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Publication Date

2026-06-13

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Santa Barbara

Genetic Orientation Modulates Statistics of Single Cell Gene Expression

A Thesis submitted in partial satisfaction of the
requirements for the degree Master of Science
in Mechanical Engineering

by

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June 2026

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June 2026

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Annie Nguyen

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to Enoch Yeung, my PI, for his unwavering supervision, guidance, and patience through my academic and research journey. Despite the many challenges that ensued, his encouragement and support was invaluable, and I am grateful for everything I learned at my time in the lab. I would also like to thank all my fellow labmates for their endless selflessness in assisting me in my project and for all the fun camaraderie in and outside of the lab. My time here was especially fulfilling because of such an incredible group of researchers, and I am eternally grateful to have experienced my science adventure with the most inspiring people.

I also extend my biggest thanks to all my friends, Naya Reyes, April Massey, Camila Jimenez, and Tamara Massoud, who have been pillars of support for me through the years. From late-night studying to movie nights, and to all our fun and wild days in Santa Barbara, I am immensely thankful for all the experiences that have shaped me to become the person I am today. Last, and most importantly, I'd like to thank my family for their endless love and support that I am honored to receive. Thank you to my parents, who constantly believed in me and my journey, offering their never-ending support even when I didn't know what I was doing. And with the biggest thanks to my sister, Mimi, who has been my supporter from the very beginning, the person I can always rely on, and the best sister I can ever ask for.

ABSTRACT

Genetic Orientation Modulates Statistics of Single Cell Gene Expression

by

Annie Nguyen

Among the mechanisms of cellular regulation, DNA supercoiling is crucial in modulating gene expression; whether DNA is underwound (negative supercoiling) or overwound (positive supercoiling) directly affects the accessibility of polymerases to initiate transcription. As transcription occurs, the opening of the transcription bubble and the subsequent movement of RNAP have an immense influence on the state of local supercoiling. Recent studies have shown how the natural propagation of negative and positive supercoils via RNAP movement leads to significant changes in gene expression relative to the nearby genetic syntax (convergent, divergent, and tandem gene orientations). While this discovery has offered great insight into the importance of genetic syntax on gene expression, there is much to know about the extent of these different orientations on cell fate, especially among pathogenic bacteria. Here, we explore how cell fate of bacteria is determined by modeling virulence-associated gene clusters, *bfmRS* and *tatABC*, found in the pathogenic *A. baumannii* strain using fluorescent genes in different genetic orientations.

Through the use of epifluorescence microscopy, our results reveal that even when the native intergenic makeup of each gene cluster is preserved, the difference in genetic orientation produces polyphenic variations between and within different bacterial populations. Whereas one genetic orientation will display a favoritism towards one fluorescent gene, another orientation brings rise to multimodal expression of each fluorescent gene at varying degrees. With exposure to identical growth conditions, it is evident that the emergence of phenotypic variance is intimately connected to the genetic syntax rather than external perturbations. These findings provide fundamental understanding of the influence of DNA supercoiling on cell fate and multimodal behavior, providing not only insight into the relationship between gene syntax and expression, but also an explanation of how virulence activity is initiated and persists over time.

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I. Introduction

Cell populations are perpetually evolving, complex mixtures of millions of individuals, often splintering into subgroups with distinct categorical qualities. This property is often referred mathematically as multi-modality, the idea that multiple, distinct thematic or phenotypic properties are present in a population of single cells. One way that multi-modality arises in cell populations is through the process of genetic diversification. As cells mutate, certain mutations cause changes in cellular phenotype that give advantages over selection or increases in fitness of a subpopulation, leading to the emergence of dominant phenotypes and repression of recessive ones¹. For bacterial populations specifically, phenotypic dominance due to spontaneous mutation is often coupled with the natural advantage of horizontal gene transfer (HGT). Through this process, bacterial cells are susceptible to the acquisition of new genetic material that introduces or enhances traits useful for survival. In recent years, HGT has contributed immensely to bacterial survival by developing environmental adaptations such as resistance to heavy metals like arsenic² or by procuring new metabolic processes that enable novel enzymatic capabilities³. Therefore, through mutation, bacterial cells are able to derive varying levels of diversification within a single population.

