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Investigating the Effects of DNA Packaging on Natural Mutation Frequency

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DNA gyrase causes negative supercoiling in DNA and plays an important role in DNA packaging. We hypothesized that DNA treated with a gyrase inhibitor would be less supercoiled, and thus more accessible to repair enzymes. This would lead to a lower natural mutation rate in treated cells compared to untreated cells. To test this hypothesis, *Escherichia coli* DH5α pUC19 containing a plasmid which carried ampicillin resistance was treated with a gyrase inhibitor and its plasmid analyzed for evidence of elimination of supercoiling. The antibiotic novobiocin was used as a gyrase inhibitor and ampicillin resistance was used as an indication of mutation, and hence formed the basis of calculating mutation rates. The methods of experimentation were: (a) Dilution susceptibility test to determine what concentration of novobiocin to use; (b) Gram stain analysis to confirm the effects of novobiocin on cell size; and (c) Luria-Delbruck fluctuation assay to determine the natural mutation rates of treated and untreated cells. The results obtained were not as expected. Treated cells had a higher mutation rate than untreated cells. This result is contrary to our hypothesis. Inhibition of DNA gyrase might actually inhibit, not enhance the accessibility of DNA to repair enzymes. Alternatively, these results could be affected by other factors not taken into account, such as the possibility of inhibition of transcription in unwound DNA, and thus the inhibition of the synthesis of repair enzymes, or the concentrations of novobiocin used might have been too high.

The packaging of bacterial DNA is the result of the reversible binding of histone-like proteins (HU, IHF and FIS) and the introduction of negative supercoils by topoisomerases such as gyrases. Supercoiling is an important and intrinsic aspect of DNA-tertiary structure and cellular DNA conformations are highly regulated by each cell (24). Bacterial circular duplex DNA is negatively supercoiled, which is important for the packing of long DNA molecules into highly restricted spaces in the nucleus. The normal biological functioning of DNA occurs only if it is in the proper topological state (3). The negatively supercoiled DNA also prepares the DNA for processes requiring the separation of strands such as DNA replication, repair and transcription (3).

DNA gyrases are composed of heterotypic tetramers (A2B2). Subunits A (~100kD) and subunits B (~90kD) are encoded by the *gyrA* and *gyrB* genes, respectively (17, 23). Subunit B is involved in ATP hydrolysis and energy transduction, while subunit A plays a role in DNA breakage and reunion (17, 23). The DNA gyrase of *E. coli* facilitates the initiation step by its negative supercoiling of DNA that promotes the unpairing of strands in front of a helicase (23). A variety of inhibitors have been found to interfere with specific enzymatic reactions of DNA gyrases, rendering it inactive (8, 9, 15). There are two major classes of gyrase inhibitors: quinolones and coumarins. Other gyrase inhibitors include ribosomally synthesized proteinaceous poisons-like microcin B17, CcdB and cyclic peptide cyclothialidines (8, 9, 15).

Coumarins and cyclothialidines are two classes of natural products isolated from *Streptomyces* that are known to inhibit enzymatic activity of DNA gyrase by interfering with ATP hydrolysis (14). Both classes of molecules compete with ATP for binding to GyrB, and thus inhibit the ATPase activity of the enzyme (6, 14). The best-known antibiotic from the coumarin class is novobiocin which is an excellent inhibitor of the enzyme with $K_d \approx 10$ nM (14).

Since mutations result from changes in DNA sequence that evade the cellular repair mechanisms, enhancing the access of repair enzymes to a damaged lesion or mispairing should decrease mutation rates as these enzymes function by interacting directly with DNA (4). To test our hypothesis, we used the gyrase inhibitor, novobiocin (1, 10) to block and/or reduce the degree of DNA packaging (supercoiling) in an attempt to make it more accessible to repair and less prone to mutation. We investigated the effects of genome packaging on the interaction of repair enzymes by measuring the natural mutation rate of prokaryotic chromosomal DNA. The *E. coli* DH5α, which can develop ampicillin resistance due to mutations in penicillin binding proteins or changes in membrane permeability, was the focus of our experiment. Our work consisted of the following: (a) determining the concentration of

