

Chapter 2

Measuring In Vivo Supercoil Dynamics and Transcription Elongation Rates in Bacterial Chromosomes

N. Patrick Higgins

Abstract

DNA gyrase is the only topoisomerase that can catalytically introduce negative supercoils into covalently closed DNA. The enzyme plays a critical role in many phases of DNA biochemistry. There are only a few methods that allow one to measure supercoiling in chromosomal DNA and analyze the role of gyrase in transcription and its interaction with the other three bacterial topoisomerases. Here, we provide molecular tools for measuring supercoil density in the chromosome and for connecting the dots between transcription and DNA topology.

Key words Supercoiling, Transcription, Resolvase, Gyrase, Salmonella, Chromosome

1 Introduction

Site-specific recombination assays based on the $\gamma\delta$ resolution system have provided information about nucleoid structure and quantitative estimates of supercoil density in living bacterial cells [1]. The $\gamma\delta$ resolvase system requires supercoiled substrates, as do many transposons and other site-specific recombination systems. There are three requirements for the $\gamma\delta$ system. First, two 114 bp $\gamma\delta$ Res sites must be present in the DNA substrate, arranged as direct repeats. Second, sufficient resolvase must be present to saturate all chromosomal Res sites. A dimer of resolvase must bind to Res sub-sites I, II, and III. Third, the DNA substrate must be negatively supercoiled. Supercoiling is needed to drive formation of a 3-node synapse (Fig. 1a), which precisely aligns two directly repeated Res I sites for DNA breakage and reunion cycles [2, 3]. Only resolvase dimers bound to Res sub-site I can catalyze strand exchanges. Once the supercoiled resolvase synapse is formed, the complex deletes the intervening circular DNA segment and rejoins the substrate using no external energy and leaving a single Res site scar. Formation of a three-node

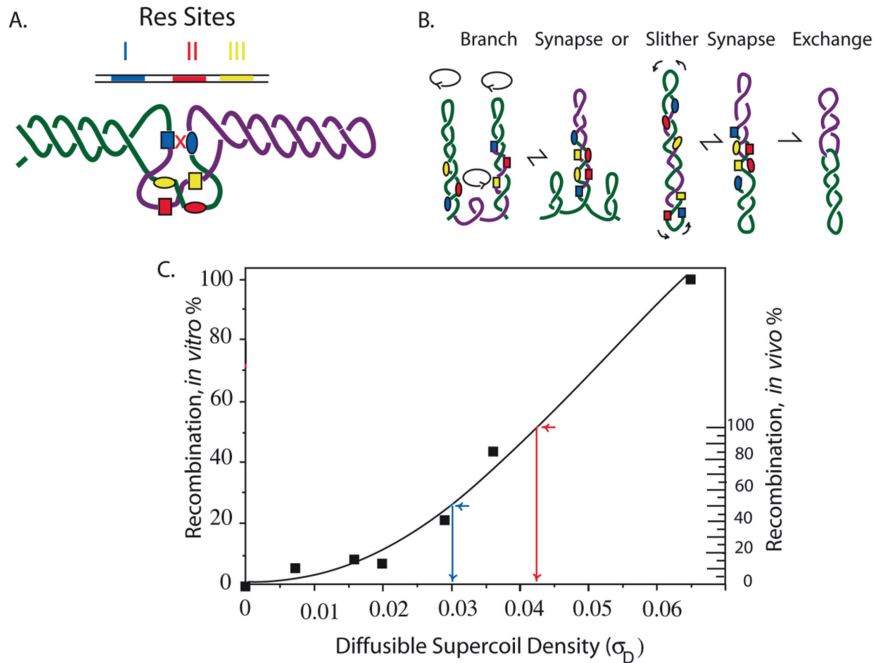


Fig. 1 Supercoil dynamics of the autocatalytic reaction of the $\gamma\delta$ resolution system. (a) The topological structure required for resolution juxtaposes two blue I sub-sites that become cleaved and rejoined by resolvase within the three-supercoil synapse. (b) Dynamic DNA movements of branching and slithering promote the three-node tangle (a) and release two singly catenated supercoiled circles as end products at *right*. (c) The negative supercoil dependence is shown for *in vitro* (*left*) and *in vivo* (*right*) resolution reactions