Rather than pure mutational chance, multi-modality also occurs through native genetic pathways. In nature, multi-modality is a manifestation of biological robustness, in which multiple subpopulations of cells adapting differently to the environment increase the chances of one subpopulation surviving a black swan event. This phenotypic property is a key mechanism in the robustness of persister cells, a dormant subpopulation of bacterial cells that resist bactericidal agents such as antibiotics⁴. The consequences of this multi-modal state of persistence creates concern for aggressive, recurring bacterial infections in patients, most

notably seen in chronic cystic fibrosis infections caused by *Pseudomonas aeruginosa* persists in the lungs⁵. Research on the formation of persister cells reveals the role of specific persister gene pathways that enable bacterial cells to enter a state of dormancy and resist antibiotic treatments. Specifically, studies on the type II toxin/antitoxin system (TA) show how persisters use its toxic protein counterpart to inhibit important cellular functions such as translation and replication to reduce metabolic activity⁶. As such, the mechanisms of pathogenesis often rely on bacterial multi-modality to make decisions about when to mount a virulent response or when to maintain a vegetative, docile state.

Rather than the sequence identity of the genes themselves, multi-modality in bacteria can also be a programmed outcome of how genes are expressed. Specifically, this type of programming is mediated through chemical modifications to the DNA structure, e.g. acetylation and methylation. Such modifications are the key mechanisms of the field of epigenetics, the idea that structural modification to genetically intact and identical sequences give rise to different phenotypes. For example, the process of DNA methylation prompted by a high-fat diet led to the heterogeneous expression of the *Dlk1* gene in successive mouse populations⁷. Without changing the genetic material itself, the process of methylation effectively silences the expression of certain genes by acting as a physical barrier that blocks transcription. In a similar fashion, we can apply this sentiment to the mechanisms of DNA supercoiling, the event in which the DNA helix becomes under- or over-wound as genes are transcribed.

DNA supercoiling is a well-known regulator of gene expression. As transcription is initiated and the transcription bubble opens, two different supercoils are produced: righthanded, positive supercoils and left-handed, negative supercoils. The rise of these

supercoils are a result of increasing torsional stress generated by the bending or movement of binding proteins or polymerases. In particular, as RNAP moves down a strand of DNA, it is unable to freely rotate due to the tight helical path, causing the DNA to become underwound upstream (negative) and overwound downstream (positive) the transcription bubble⁸. Resultantly, gene regulation can occur based on the level of both positive and negative supercoiling among a set of genes. In a state of negative supercoiling, the DNA strands are subject to extensive lefthand twists, allowing for the initialization of transcription due to the accessibility of the RNAP to the promoter regions⁹. However, excessive negative supercoiling could also inhibit transcription efficiency by promoting the formation of R-loops, which is when the nascent RNA strand from the RNAP binds to the DNA template due to overly accessible regions¹⁰. The presence of these R-loops within the transcribing strand acts as roadblocks to the RNAP, preventing transcription elongation. Similarly, positive supercoiling also decreases transcription efficiency by overwinding the DNA, thereby preventing strand separation and RNAP binding.

In relation to human health, the presence of supercoiling is critical in the regulation of pathogenic microbes, as it enables the activation of specific genes useful for resisting environmental stressors and increasing survival¹¹. Therefore, while research on the relationship between supercoiling and pathogenic gene regulation is well known, many of these studies emphasize environmental influences, leaving much to know about how the compositional context of genes contributes to supercoiling-induced changes in their expression. From research discussing the correlation between supercoiling and transcriptional bursting, it was seen that an accumulation of localized positive supercoiling resulted in a slowdown of transcription elongation, effectively demonstrating the topological significance of DNA

supercoiling in regulating different states of expression¹². Knowing this, we are curious about how supercoiling, due to genetic syntax, can alter gene expression and contribute to polyphenic variation in a population of cells.

We show in this thesis that DNA supercoiling acts as a biophysical mechanism for changing the nature of multi-modality in populations of genetically identical cells. Using fluorescent genes alongside the intergenic makeup of the virulence-regulating genes *tatABC* and *bfmRS*, we observed clear phenotypic variations of our gene constructs as they took on different genetic orientations. Specifically, time-lapse microscopy imaging was able to reveal how particular orientations produced different rates of multi-modal expression, as some cells displayed varying levels of single or co-expression of each fluorescent gene. Given the identical experimental conditions, this highly suggests the role of fluctuating supercoiling dynamics as the composition of each gene differs.

Additionally, experimentation using novobiocin also revealed that to some degree, tuning supercoiling does alter population-level responses, although more extensive research is required to identify definitive trends. Ultimately, our findings infer that amidst a variety of external inducers, DNA supercoiling is an essential gene regulator that is highly influenced by the local genetic syntax to produce multi-modal states. As a result, this research helps us gain an understanding of how supercoiling can influence the degree of expression among virulent genes, subsequently creating a foundation that enables us to better comprehend and predict individual and population-level cell fates among bacterial pathogens.