antibiotic that is both non-lethal and inhibitory; (b) confirming that the inhibitory effects of the antibiotics are due to gyrase inhibition that creates changes in DNA supercoiling; and (c) comparing the natural mutation rates between treated and untreated cells. We expected to observe lower mutation rates in treated cells relative to the controls.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study were *Escherichia coli* DH5α and *E. coli* DH5α pUC19. These strains were obtained from Dr. William Ramey, University of British Columbia, Department of Microbiology and Immunology (16). Both strains were incubated at 37°C in a water bath (Metabolyte Water Bath Shaker, New Brunswick Scientific) shaking at 100 rpm for 12-16 hrs and were then placed at 4°C to serve as stock culture. DH5α was routinely grown in Luria-Bertani (LB) broth (18) or M9-glucose minimal media (18). DH5α pUC19 was grown in LB or M9-glucose media supplemented with ampicillin (50 ug/ml). DH5α carries the following mutations: *ton*A, *end*A1, *rec*A1, *hsd*R, *lacZ*ΔM15, *mcr*A, and *hsd*RMS. The pUC19 plasmid carries an ampicillin resistance gene and the α-fragment for β-galactosidase.

Dilution susceptibility test. For each of the two strains, 11 tubes containing LB were serially diluted with novobiocin with a starting concentration of 10 mg/ml. Novobiocin concentrations tested were 10 000, 5 000, 2 500, 1 250, 625, 313, 166, 78, 39, 20 and 0 μg/ml. Tubes were then inoculated with roughly 4000 cells of either DH5α or DH5α pUC19 and incubated in a shaking water bath at 37°C for 12-16 hrs. After the incubation period, cell number was assessed through optical density measurement at 660 nm (Biochrom Ultraspec 3000).

Plasmid supercoiling analysis. Twelve 5ml tubes of DH5α pUC19 were grown for 12 hrs in a shaking water bath at 37°C in LB containing 50 μg/ml ampicillin at varying concentrations of novobiocin (250, 200, 150, 100, 50, 25, 10, 5, 1, 0.1, 0.01, 0 μg/ml). Plasmids were extracted from the overnight cultures using the Concert™ Rapid Plasmid Miniprep protocol (Life Technologies).

Plasmids were run on two 0.9% agarose (BioRad Laboratories) 1X TAE gels (18) with the λ *Hin*dIII DNA ladder (Invitrogen); one gel contained 0.02 μg/ml ethidium bromide (Sigma), while the other gel was stained with 1 μg/ml ethidium bromide for 20 min after it was run. Each gel ran for 90 min at 120 V in a gel box (Horizon 11-14 Horizontal Gel Electrophoresis System).

Luria-Delbruck fluctuation assay. The MSS algorithm (19) was used to calculate the most probable *m* based on the observed data by maximizing the joint Luria-Delbrück distribution of mutant numbers:

$$\hat{m} = \max_{m} \prod_{i=1}^{35} f(r_i \mid m), \text{ where } f(r"m) = p_r = \frac{m}{r} \sum_{i=0}^{r-1} \frac{p_i}{(r-j+1)} \text{ with } p_0 = e^{-m}$$

This is currently the most computationally efficient method and is valid for all values of *m*. Also, unlike other methods, MSS allows the calculation of standard deviations and applications of common statistical analyses. TNTC is given the value of 300 in later analyses since for most $m \leq 16$, mutant counts over 300 contribute only a minute portion of a Poisson distribution. Detailed sample computations and calculations are included in Appendix A. Mutation rate, μ, is determined by dividing *m* by the number of cells at risk, which approximately equals to the final number of cells in a culture, assuming all replicates reached saturation and the initial inoculation is less than 1/1000 of the final cell number. A single replicate is defined as allowing 1 ml of minimal medium or LB with an initial inoculation of 4,000 cells to grow to saturation ($\approx 2 \times 10^9$ cells).

Two fluctuation assays were performed. For the first fluctuation assay, 35 tubes containing 1 ml M9-glucose and 35 tubes containing 1 ml M9-glucose-novobiocin (50 μg/ml) were inoculated with DH5α and incubated in a shaking water bath at 37°C for 72 hours. After the incubation period, cells were spun down at 13,000 rpm in a micro-centrifuge (Eppendorf Centrifuge 5415C, Brinkman Instruments), and resuspended in 100 μL supernatant. Each 100 μL sample was then plated onto M9-glucose plates containing 25 μg/ml ampicillin. Plates were incubated at 37°C for 48 hrs. Colonies arising on the plates were then enumerated.