synapse involves movements called slithering and branching (Fig. 1b). Branching rearranges the interwound DNA structure by starting new loops that grow and recede laterally. Slithering is a reptilian mode of writhe that displaces two opposing strands along an axis of interwound loops. When branching and slithering are unobstructed, resolution efficiency increases with the level of diffusible negative supercoiling *in vitro* [4] and *in vivo* [1, 5] for distances of 10 kb in log phase cells and up to 100 kb in stationary phase cells [6]. This *in vitro/in vivo* relationship provides the basis for estimating the *in vivo* supercoil density from resolution frequencies [7].

Initially, the $\gamma\delta$ resolvase system for *in vivo* chromosome analysis had three of four characteristics that were essential for measuring accurate supercoil differences over a 100-fold range of sensitivity: 1—Tight repression of resolvase synthesis that generates a low background in resolution assays; 2—A temperature-sensitive repressor that dissociates from DNA at 42° and promotes rapid high-level resolvase expression; 3—A cI^{857} repressor that refolds rapidly to reestablish repression when cultures are shifted back to 30°.

The fourth property required for an optimal system became clear when cells induced for resolvase expression were tested for the ability to make deletions in a plasmid substrate that was introduced into cells by electroporation. WT resolvase catalyzed resolution reactions for several hours after induction followed by growth at 30°. The enzyme was immortal and only became depleted by cell division dilution. The significance of this result was that we could not detect barriers that form and disappear over the time frame of a cell generation, much less measure the time frame of gene expression [6]. Colony assays carried out with a WT resolvase were blind to many of the most interesting temporal supercoil changes that occur in a specific genome region.

Robert Stein solved this problem by making 5 min, 15 min, and 30 min time-restricted derivatives of $\gamma\delta$ resolvase. He first added a C-terminal extension to WT resolvase, which is the 11-amino acid sequence that the SsrA system adds to translation fragments that result from a stalled ribosome [8]. This sequence normally releases the stalled ribosome by making ribosomes hop to a special RNA that has a translation stop codon at the end of an 11 amino acid sequence. Proteins with the 11 amino acid tag are efficiently targeted for degradation by the ClpXP protease. Stein's mutant resolvase with C-terminal extension of Ala-Ala-Asn-Asp-Glu-Asn-Tyr-Ala-Leu-Ala-Ala had a 5 min half-life in both *E. coli* and *Salmonella*. He made a second mutant resolvase with the change in the SsrA tag from A8-D, which produced a 15 min resolvase. A third change of L9-D yielded a 30 min resolvase. The 30-min resolvase provided a method to analyze the consequences of transcription throughout a chromosome by transiently halting most transcription for 30 min with rifampicin.

$\gamma\delta$ resolvase experiments have provided critical data for five discoveries in bacterial chromosome physiology. (1) Resolution studies using the 5-, 15-, and 30-min derivatives provided the compelling evidence that bacterial chromosomes are organized into about 400 domains (@ 10 kb), which are stochastic in both length and boundary positions during normal cell growth [1, 5, 9]. (2) Experiments using the 5-min resolvase showed that gene transcription creates a supercoil diffusion barrier and that temporal appearance and disappearance of boundaries coincide precisely with transcription [10, 11]. (3) Resolution studies at the *rrnG* operon proved that the model of transcription proposed by Liu and Wang [12] generates a gradient with excess (–) supercoils upstream and depleted levels of (–) supercoils in a 10 kb domain downstream of ribosomal RNA operons in WT cells [7, 13]. Experiments with the 30 min resolvase demonstrated that the loss of (–) supercoiling generated by RNA polymerase downstream of a transcription terminator must match the rate of (–) supercoiling by gyrase to maintain supercoil density throughout a chromosome [7]. In this paper, we describe the strategy, basic modules, and

methods that can be extended to other bacteria for systematic analyses of chromosome mechanics [7, 14].

