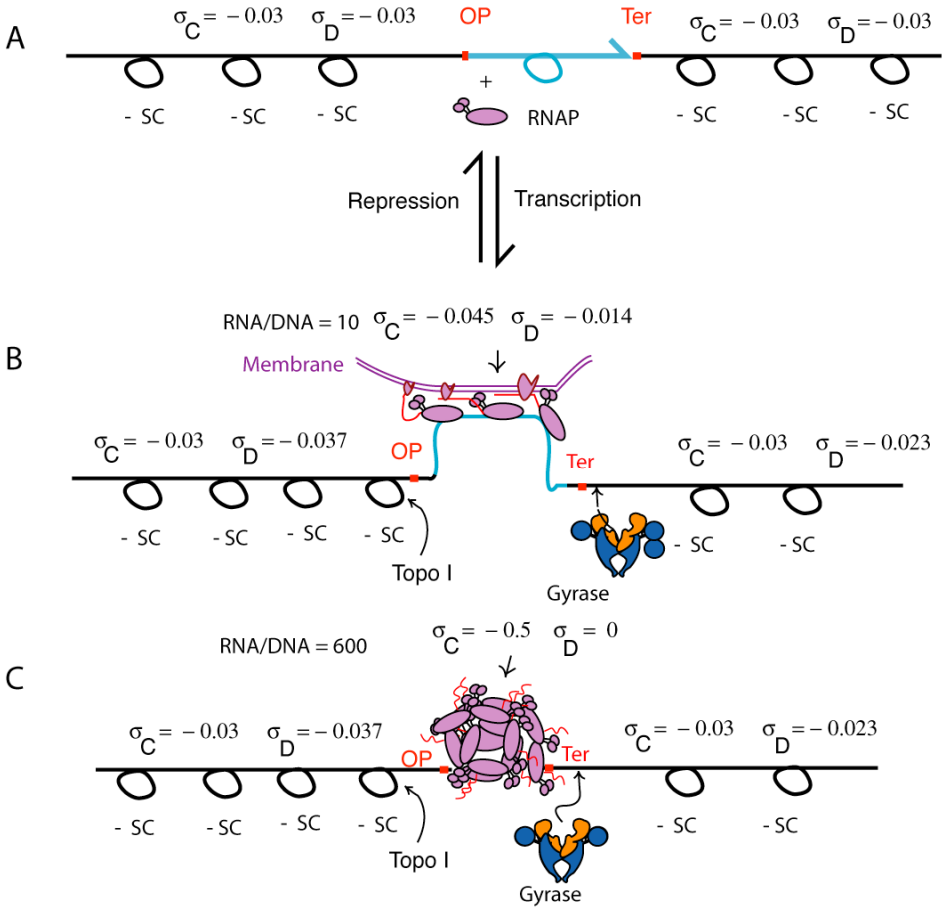

Fig. 6. Model of Transcription-Induced Supercoil Domains. A. An operon is shown with the operator/promoter region (OP) and the transcription terminator (Ter) in red. Under low transcription, supercoiling is organized into stochastic domains with the median values of constrained and diffusible supcoiling being equal (σ = - 0.060). B. Moderate transcription of the ATP operon (RNA/DNA ratio of 10), increases negative supercoil density upstream of the promoter to σ_D = - 0.37 so that σ = -0.067. Within the transcribed region, supercoiling constrained by RNAP increases to σ_C = - 0.045, whereas the effective diffusible supercoiling across the transcription zone drops to σ_D = - 0.014. Beyond the terminator the σ_D drops from -0.03 to -0.023 for a combined σ = - 0.052. C. At loci with very high transcription rates (*rrn* genes), supercoil density upstream and downstream are the same as in B. Constrained supercoiling rises to σ_C = - 0.50 and diffusible supercoiling in the transcribed region drops to 0. See text for details.