Results

A. Relative Gene Orientation Changes the State of DNA Supercoiling

To understand the extent of gene regulation by genetic syntax and DNA supercoiling, we first examined a simple gene network comprised of the RFP and CFP fluorescent reporter genes in three different orientations: convergent, divergent, and tandem (**Figure 1A**). As preexisting plasmids designed and assembled in the lab, these genetic constructs, also referred to as the RFP-CFP expressing cells, use Lac and Tet promoters to observe the degree of gene expression variation as their respective inducers (IPTG and ATC) are introduced. However, since our intention is to simply observe the correlation between genetic orientation and gene expression, we utilize the bacterial strain *E. coli* MG1655 Δ LacI, rather than the *E. coli* MG1655 Z1 strain they were originally transformed in. Unlike MG1655 Z1, MG1655 Δ LacI does not express the LacI and TetR repressor proteins, thereby promoting constitutive expression of both the RFP and CFP genes.

Visualization of the cells is carried out using agarose pads as the vessel of our cell cultures for subsequent microscopy experiments (**Figure 1B**). Agarose pads prepared with cultures of the three different orientations underwent a 7-hour time-lapse fluorescence microscopy experiment where distinct gene expressions arose between orientations (**Figure 1C**). Analyzing the cells at hour 7, the timepoint with the best overall gene expression, we see that in the convergent orientation, barely any RFP or CFP is expressed among the population of cells. In contrast, the divergent orientation delivers the highest expression of both RFP and CFP, as demonstrated by its intense magenta color. The same trend is extended to the tandem