2 Materials

2.1 Res Modules

Four selectable drug modules containing Res sites are illustrated in Fig. 2. Modules in A, B, and C include IS10 terminal ends [15]. These modules can be introduced in to bacterial chromosomes using *Tn10* transposition reactions (1) Alternatively, they can be introduced using homology-dependent λ red recombineering [16]. Recombineering of modules allows one to perform chromosome walks along the bacterial genomes to search for supercoil barriers [10, 11]. A chromosome walk can also identify essential genes that block recovery of recombinants because the deletion is lethal. With long inexpensive DNA synthetic blocks and Gibson assembly [17], one can easily create new Res modules for many purposes.

The fourth element in Fig. 2 (D) is an 8998 bp supercoil sensor that can be used to measure supercoil density of WT or mutant strains at a specific point and to determine the speed of RNA elongation at that same chromosomal location [18]. The sensor has an entire lac operon (*lacIZYA*) plus a selectable Gentamycin resistance gene (Gn) flanked by directly repeated res sites. Directly repeated FRT sites make the ends of the element. the supercoil sensor can be inserted into any chromosomal locus that has a single FRT site using the yeast 2 μ FLP recombinase [18, 19]. The resolution efficiency of the sensor increases in proportion to local negative supercoil density, and the in vivo range of the sensor spans

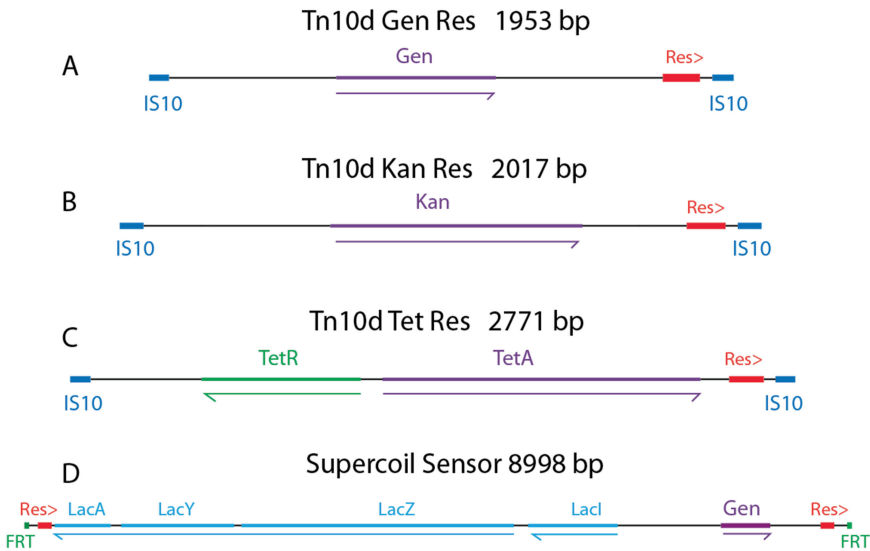


Fig. 2 Modules for chromosome tagging with a single 114 bp $\gamma\delta$ Res site (a–c) or the supercoil sensor which has two Res sites (d)

2 orders of magnitude from <1% to 100% resolution (Fig. 1). This sensor is superior to supercoil-dependent promoter modules and supercoil-dependent psoralen crosslinking methods that have a 2 or 3-fold range of detection [20–23].

2.2 *Resolvase Expression Plasmids*

Four plasmids that encode time-restricted or WT $\gamma\delta$ Resolvase under λ cI^{857} are: pJB Res cI^{857} -SsrA (5'') Cm; pJB Res cI^{857} -SsrA-A8D (15'') Cm; pJB Res cI^{857} -SsrA-L9D. (30 ") Cm; and pJB RES cI^{857} (> 2 h).

2.3 *Growth Chambers, Culture Media, and Biochemical Solutions*

Most of our experiments have been done with LB medium, which results in very rapid growth. Supercoiling activity at certain chromosomal positions changes dramatically when cells are grown in minimal medium (*see Note 1*).