For the second fluctuation assay, 35 tubes containing 1 ml LB and 35 tubes containing 1 ml LB-novobiocin (78 μg/ml) were inoculated with DH5α and incubated in a shaking water bath at 37°C for 24 hrs. After the incubation period, cells were spun down at 13,000 rpm in a micro-centrifuge, and resuspended in 100 μL supernatant. Each 100 μL sample was then plated onto LB plates containing 40 μg/ml ampicillin. Plates were incubated at 37°C for 48 hours. Colonies arising on the plates were then enumerated.

Gram stain analysis. DH5α and DH5α pUC19 grown for 12-16 hours at 37°C with and without novobiocin were examined microscopically using the Gram stain procedure. Observations at 400X and 1000X were taken using a Kyowa UNILUX-12 light microscope.

RESULTS

Quantitative effects of novobiocin on DH5α and DH5α pUC19. Novobiocin is an antibiotic which halts cell replication through the inhibition of DNA gyrase. To determine the concentration at which cell replication was inhibited by novobiocin but not killed, a dilution susceptibility test was performed (Fig. 1). As novobiocin concentration increased, the OD_{660} of overnight cultures steadily decreases. A sharp drop of OD between novobiocin concentrations of 166 μg/ml and 313 μg/ml was observed.

Qualitative effects of novobiocin on DH5α and DH5α pUC19. To determine if novobiocin indeed had an effect on the supercoiling of DNA, plasmids were isolated from DH5α pUC19 grown overnight in the presence of varying concentrations of antibiotic. As ethidium bromide induces supercoiling (16), the plasmids were run on two gels, one including ethidium bromide (Fig. 2a) and one stained with ethidium bromide after electrophoresis (Fig. 2b), to observe the differences in banding patterns. Fig. 2a shows that as novobiocin concentration decreased, a large band at roughly 2.5 kb increases in intensity. Fig. 2b shows that as novobiocin concentration increases, a large band at roughly 1.8 kb increases in intensity, as do two faint bands at roughly 2.5 and 2.8 kb. The untreated sample (lane

13) is supercoiled and as expected it travels the furthest down the gel. This shows that novobiocin does indeed have an effect on the supercoiling of DNA.

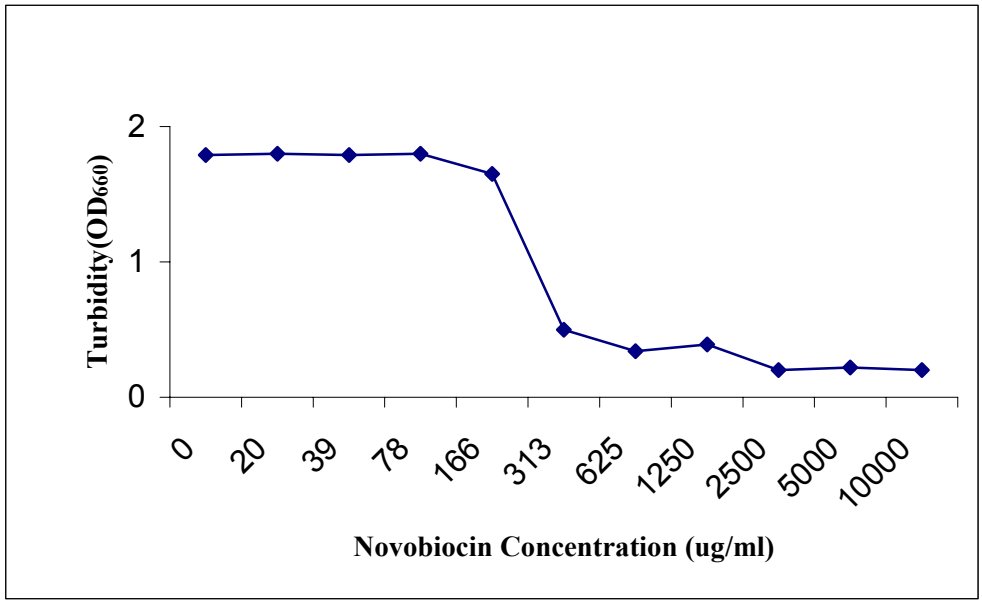

Fig. 1. DH5α Dilution Susceptibility Test. DH5α cells were treated with varying concentrations of novobiocin and incubated on a shaking water bath at 37°C overnight for 12-16 hours.