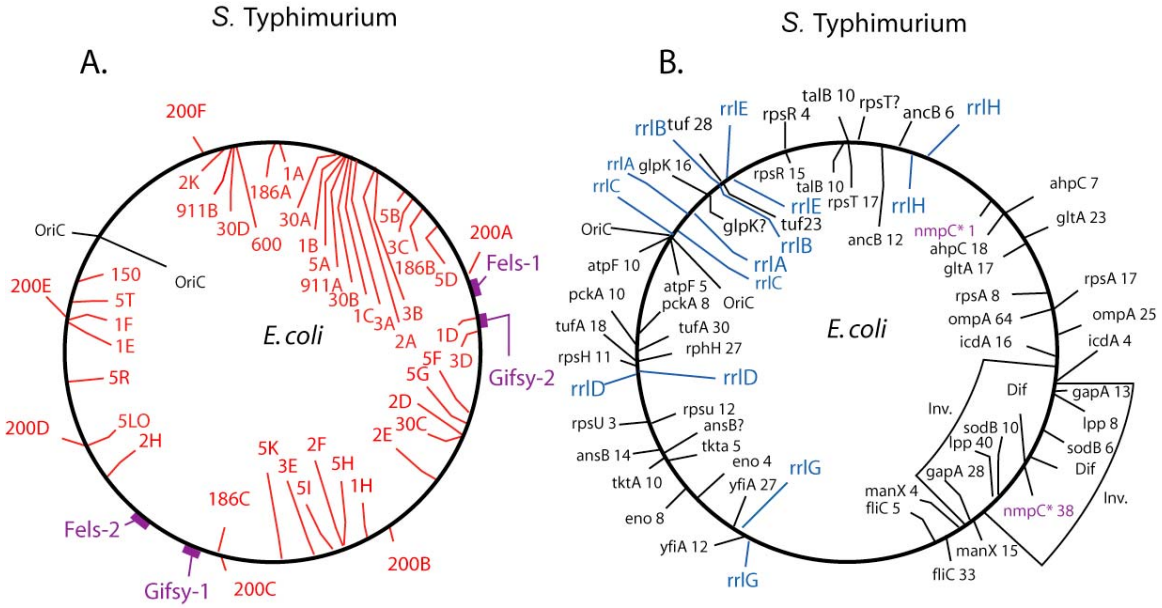

Fig. 7. Maps of *E. coli* (inside) and *S. typhimurium* (outside) showing the positions of autonomously mobile DNA elements (A) and highly transcribed genes (B) for chromosomes aligned at *oriC*. A. In *E. coli*, there are multiple copies of IS1 (1A-H), IS2 (2A-H), IS3 (3A-F), IS5 (5A-R), IS30 (30A-C), IS150 (150), IS186 (186A-C), IS600 (600), and IS911 (911A-B). Salmonella *Typhimurium* LT2 has only 6 copies of IS200 (200A-F) and four lysogenic and plaque-forming prophage, Fels-1, Gifsy-2, Gyfsy-1, and Fels-2, shown in purple in clockwise order. By contrast, the transcription map is very similar. The only major differences in gene order involve a symmetric inversion around the Dif site and the appearance of the *nmpC* gene (purple), which is highly transcribed only in Salmonella, at the terminus near the Dif site. In each map the, location of the top 27 protein encoding genes (black) and the 7 ribosomal RNA operons (blue) are shown. The steady state RNA/DNA level for the protein encoding genes from microarray experiments conducted on cells growing in exponential phase on LB medium are shown in B.

Table S1. Oligonucleotides used for strain construction

Precursor of Frt site in NH3868
clpB Frt Kn Frt For
CACAAATTTGAATAAAACGGGCCCTGACGGGCTCGTTTTT/ TGTAGGCTGGAGCTGCTTCG

clpB Frt Kn Frt Rev
ACATTTGAACCAAAAGCCTATTTTCGCCTGGTTTTTAAAC/ CATATGAATATCCTCCTTAG

Precursor of Frt site in NH4028
STM2655Frt Kn Frt F
TGAACTATCCTTTACACGTGCTTATATAAGCAGTGAGGAT/ CATATGAATATCCTCCTTAG