1. LB Medium: Dissolve 5 g NaCl, 10 g Tryptone, 5 g yeast extract in 1 L of deionized H₂O. Add 15 G Difco Agar to 1 L of LB in a 2 L flask for plates. Autoclave liquid and solid LB for 20–30 min and pour plates when the agar is cool enough to be handled without a glove (about 45 °C).
2. AB minimal medium: $5 \times A = 2$ g (NH₄)₂SO₄; 6 g Na₂HPO₄; 3 g KH₂PO₄; 3 g NaCl dissolved in 200 ml H₂O and autoclaved for 30 min. $B = 797$ ml H₂O autoclaved for 30 min. Add 1 ml 0.1 M CaCl₂ (sterile); 1 ml 1.0 M MgCl₂ (sterile); and 1 ml 0.003 M FeCl₃ (sterile). Mix the 200 ml of $5 \times A$ with 800 ml of B . Add a sterile carbon source of 0.05% Glucose, 0.2% Alanine, or 0.2% Succinate plus any strain-required amino acids and vitamins to support growth rate doubling times of 90 min, 125 min, and 300 min, respectively.
3. Antibiotics Add antibiotics to LB liquid and solid medium at a concentration of 20 µg/ml Tetracycline HCl; 50 µg/ml Kanamycin SO₄; 20 µg/ml Chloramphenicol; and 10 µg/ml Gentamycin. In liquid and solid minimal media, add drugs at concentrations of 10 µg/ml Tetracycline HCl; 125 µg/ml Kanamycin SO₄; 5 µg/ml Chloramphenicol; and 10 µg/ml Gentamycin.
4. Culture and spectrometer equipment and supplies. For culture growth and easy measuring of cell concentration, 125 ml side arm flasks and a Klett spectrophotometer permit rapid monitoring of culture growth rates without removing flask lids or caps. However, one can monitor culture density using any type of culture flask or tube and read the A₆₅₀ in a standard spectrophotometer with glass or plastic cuvettes or a nanodrop instrument. An OD of 0.4 is a reasonable target for mid-log stage experiments. For experiments that demand short time sampling (transcription elongation), 50 ml media bottles with reusable polypropylene screw tops removed during fast manipulation are ideal.

5. Fisher blue cap snap on culture tubes (17 × 100 mm) are ideal for growing overnight cultures and β -Gal assays.
6. Two shaking incubators are required, preferably near each other. Set one at 30° and set a shaking water bath incubator at 42°.

3 Methods

$\gamma\delta$ Resolution Assays.

3.1 Drug Module Deletion Protocol

1. Streak out strains to be tested on LB plates containing the drugs that select for Res module(s) plus chloramphenicol, which selects for stable maintenance of the appropriate Resolvase expression plasmid.
2. For each strain to be tested, inoculate three blue cap tubes containing 2 ml of the same medium in step one. Incubate overnight at 30°.
3. For each test culture, preload the appropriate number of flasks with 5–10 ml of LB + Cm medium. Inoculate each flask by adding a portion of the fresh overnight culture at a ratio of 1:100 (50 μ l overnight culture into 5 ml of LB + Cm).
4. Incubate cultures in a 30° shaking incubator and monitor the cell growth by measuring culture OD₆₀₀. For strains like WT *E. coli* or *Salmonella*, it takes 4–5 h for cultures to reach mid log phase, which equates to an OD₆₀₀ of 0.4–0.5.
5. When cells reach the desired OD, take a sample of cells for an un-induced control and place each flask in the 42° incubator for 10 min.
6. Return each temperature-induced flask to the 30° incubator and incubate for at least 2 h to allow recombinant chromosome to segregate into daughter cells. Alternatively, let the flasks incubate overnight, since the ratio of recombinants is stable after the Resolvase enzyme is degraded.
7. Spread cells on plates after making appropriate dilutions to get plates with 100–300 colonies/plate and incubate at 30°.
8. Use one sterile toothpick to patch a single colony on two plates that contain the antibiotic for each drug module. Patch 100 colonies in this way and calculate the deletion efficiency.

3.2 Lac Deletion Protocol

Steps 1–6 are the same.