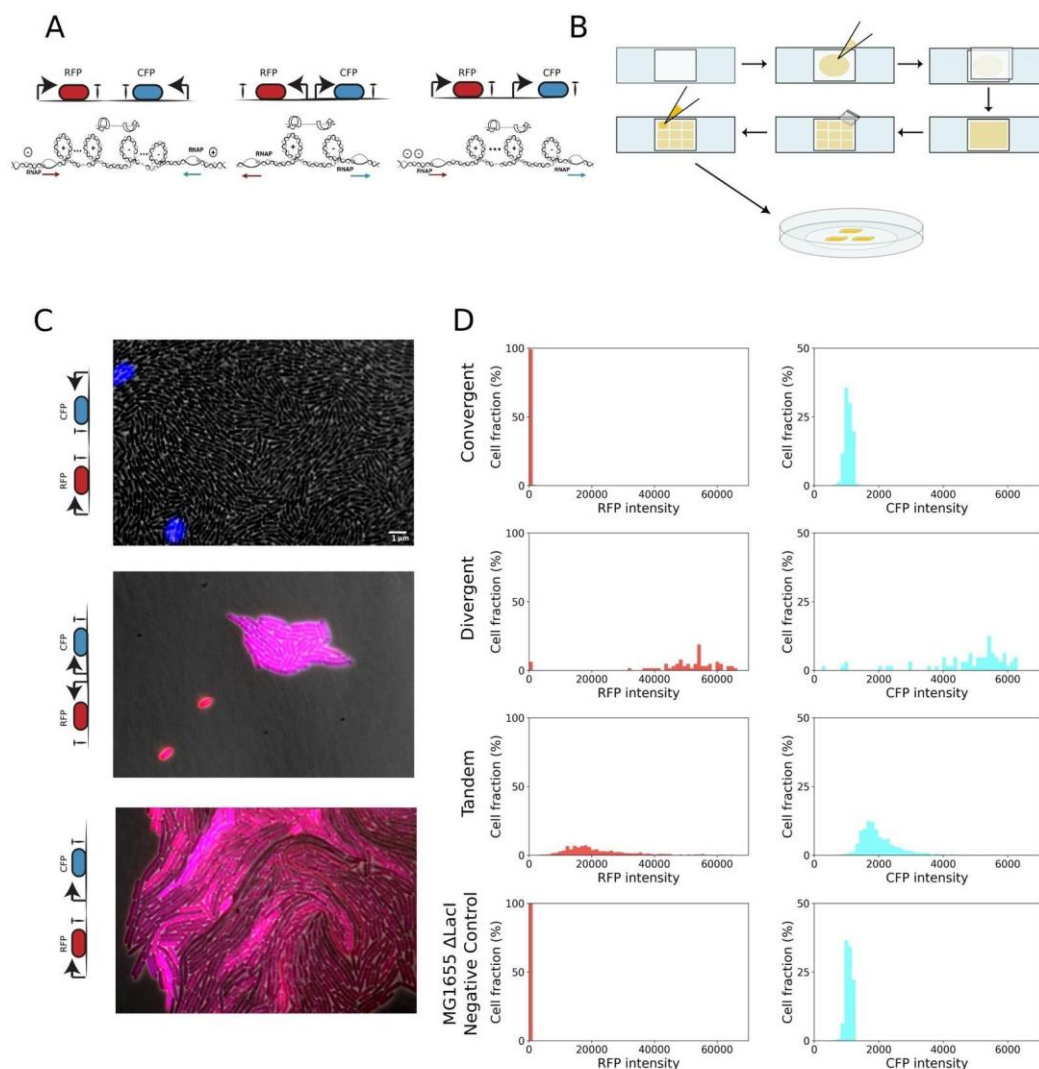


Figure 1. Understanding the context of supercoiling dynamics in a simple gene construct. **A)** Designs of RFP and CFP reporter genes in convergent, divergent, and tandem orientations to observe gene expression differences as a result of DNA supercoiling. **B)** Simple agarose pad demonstration illustrating the production of a thin, 1.5% agarose pad followed by the addition of a prepared cell culture prior to plating for microscopy. **C)** Snapshot at $t = 7\text{hr}$ of a 7-hour time-lapse microscopy experiment for each gene orientation. The expression of a magenta color represents co-expression of RFP and CFP. Scale bars represent $1\ \mu\text{m}$. **D)** Histogram plots presenting the fraction of cells vs. specific fluorescence intensities at time reflecting microscopy images ($t = 7\text{hr}$).

orientation as well, where we see a significant population of varying widespread magenta expression. To better understand the degree of gene expression variation, more detailed analysis is obtained by measuring the cell count of each microscopy image and their respective fluorescent intensities.

Plotting the cell count and fluorescence intensity leads to the generation of histograms that more accurately detail the expression trends within a single population (**Figure 1D**). For example, while we can validate the low RFP and CFP expression in the convergent orientation, the histograms confirm that between the divergent and tandem orientations, one orientation produces higher fluorescence intensity than the other, despite co-expression of both RFP and CFP. In this case, the histogram data reveals that higher fluorescence of RFP and CFP occurs in the divergent orientation than the tandem (4000-7000 AFU versus 10004000 AFU), implying the significant role of gene orientation on gene expression. Therefore, although we can confirm that a correlation exists between gene orientation and expression, there is still much to know about the underlying mechanisms behind its regulation and how these may change in the presence of specific genes.

B. Phenotypic Variance in Virulence-Regulating Simulated Genes

The clear relationship between genetic orientation and the degree of gene expression prompted us to inquire how the natural genetic architecture of virulent gene clusters contributes to the cell fate of bacterial pathogens. Specific focus on the nosocomial pathogen *A. baumannii* led to the study two gene clusters, *bfmRS* and *tatABC*, which have been shown to have major contributions to the degree of pathogenicity by regulating cellular survival, motility, antibiotic resistance, adhesion to host cells, and biofilm production^{13,14,15,16}. As a common source of aggressive healthcare-associated infections (HAIs), extensive research on the virulent mechanisms of *A. baumannii* is crucial in combatting possible life-threatening conditions. We explore the significance of genetic syntax by replacing the coding sequences of the naturally occurring genes in the *bfm* and *tat* clusters. As such, we study the importance of