STM2655Frt Kn Frt R
AAGTCCTGCTGGTAGTTAAAAGGTTTGATAGCCAATGAAA/ TGTAGGCTGGAGCTGCTTCG

NH4029 and NH4031
rluD Kn Res For
TCGGCGCGCATCGCATCGATAAGGTCCACCATATCTTGTGGAAT/ AGCAGGTAGCTTGCAGTGGGC

rluD Kn Res Rev
ACCATCCTGTATCGGGTATCGAAATGGAGTGGCACGCGCCG/ CGGAATTAGCTTGCATGCCTG

yfiM Gn Res For
GCCATCTGGCTAACGACCACTGGAGCGGTCAGGATAAAG/ GACCCAGTTGACATAAGCCTG

yfiM Gn Res Rev
ACCGGCTGCCGATAGCATCGCTGATGCCATAAAATGCTG/ GCGGAATTAGCTTGCATGCCTG

NH4030 and NH 4032
yaeD Gn Res For
TTTCGTCCTAATACGGTCAACGCCGTCAACAGAATG/ GACCCAGTTGACATAAGCCTG

yaeD Gn Res Rev
TTATAGCTCCTGAGTAAAGCAAGTGGCGCTAGTATCGCAT/ GCGGAATTAGCTTGCATGCCTG

STM0257 Kn Res F
CACTGTTAGGCGTGATTGCGCTGATGAGCAGTCAATTGCT/ AGCAGGTAGCTTGCAGTGGGC

STM0257 Kn Res R
CAGCAACAGACGAGGATGAGTCAGTACTTTCACCTGGTCA/ CGGAATTAGCTTGCATGCCTG

NH4057
STM0860 Tc-For
CCCGGAAAAAGGCAAAAATTATGGCTGGCCGCTGGCCAC/TATGATTCCCTTTGTCAACAGC

STM0860 Tc-Rev
CGACAATTTCACCTTTGGCTTCCGGCACTTTCAGACCACT/TTAAGGTGGATACACATCTTGTC

mdaA Kn Res For
GCCTGCCGAGCTTGTGGTTCATGAGAACCAGTATCAACC/TGTAGGCTGGAGCTGCTTCG

mdaA Kn Res Rev
CAGATAATACTCAGCCAACTGCTCGTCATAGCGCGCCAGCAG/TTAAGGTGGATACACATCTTGTC

NH4058
Gn Res For
CGCCTTCAGCCGCTTGTTTTTGTCGTTGCTAGTTTATCG/ACCCAGTTGACATAAGCCTG

Gn Res Rev
CCGGCTTGAAAGCATAAAACCAGTCTGATTGCGGACTGG/CGGAATTAGCTTGCATGCCTG

gidB Kn Res For
TCATGCTTACGCAATTCATTTACGTGTCAATTCATGCCT/AGCAGGTAGCTTGCAGTGGGC

gidB Kn Res Rev
CGACAGGTATAACGCATTTCAGCGTTGTTACATTTAACTC/GGAATTAGCTTGCATGCCTG

The 5' end of each primer matches target DNA (35~45 bases) and the 3' end matches a DNA cassette (~20 bases) to prime PCR amplification. The "/" in each primer sequence is the junction between the target and cassette sequences, which are underlined.