7. Plate diluted cells on Lac indicator plates. Resolution efficiency is determined by color. On XGal, whites are recombinant and blues are non-recombinants. On MacConkey the pattern is white and

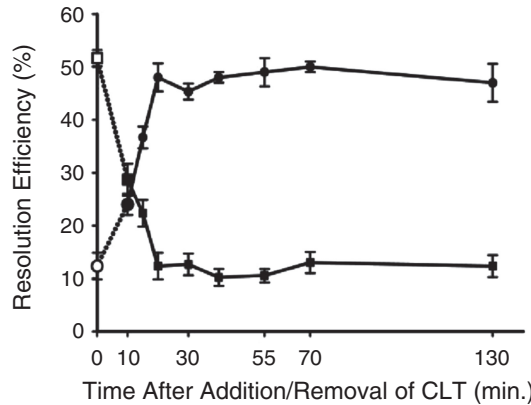


Fig. 3 Find supercoil barriers caused by gene expression [10]. An example of the lac method for measuring deletion efficiencies is shown above. The *Salmonella* strain NH3492 has a 12 kD chromosomal segment flanked by a Gn Res module on the left and a Lac Res module on the right in addition to the 5'' resolvase plasmid. *Tn10* repressor, TetR, regulates expression of β -Gal. The *lacZ* gene becomes deleted by $\gamma\delta$ resolution). In the experiment shown in Fig. 3, three cultures (*open squares*) were initiated by diluting a fresh overnight culture 100-fold into LB + Cm medium and grown to 50 Klett in the absence of the inducer chlorotetracycline (CLT). Each culture had a $\gamma\delta$ resolution efficiency of 50%. CLT was added to the cultures (filled squares) and thermo-induced at 5 min intervals. Resolution rates dropped fivefold to 10%. The reciprocal experiment was carried out by growing three cultures to 50 Klett in LB + CLT. Assays of resolution in these cells occurred at a rate near 10% (*open circles*). CLT was washed out of the cells and incubation continued at 30° in LB for 130 min (*filled circles*). The resolution efficiency rose quickly to 50% and remained at this level for 2 h. This experiment was the first demonstration that transcription creates its own barrier to supercoil diffusion in a bacterial chromosome

red, but colonies can be counted in 20 h whereas it takes 2–3 days to develop good color at 30° on XGal (*see Note 2*).

An example of the lac method for measuring deletion efficiencies is shown above (Fig. 3) (*see Notes 3 and 4*).

3.3 Measure Supercoil Dynamics with the Supercoil Sensor (Fig. 2d)

The supercoil sensor provides a way to monitor topological dynamics at multiple positions in a chromosome. The sensor enabled us to demonstrate that the Liu and Wang model of twin domains of supercoiling [12] causes a gradient of supercoiling with an excess upstream and a deficit downstream of highly expressed genes even in WT cells [13]. The 30'' resolvase (pJB Res ϵI^{857} -SsrA-L9D Cm) is ideal for this experiment. One variation in the standard experiment showed that the enzymatic rates of RNA polymerase elongation and gyrase supercoiling are linked [7].

To test the hypothesis that transcription can produce a flat chromosome without diffusible supercoils, a simple modification