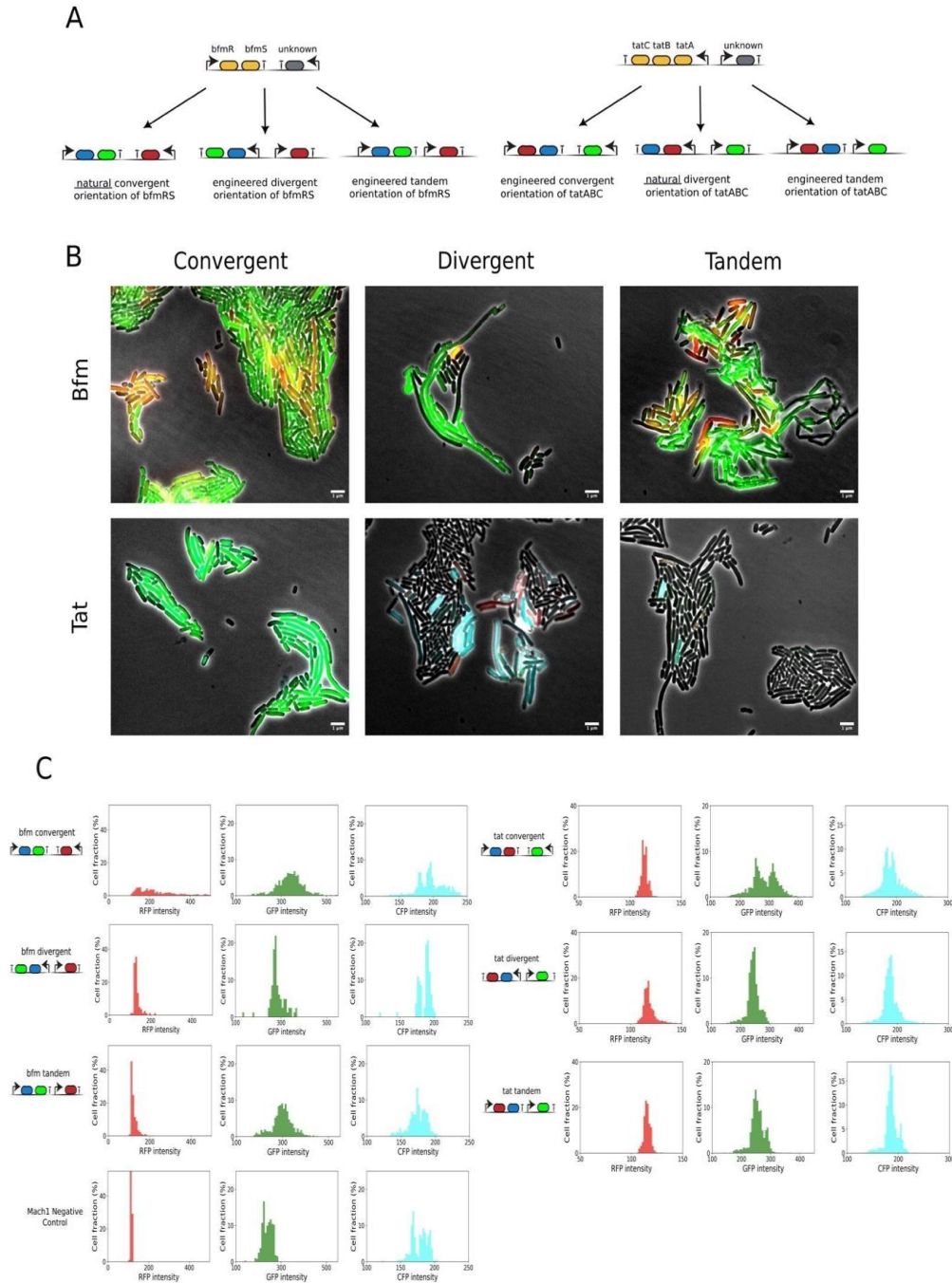


Figure 2. Studying supercoiling effects on virulence-regulating genes, *bfmRS* and *tatABC*. **A)** Refactored designs of the *bfmRS* and *tatABC* genes using three fluorescent proteins, RFP, GFP, and BFP in convergent, divergent and tandem orientations. **B)** Snapshot at $t=4$ hr of a 7-hour time-lapse microscopy imaging of each refactored gene cluster and their different orientations. Yellow-orange cell expression represents coexpression of RFP and GFP while bright green/teal cell expression represents coexpression of GFP and CFP. Scale bars represent 1 μ m. **C)** Histogram plots presenting the fraction of cells vs. specific fluorescence intensities at time reflecting microscopy images ($t=4$ hr).

genetic syntax by constructing these fluorescent genes into the convergent, divergent, and tandem orientations (**Figure 2A**).

By replacing the native genes with fluorescent genes and preserving their promoter sequences, we can theoretically model how virulence-regulating genes are affected by directionally altered DNA supercoiling based on the extent of their cellular fluorescence. To visualize these effects, each genetic construct was assembled into plasmids and transformed into Mach1 *E. coli* cells, where they are then used in a 7-hour fluorescence agarose pad microscopy experiment. Due to the phototoxicity mediated by the light required to fluoresce BFP (excitation/emission: 402/457nm), the CFP channel was used instead due to its low phototoxicity and overlapping excitation-emission range. As a result, the BFP expression will be represented as CFP for our *tat* and *bfm* strains.