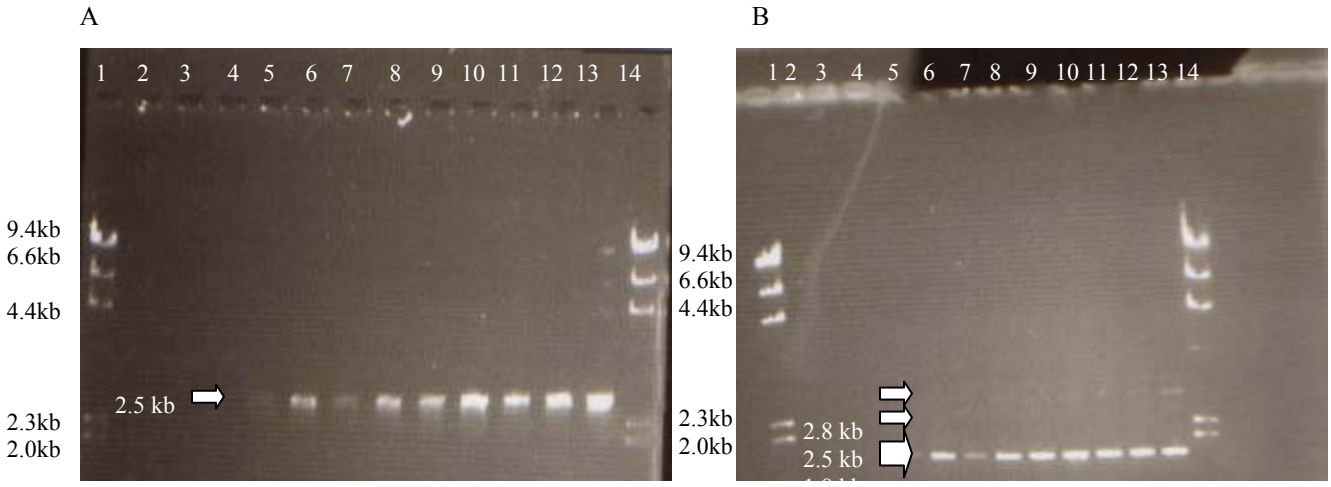

Fig. 2. Comparison of plasmids from DH5α pUC19 cells treated with varying concentrations of novobiocin. Lane 1, λ Hind III standard; lane 2, 250 µg/ml novobiocin treatment; lane 3, 200 µg/ml novobiocin treatment; lane 4, 150 µg/ml novobiocin treatment; lane 5, 100 µg/ml novobiocin treatment; lane 6, 50 µg/ml novobiocin treatment; lane 7, 25 µg/ml novobiocin treatment; lane 8, 10 µg/ml novobiocin treatment; lane 9, 5 µg/ml novobiocin treatment; lane 10, 1 µg/ml novobiocin treatment; lane 11, 0.1 µg/ml novobiocin treatment; lane 12, 0.01 µg/ml novobiocin treatment; lane 13, 10L 0 µg/ml novobiocin treatment; Lane 14, λ Hind III standard. (A) Ethidium bromide was added to the gel prior to electrophoresis. (B) Gel stained with ethidium bromide post-electrophoresis.

Because novobiocin inhibits DNA gyrase and not cellular metabolism, cells treated with this antibiotic should be able to grow, but not divide since the initiation of cell division depends on completion of DNA synthesis. This

should produce a population of abnormally large cells. This phenomenon was analyzed through the Gram stain procedure in both DH5α and DH5α pUC19 (Fig. 3). Gram stains were prepared from cultures incubated with novobiocin at 0 μg/ml, 50 μg/ml, 78 μg/ml, and 166 μg/ml. Cells treated with novobiocin at concentrations below 50 μg/ml did not show an increase in cell size relative to untreated cells.