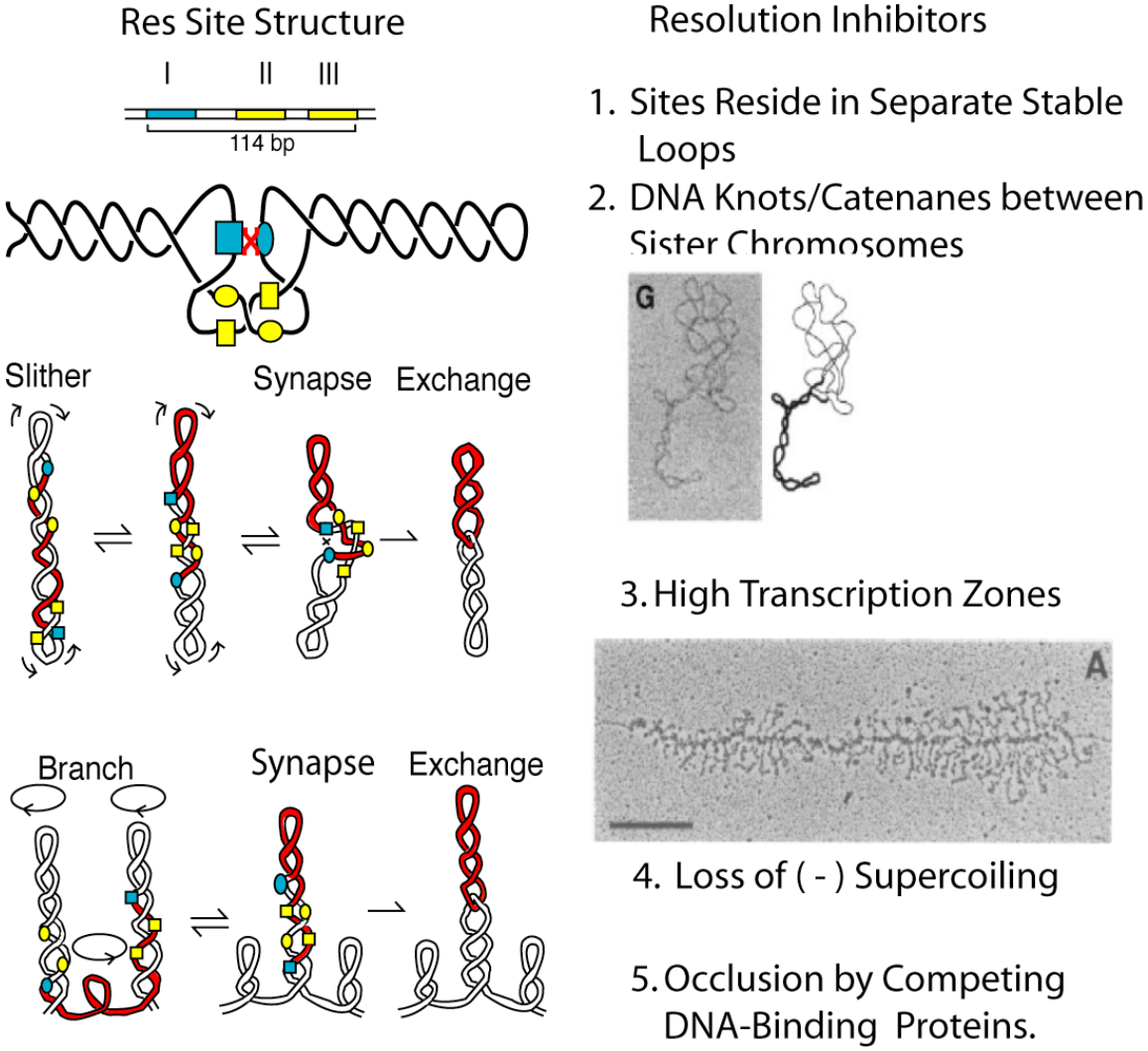

Fig. S1 The γδ resolvase mechanism, and conditions that block synaptic pairing. A 114 bp γδ Res site includes 3 sub-sites labeled I (blue), II, & III (yellow). Each sub-site binds a resolvase dimer, shown as ovals or boxes on different directly repeated sites. Recombination requires a highly supercoiled synapse with 3 negative crossing nodes. Enzymes at Res I (blue) can recombine while the other two sites (II & III) stabilize the complex during strand transfer. Two DNA movements bring sites into synapse: slithering (middle) or branching (bottom). Conditions that block synaptic pairing for recombination include: 1, sites separated in different loop domains. 2. Pre-catenane tangling of strands during DNA replication (Peter, Ullsperger et al. 1998). 3. Genes undergoing high transcription (French and Miller 1989). 4. Loss of (-) supercoiling. 5. Occlusion of Res sites by other DNA binding proteins.