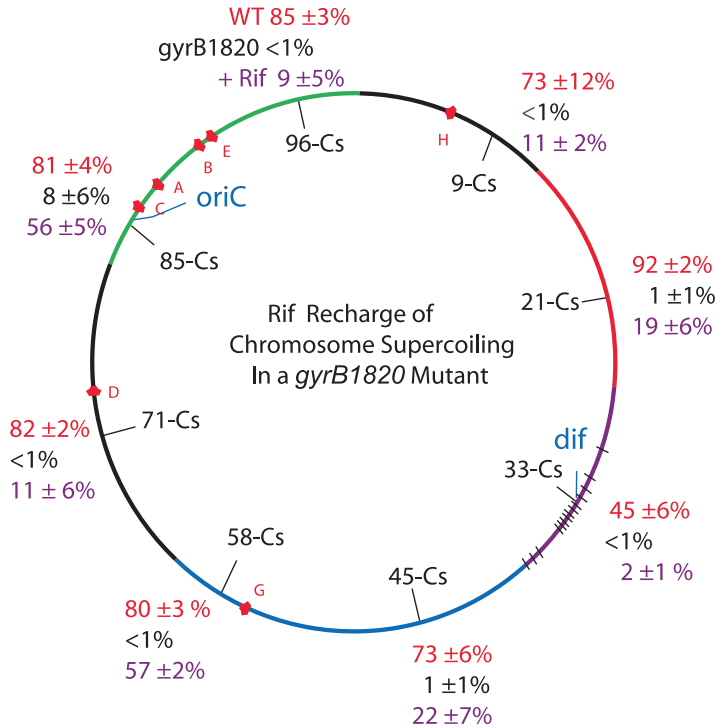


Fig. 4 Chromosome supercoiling linked to RNA transcription and gyrase

after the 10 min incubation at 42° provides the experimental answer (Fig. 4). The test is to split the culture and add rifampicin to one half during the post-induction 30 min incubation at 30°. Rifampicin blocks initiation of RNA synthesis but not elongation and termination. If transcription is responsible for running the chromosome flat, supercoiling might restore resolution during the 30 min half-life of resolvase. At 30 min, rifampicin was removed from cells by centrifugation and resuspension in LB + Cm. Then both cultures were handled with the normal protocol. For the *gyrB1820* strain in Fig. 5, 60% of the resolution efficiency was restored by the Rif treatment. Although not all parts of the genome recovered equally, this result proves that gyrase catalytic rates and RNA polymerase rates must match to maintain WT supercoil levels in a *Salmonella* chromosome.

3.4 Measuring Transcription Elongation

The observation that transcription can run the chromosome flat in cells with a slow gyrase leads to a question. Does a mutant gyrase affect the rate of transcription?

To measure transcription elongation rates, the Lac operons at each location provides a way to test transcription elongation rates at the same sites that were used to measure supercoiling.

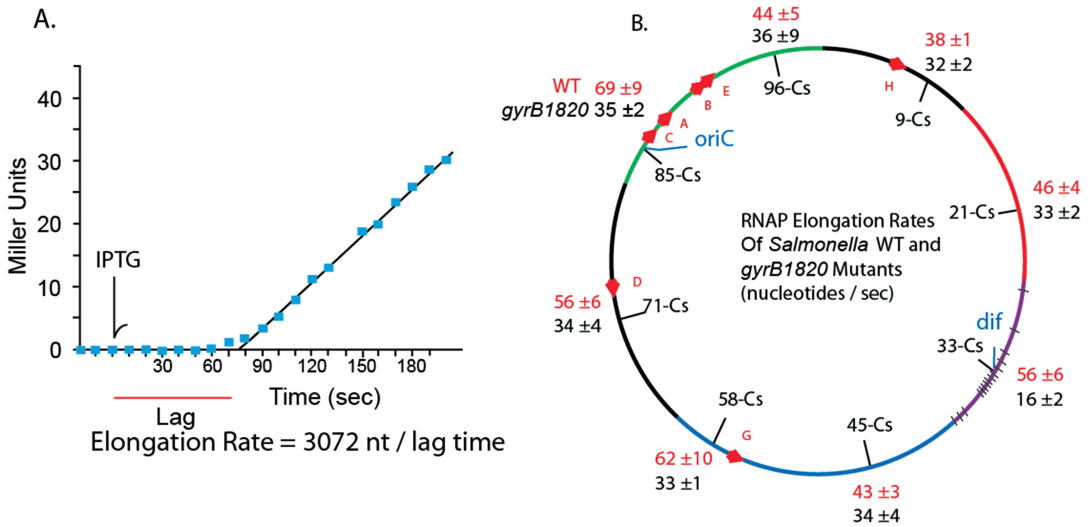


Fig. 5 Transcription elongation rates measured in WT and *gyrB1820* strains of *Salmonella*