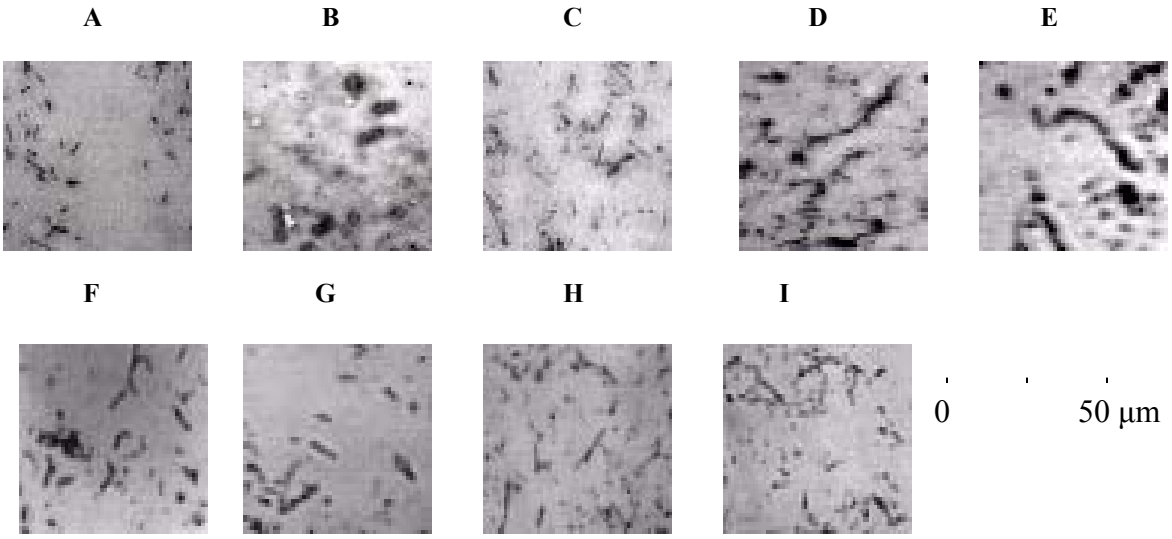

Fig. 3. Gram stain analysis of DH5α and DH5α pUC19 treated with varying concentrations of novobiocin. (A) untreated DH5α. (B) DH5α treated with 39 μg/ml novobiocin. (C) DH5α treated with 50 μg/ml novobiocin. (D) DH5α treated with 79 μg/ml novobiocin. (E) DH5α treated with 156 μg/ml novobiocin. (F) untreated DH5α pUC19. (G) DH5α pUC19 treated with 39 μg/ml novobiocin. (H) DH5α pUC19 treated with 78 μg/ml novobiocin. (I) DH5α pUC19 treated with 156 μg/ml novobiocin.

Mutational analysis. The number of mutation events (*m*) that gave rise to ampicillin resistant *E. coli* DH5α cells during population growth is estimated by the MSS maximum-likelihood method for two sets of parallel cultures. Each set contained both control and treatment cultures with 35 replicates each and the growth conditions were different for each set. The number of mutants observed on selective ampicillin plates and colony count on non-selective plates are summarized in Table 1, where TNTC represents counts greater than 300.

Table 2 states the results of the fluctuation assay analysis. The MSS method allowed 2 decimal points of precision and total cell counts are assumed to be exact. Cells treated with 78 μg/ml novobiocin and grown on LB showed a higher mutation rate than cells not treated with novobiocin and exposed to identical conditions. Cells treated with 50 μg/ml novobiocin and grown on M9-glucose showed similar results which were a higher mutation rate than cells not treated with novobiocin.

DISCUSSION

Based on the dilution susceptibility tests on the effects of novobiocin on DH5α, cultures treated with 50 μg/ml and 78 μg/ml novobiocin have approximately 86% and 99% less cells than the control, respectively. Microscopic observations based on Gram stains and the supercoiling assay confirm that the inhibitory effect of novobiocin at the above concentrations is due to interference with DNA replication, not DH5α metabolism. In the supercoiling assay, a supercoiled DNA molecule is more compact than a relaxed DNA molecule of the same length. Supercoiled DNA moves faster than relaxed DNA molecules when electrophoresed. Hence they can be separated by agarose gel electrophoresis or by equilibrium centrifugation. Figures 2a and 2b show this difference in migration of plasmids treated with different concentrations of novobiocin. The untreated sample (lane 13) is supercoiled and, as expected, travels the furthest down the gel. This shows that novobiocin indeed has an effect on the supercoiling of DNA.

At both 50 μg/ml and 78 μg/ml of novobiocin, the turbidity readings did not differ significantly from the control (Fig. 1), yet their average cell size was nearly 2X that of the untreated cells (Fig. 3). This may suggest that treated

cultures had approximately 2X less cells than the untreated culture, since in order to maintain a constant turbidity measurement, the percent increase in cell size as result of novobiocin treatment should be accompanied by an equal decrease in cell concentration. These two antibiotic concentrations were chosen for the fluctuation assay since they allowed sufficient population growth for a quantitative investigation of natural mutation rates.