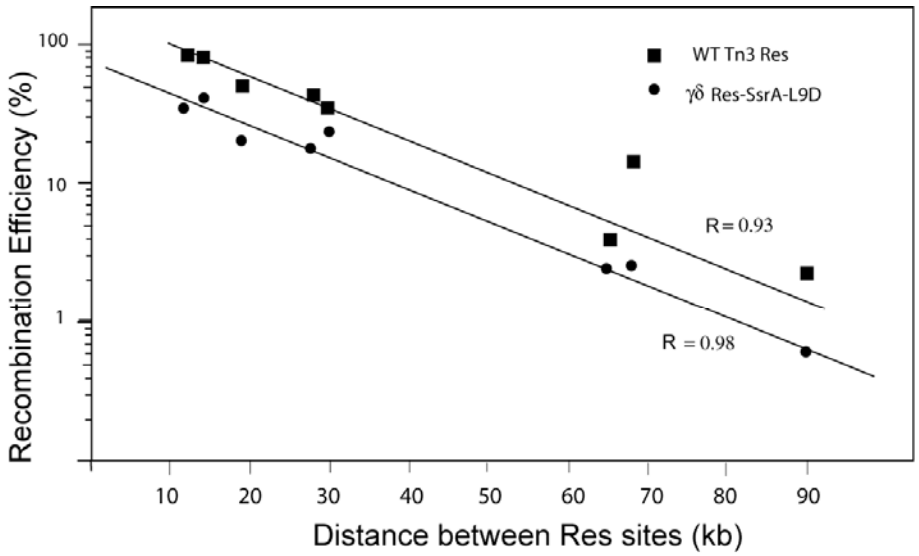

Fig. S2. Dependence of recombination efficiency on distance for WT Tn3-Res and γδ -Res-SsrA-L9D resolvases.

SUMMARY

Many factors affect DNA supercoiling. These include: transcription, replication, recombination, repair, and the growth rate of the cell (Cozzarelli 1980). Topoisomerases act upon DNA when global or local supercoiling levels are altered. The effect of transcription on topology has been widely studied, yet still lacks a unanimous model. One reason is because of a lack of *in vivo* studies demonstrating the changes of chromosome structure with transcription. Most DNA topology studies have been conducted on small plasmids that are isolated and assayed for changes in topoisomer distribution (Wu et al, 1988, Cook, D et al 1992). My work used γδ-resolution assays to analyze chromosome supercoiling, which drives the movement between two Res sites for a deletion recombination reaction (Deng, Stein et al. 2004; Pang, Chen et al. 2005).

For the first time, DNA topology has been measured in the ribosomal regions of the chromosome. Our results show very low (≤ 1%) resolution efficiency of the rRNA operons. The decrease in resolution indicates a lack of diffusible supercoiling. Resolution efficiency was restored by addition of Rifampicin. We also developed a system to quantify supercoiling changes upstream and downstream of a ribosomal operon. Contrary to the predictions of the popular twin domain model, only a moderate decrease in negative supercoiling was found downstream of transcription. We postulate RNAP confines highly transcribed regions and serves as a barrier to supercoil diffusion. The formation of the domain barriers in the *Salmonella* chromosome reduces changes in supercoil diffusion. These types of domain barriers are directly related to transcriptional activity. My results, together with Deng's initial report (Deng, Stein et al. 2004), suggest

highly transcribed genes are responsible for about 10% of the constrained supercoiling on the chromosome. But overall, highly transcribed genes have strong global effects on supercoiling.

It is now important to consider how DNA replication is affected by highly transcribed regions. From our findings, we propose that highly transcribed genes are confined by RNA polymerase. However, this confinement would present a dilemma for the replication machinery. DNA Pol III replicates at 1000 nt/sec while RNAP synthesizes 40-45 nt/sec. For viability, all cells must have evolved with the difference in the rate of synthesis (Brewer 1988). If RNAP confines the transcribing region, how will DNA become accessible for replication? Three scenarios are possible to solve this problem. First, DNA polymerase III waits for transcription to end and then resumes. Another option would be DNA Pol III displaces RNAP without interrupting DNA synthesis. And lastly, the two machinery could work together to solve the problem. DNA polymerase slows down and tracks behind RNAP. Liu et al reported that upon collision with RNAP, DNA Pol III would simply pass the ternary complex without disturbance (Liu, Wong et al. 1993; Liu, Wong et al. 1994). A problem with this model is that the bacterial rRNA operons can have up to 150 RNAP molecules during fastidious growth (Mueller, Oebbecke et al. 1977; Schneider, Ross et al. 2003). The space crowding would not only pose a problem for DNA Pol III, but also for completion of rRNA transcripts for ribosome biogenesis.