3.5 Protocol to Test RNA Elongation Rates, Adapted from Vogel [24]

1. Inoculate a flask with 20 ml of minimal AB medium supplemented with selective drugs for the modules + Cm and 0.02% glucose with 1 ml of an overnight culture.
2. Measure cell density and carry out the assay when the OD₆₀₀ is between 0.2 and 0.4. Record the OD₆₀₀. It is important to use an open culture vessel that allows rapid pipetting of 500 µl samples from a culture every 10 s for 4 min.
3. Prepare enough blue cap tubes for the number of samples needed plus 10% extras for mistakes. Add 500 µl of ice-cold ZS-Cm buffer = 60 mM Na₂HPO₄; 40 mM NaH₂PO₄ 10 mM KCl; 1 mM MgSO₄; 50 mM β-Mercaptoethanol; 0.01% SDS; and 200 µg/ml chloramphenicol to block further protein synthesis at 4°. Keep these tubes in an ice-cold bath.
4. Start by recording the culture OD₆₀₀ and take three 500 µl samples at 10 s intervals to establish the background subtraction. Place these tubes in a separate ice bath.
5. Add IPTG to the remaining culture in a shaking water bath at a final concentration of 1.5 mM.
6. Remove 500 µl aliquots at 10 s intervals, placing each tube in the second ice bath for the next 4 min. Once all samples are in the ice bath, add 100 µl of chloroform, vortex, and incubate the tubes for 2 min at 30°.
7. Start timed B-Gal assays on each set by adding 200 µl of ONPG 4 mg/ml. Place the tubes in a stationary 30° incubator for 1.5–4 h to allow development of appropriate levels of color. Note the start time.

8. Stop each reaction by adding 500 μl of 1 M Na_2CO_3 and mix each tube. Centrifuge tubes for 5 min and then pipette the top 750 μl to a disposable plastic cuvette (*see Note 5*).
9. Measure OD_{420} and OD_{550} values of each reaction. Calculate standard units as described by [25].
10. Calculate the standard LacZ Miller units [25] and divide 3072 nucleotides (nt) by the lag time in sec to get the elongation rate.

When transcription elongation rates were measured at 8 positions in WT *Salmonella* (Fig. 5 red numbers), the WT average for all eight sites was 55 nr/s. However, there was significant variation in rates at different sites. For the supercoil sensor at Cs 85, which is in a hyper-supercoil zone because the sensor lies just behind the highly transcribed ATP operon, the transcription rate was 69 nt/s. At Cs 9 the transcription rate was about half that at 38 nt/s. This suggests that supercoiling gradients in the WT chromosome influence RNA polymerase. Data from the flat chromosome gyrB1820 proves the case (Fig. 5 black numbers). Transcription rates at 7 positions fell to 32 nt/s. The sensor at Cs 33 is very near the Dif site, and at this position transcription falls to 16 nt/s. This position may become positively supercoiled due to convergent replication forks.

4 Notes

1. Use fresh medium made within 1 month of an experiment. Complex media like LB stored on lab a shelf develops toxic-free radicals that result from exposure to fluorescent light. Diluting cells into such medium changes cellular metabolism, and cells can become induced for the RecA SOS response that can cleave λ repressor.
2. Most strains show ratios of recombinant to non-recombinant cells that remain stable after the resolvase is degraded. Therefore, resolution rates measured 2 h after induction agree with rates measured the following day in stationary culture.
3. Most strains give similar efficiencies from 1 h at 30° until they reach mid log phase. As culture densities become high, growth rates slow as cells enter stationary phase. Resolution efficiency increases as transcription rates decrease going into stationary phase.
4. When experiments are carried out with *E. coli*, the high supercoil density of this organism (15% higher than *Salmonella* Typhimurium) results in average dilution efficiencies of the 9 kD sensor of >80% throughout most of the genome. To lower this rate it is advisable to use the 15'' or 5'' resolvase and construct supercoil sensors longer (15–20 kD) than the one used in *Salmonella*.

5. For the LacZ-transcription elongation assay, disposable 1.5 ml plastic cuvettes for measuring a large number of color changes are useful. Fisher supplies these cuvettes in convenient boxes that are easy to handle (Cat no. 14-955-127 includes 500 cuvettes).

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