Table 1. Plate Counts - Ampicillin Resistant Mutants

Plate #	Number of Amp^R Colonies [a]	
	Without Novo.	With Novo.
1	TNTC	4
2	TNTC	28
3	TNTC	2
4	TNTC	9
5	TNTC	3
6	TNTC	1
7	TNTC	132
8	TNTC	81
9	TNTC	2
10	TNTC	4
11	TNTC	TNTC
12	TNTC	TNTC
13	TNTC	12
14	TNTC	14
15	TNTC	187
16	TNTC	TNTC
17	TNTC	TNTC
18	TNTC	TNTC
19	0	0
20	0	TNTC
21	0	TNTC
22	TNTC	TNTC
23	TNTC	10
24	TNTC	10
25	TNTC	TNTC
26	TNTC	214
27	TNTC	TNTC
28	TNTC	TNTC
29	TNTC	251
30	TNTC	5
31	TNTC	94
32	TNTC	41
33	TNTC	31
34	TNTC	TNTC
35	TNTC	104

Plate #	Number of Amp^R Colonies [b]	
	Without Novo.	With Novo.
1	10	0
2	0	0
3	0	TNTC
4	71	0
5	0	0
6	11	0
7	0	0
8	0	0
9	0	TNTC
10	0	0
11	8	TNTC
12	10	0
13	0	0
14	5	0
15	0	0
16	0	TNTC
17	5	0
18	0	TNTC
19	15	0
20	0	0
21	0	TNTC
22	5	TNTC
23	12	0
24	8	TNTC
25	0	TNTC
26	0	TNTC
27	20	TNTC
28	5	TNTC
29	0	TNTC
30	21	0
31	0	TNTC
32	0	TNTC
33	14	0
34	0	0
35	3	0

[a] DH5α plated on LB-novobiocin (78 µg/ml) and ampicillin (40 µg/ml)
[b] DH5α plated on M9-novobiocin (50 µg/ml) and ampicillin (25 µg/ml)

Both sets of fluctuation assays (Table 2) confirm that novobiocin has an inhibitory effect on cell growth based on total cell counts. Contrary to our expectations, cultures treated with 50 µg/ml of novobiocin had approximately 100X the mutation rate of the controls, while cultures treated with 78 µg /ml novobiocin had approximately 2X the mutation rate of the control. This suggests that the inhibition of DNA gyrases interferes with, rather than facilitates,

the accessibility of DNA to repair enzymes. One possible hypothesis is that DNA gyrases underwind bacterial DNA and open up the double helix, exposing its nitrogen bases to repair enzymes (13). The resulting changes in supercoiling are compensated by the coiling/packing of the chromosome that may not decrease the diffusion of repair enzymes to damaged DNA lesions. Therefore an increase in mutation rate will not be observed in antibiotic treated cells. Also, when DNA is unwound, transcription may be inhibited. This blockage of transcription could explain the increased mutation rate in our treated cells since repair enzymes may not be present or functional.

Table 2. Fluctuation Assay Results For *E. coli* DH5α

Set #		Concentration of Mutants (mutants/ml)			Final Cell Concentration (10^8 cells/ml)	Mutation Rate (10^{-8})		
		LCL[c]	Est.	UCL[d]		LCL	Est.	UCL
1[a]	**Control**	12.91	15.32	18.21	3.48	3.78	4.40	5.23
1	**Novobiocin (78 µg/ml)**	3.65	5.26	7.58	0.5	7.3	10.05	15.2
2[b]	**Control**	0.58	0.89	1.36	> 3000	< 0.0023	< 0.0030	< 0.0045
2	**Novobiocin (50 µg/ml)**	0.49	0.77	1.21	2.69	0.18	0.29	0.45

[a]Cells grown in LB and plated on LB with 40 µg/ml ampicillin
[b]Cells grown in M9-glucose and plated on M9-glucose with 25 µg/ml ampicillin.
[c]Lower 95% confidence limit.
[d]Upper 95% confidence limit

In Table 1, the cell density of cultures treated with 50 µg/ml novobiocin was approximately 100× higher than the control and the cell density of cultures treated with 78 µg/ml novobiocin was 7× higher than the control. As discussed earlier, we only expected a 2× decrease in cell number in novobiocin treated cultures compared to the untreated ones based on the dilution susceptibility tests. This deviation in viable plate count ratio suggests that some cells in the novobiocin treated cultures were probably novobiocin-resistant. Also, the actual cell numbers determined by viable plate counts were lower than those obtained from turbidity readings (data not shown). This could be explained by the fact that novobiocin treated cells may fail to divide and may not be able to grow into visible colonies. Accordingly, the number of colonies per ml on non-selective plates may underestimate the actual number of cells in treated cultures and in turn overestimate the mutation rate for the novobiocin treated samples. This could provide an alternative explanation of the observed results.