A current model by Pomerantz & O'Donell suggests that both co-directional and head-on collisions displace the RNAP ternary complex (Pomerantz and O'Donnell 2008; Pomerantz and O'Donnell 2010). It is conceivable that these collisions provide a pausing

for replication machinery to slow down. When DNA polymerase slows down, the mutation rate also decreases and preserves highly transcribed genes (Parkhomchuk, Amstislavskiy et al. 2009).

The head-on collisions appear most damaging to the cell due to longer pausing by the replication apparatus (Liu and Alberts 1995; Mirkin and Mirkin 2005). This effect may be the reason why over 70% of *E.coli's* essential genes are transcribed in the same direction as replication and accumulates fewer mutations on the leading strand (Tillier and Collins 2000; Rocha 2002; Rocha and Danchin 2003; Srivatsan, Tehranchi et al. 2010). Studies indicate a strong directional bias of rRNA genes transcription with replication. Upon inversion of rRNA genes, there is an increase in genome instability in *Bacillus* (Srivatsan, Tehranchi et al. 2010). In *S.cerevisiae*, Chr XII has 150 rDNA repeats that play a role in genome integrity. Reduction in rDNA copy number causes a loss in chromosome organization leading to cell death (Kobayashi 2006; Ide, Miyazaki et al. 2010). It is likely that RNAP may have a role in this instability as it blocks condensin binding and disturbs the normal architecture of the cell (Freeman, Aragon-Alcaide et al. 2000). This instability may be due to an increase in head-on collisions with DNA pol III or the alteration of native topology. The top 100 active genes for *E.coli* and *Salmonella* are conserved for function, activity, and location (Manna, Porwollik et al. 2007). It should be considered that highly transcribed genes are located around the chromosome, thereby limiting the amount of collisions with DNA Pol III and preserving genes. The gene pattern can offer a logical explanation of how high transcription affects chromosome structure or provides genomic stability (Higgins, Booker et al. 2010)

Transcription changes the structure of the chromosome probably by constraining DNA supercoils. In highly transcribed regions, RNAP binding accounts for the inhibition of two Res sites to form a synaptosome. We have outlined a model in which transcription-induced domain barriers are a result of RNAP binding. Botchan *et al* first described a model of how RNAP affected DNA structure by DNA unwinding with a preference for negatively supercoiled templates (Botchan, Wang et al. 1973). Later Liu and Wang detailed a model of transcription-coupled DNA supercoiling ahead and behind the transcribing RNA polymerase in plasmids. This model has been widely studied and accepted as standard for topology in highly transcribed regions. Our data suggests a moderate decrease in negative supercoiling downstream of highly transcribed genes instead of a complete modification to positive supercoiling. Transcription-induced supercoiling is a transient and local process that is isolated from other processes. Highly transcribed genes appear to be under selection for activity and function, as the top 100 genes in *E. coli* and *Salmonella* are conserved. Highly transcribed genesa are also insulated from mobile elements, such as phage Mu (a highly replicative transposon) (Manna, Breier et al. 2004). Mobile/transposable elements and laterally acquired genes can also be regulated by DNA-binding proteins like IHF or H-NS (Haniford 2006; Fass and Groisman 2009). HN-S and IHF both bind DNA and alter DNA structure (Ellenberger and Landy 1997; Becker, Kahn et al. 2007) possibly by inducing positive supercoiling (Dorman 2009).

As more information about DNA twists, kinks, and knots are revealed, there are more intriguing questions that arise. How did *Salmonella* and *E. coli* evolve with 100% conservation of the top 100 genes yet at a different supercoiling level? If supercoiling is

critical for cell survival, you would expect the same supercoiling levels in two closely related organisms. Will there be a difference in upstream and downstream regions in topoisomerase mutants (*gyrA, gyrB, topA*)? And lastly, can we use our data as a quantitative model for upstream and downstream regions in all highly transcribed genes?

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