The control sample in Trial 2 showed a final cell population greater than 3×10^{11} cells/ml that is unlikely to be valid since *E. coli* grown in M9 is not expected to reach such a high population and the associated paste-like characteristic of the culture was not observed. Apart from all other fluctuation sets, dilution plating ($10^5 \sim 10^9$) used to determine total cell number in this sample all resulted in TNTC. The OD measurement of the culture was consequently used to determine the final cell concentration. Based on the mutant cell count in Table 1, no extreme high values were obtained, indicating that no mutants were present in the initial inoculum and that the Luria-Debrűck assumptions should have been satisfied for the use of the MSS-MLE method. Therefore, the estimated number of mutation events was valid and the lowered mutation rate found in the control sample may be underestimated due to the inaccuracy in determining the final population size. Then the significant increase in mutation rates at 50 µg/ml novobiocin compared to the control cannot be solidly supported and inferences for the conclusion will not be made based on these results.

Finally, it should be noted that most recent work on the inhibition of *E. coli* gyrases employs novobiocin at concentrations of 0.5-2 µg/ml (7). The concentrations we employed are at least 25X higher. Relatively high concentrations of novobiocin may induce an SOS response similar to that induced by quinolene gyrase inhibiters (2). This response will overestimate the mutation rate corresponding to alterations in the degree of DNA super-coiling.

Based on previous research, 35 parallels should allow our estimation of mutation rates to achieve a theoretical precision of 20% (21); however, there are several limitations of the MSS likelihood method (20). The algorithm strongly relies on the Luria-Delbruck model (11) that has several assumptions from which our experiment might have departed. The model assumes that the proportion of mutation is relatively small and that reverse mutations and death are negligible. Since ampicillin resistance may arise due to many different changes in cellular mechanisms and proper experimental growth protocol, such as time of incubation and the amount of novobiocin for treatment, the estimated mutation rates may be slightly affected. For example, sustaining cultures in lag phase may increase the variance of the final distribution of mutant numbers (12). It is possible to quantitatively assess whether assumptions for the Luria-Delbruck model were met and some deviations were observed for several mutant counts in both sets of the fluctuation assay. Also, since the estimation method is model-based, it is extremely sensitive to outliers. In our results, many parallel cultures had TNTC colony counts that may have prevented the identification of true outliers and allowed greater weight to be put on the potential outliers in the estimation process, possibly producing biased results. Finally, the construction of confidence interval based on the $\log_e$ transformation may also introduce bias especially at the ends of the distributions. The solutions to this problem are currently an active area of research.

The study gave us results opposite to the expected outcome. The novobiocin treated cells had higher mutation rates than the untreated cells. The increased accessibility of DNA did not decrease the mutation rate. The results from this study could provide alternative and additional means of generating mutants at a more convenient rate for genetic analysis and manipulations commonly used in molecular biology.

FUTURE EXPERIMENTS

Additional positively-selectable markers would strengthen the conclusion. Some examples are the ability to utilize lactose as a carbon source, the ability to grow on valine, and the ability to synthesize arginine, histidine, or leucine. If lactose utilization is used as a marker, a good strain to use would be FC40. This strain possesses a *lac* deletion on its chromosome and contains a plasmid which encodes a lac^- gene which is capable of reverting back to lac^+. To provide additional evidence regarding the effect of gyrase inhibitors, more types of these inhibitors should be used as well, such as nalidixic acid and ciprofloxacin.

The experiment could be repeated with lower concentrations of novobiocin. Steps should be taken to determine the concentration at which the SOS response of cells is induced, and effective concentrations of novobiocin below that concentration should be used.

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APPENDIX A

Sample Calculations - Determination of Mutation Rate by the MSS Maximum-Likelihood Method

The following calculations are performed on mutant counts for the 78 µg/ml novobiocin treatment with DH5α. The starting m_0 for the recursive algorithm is obtained by the Luria-Delbrűck method of the median. Then adjacent m's are used to recalculate P_r until an m is identified that maximizes the likelihood function. m_0 is obtained by solving the equation,

$$\frac{\tilde{r}}{m} - \ln(m) - 1.24 = 0$$

where Newton's method is applied and the value of m's converges to 4.44. For data set with mutant counts where the computation of the median is not valid, the Luria-Delbrűck p_0 is used and

$$m_0 = -\ln(number\ of\ cultures\ with\ zero\ mutant).$$

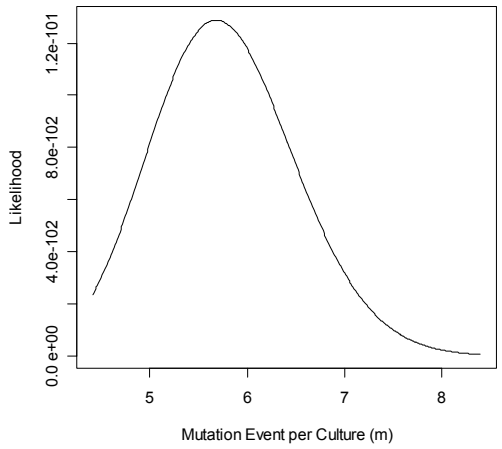
The number of mutation events, m, in the culture is defined as follow:

$$\hat{m} = \max_m \prod_{i=1}^{35} f(r_i \mid m), \text{ where } f(r \mid m) = p_r = \frac{m}{r}\sum_{j=0}^{r-1}\frac{p_i}{(r-j+1)} \text{ with } p_0 = e^{-m}$$

where r is the observed number of mutants in a culture, and i indexes culture number. For any given m, p_r's are computed for $r = 0$ to 300 and the starting value, p_0, of the recursive algorithm is computed as given above. For the obtained sample $m_0 = 4.4$,

$$p_0 = e^{-m} = 0.1228,\ p_1 = \left(\frac{m}{1}\right)\cdot\left(\frac{p_0}{2}\right) = 0.0270,\ p_2 = \left(\frac{m}{2}\right)\cdot\left(\frac{p_0}{3}+\frac{p_1}{2}\right) = 0.0387 \quad \ldots$$

Then $\prod_{i=1}^{35} f(r_i \mid m) = (p_o)^1 \cdot (p_1)^1 \cdot (p_2)^2 \cdot \ldots \cdot (p_{251})^1 \cdot (p_{300})^{12} = 5.92 \times 10^{-102}$.



The maximization results is then plotted and $m = 5.26$ that gives maximizes the above function. This computation is achieved by the statistical software S$^+$.

The 95% confidence interval can be obtained by first computing the standard deviation of the $\ln(m)$ by the formula: $\hat{\sigma} = 1.225m^{-0.315} / \sqrt{C}$, where C is the number of parallel cultures, and based on data, $\hat{\sigma} = 0.1864$. The mutations have been determined to follow approximately a standard normal distribution; therefore, the 95% confidence interval for $\ln(m)$ for our data:

$$\left[\ln(\hat{m}) - 1.96\hat{\sigma}, \quad \ln(\hat{m}) + 1.96\hat{\sigma}\right] = \left[1.29, \quad 2.02\right]$$

The upper and lower limits are then transformed by taking the exponent based *e*. The final confidence interval is then:

$$\left[e^{1.29}, \quad e^{2.02}\right] = \left[3.65, \quad 7.58\right].$$

Mutation rate, μ, is determined by dividing the mutation events by the total final cell number, which is determined base one the total cell counts (see Appendix C).

$$\frac{(52+48)\times 10^6}{2} = 5.0\times 10^7 \ cells/ml$$

Finally, the confidence interval for mutation rate is

$$\left[\frac{3.65}{5.0\times 10^7}, \quad \frac{7.58}{5.0\times 10^7}\right] = \left[7.30\times 10^{-8}, \quad 1.52\times 10^{-7}\right]$$

Sample S$^+$ Codes:

```
S <- matrix (0, 400, 1)
m0 <- 4.4
for (j in 1:400){
m <- m0+j/100
p <- matrix (0, 801, 0)
p[1] <- exp (-m)
for (r in 2:801) p[r]<- (m/(r-1))* sum  ( (p[1:(r-1)]/(r:2) ))
S[j] <- prod (p[data2+1])}
```