Through microscopy, the emergence of multi-modal behavior is evident after 4 hours of imaging (**Figure 2B**). For clarity purposes, it is important to note the genetic design differences between *tat* and *bfm*. For *bfm*, the upstream promoter regulates GFP and CFP expression while the downstream promoter regulates RFP expression. In contrast, *tat*'s upstream promoter regulates the expression of CFP and RFP, while the downstream promoter regulates GFP expression. Therefore, we look forward to observing how GFP-CFP changes against RFP in *bfm*, and how CFP-RFP changes against GFP in *tat*. Taking these into consideration, we can properly assess our microscopy results.

Analysis of the convergent *bfm* strain reveals a population of cells characterized by a multi-modal expression of green fluorescence, followed by some cells displaying light-red profiles, others displaying varying levels of yellow-orange (RFP and GFP), and others

expressing very bright light green (CFP and GFP). On the other hand, the divergent *bfm* strain demonstrates an overwhelming majority of cells fluorescing green with considerably less yellow-orange expression in the population. Although the tandem *bfm* strain shows some resemblance to the convergent strain, detailed observation shows that more cells are likely to display full red phenotypes and less yellow-orange co-expression compared to that of the convergent strain. An array of phenotypic variance also arises in the *tat* genes. Similar to the convergent *bfm*, the convergent *tat* strain produced varying levels of bright green GFP-CFP cells, coinciding with the co-expressed RFP-GFP cells in *bfm*. Interestingly, the divergent *tat* strain exhibited a complete switch in behavior, with cells developing a greater affinity towards CFP and RFP expression and very low GFP. Similarly, the tandem *tat* also exhibited a unique shift as well, as its cells produced extremely low levels of expression for all three genes with slight favoritism towards CFP.

More extensive analysis is once again provided by rendering histogram plots measuring the cell count and fluorescent intensities of each sample (**Figure 2C**). While there are resolute cellular phenotypes that are recognized among the different orientations, the histograms give in-depth insight into the expression patterns we see from sample to sample. Therefore, as we interpret this data, we make sure that the fluorescence of both the *bfm* and *tat* cells are background subtracted to account for the natural autofluorescence of the negative control (RFP = 100-115 AFU, GFP = 180-280 AFU, & CFP = 150-200). When we consider these reference values and compare the microscopy images to the histogram plots, we can confirm the pattern of behavior previously observed.

In our microscopy images, we saw a clear rise in multi-modal behavior as seen by the co-expression of each adjacent gene in the convergent *bfm* (GFP and RFP co-expression) and

tat (GFP and CFP co-expression) gene clusters. These results are well represented by their respective histograms, which give thorough insight behind these trends. For the convergent *bfm*, the large flux of GFP fluorescence and varying RFP-GFP is well represented by the substantial GFP intensity (280-480 AFU) and widespread RFP intensity range (120-450 AFU). Just as the microscopy results show, the low number of high-intensity RFP-expressing cells in our histogram corresponds to the small subset of yellow-orange cells seen amongst the sea of green cells. For the convergent *tat*, the co-expression of GFP-CFP is described by the significant GFP distribution (280-400 AFU) and the increase in cells expressing CFP (200-250 AFU) with barely any RFP expression (115-125 AFU).

As we observe the divergent and tandem orientations, we also identify the exact expression profiles previously seen in our microscopy images. For our GFP-dominant divergent *bfm* cells, the histograms reveal high numbers of GFP expression (280-350 AFU) with little to no cells expressing RFP (120-200). At the same time, the CFP-RFP dominant divergent *tat* cells also show moderate expressions of CFP (200-250 AFU) and RFP (115-150 AFU), while GFP intensity and cell count decrease (280-300 AFU). On the other hand, our multi-modal tandem *bfm* displays a dominance of cells expressing GFP (280-400 AFU) speckled with varying levels of RFP (120-180 AFU) while our weak tandem *tat* produces significant CFP expression (200-210 AFU) followed by low levels of GFP (280-310 AFU) and barely any RFP (115-125 AFU). For both divergent and tandem *bfm* orientations, CFP expression is negligible due to fluorescent intensity values within the negative control range. Therefore, as we analyze the tandem *bfm* histograms, a major question arises: how is it that despite having a lower RFP fluorescence intensity on average, we achieve more fully red cells in the tandem strain than the convergent strain?

This question corresponds directly to the development of multi-modality, in which multiple states exist within a single population despite having the same genetic makeup. In a cellular multi-modal distribution, cells have the potential to take on many phenotypes due to syntax-mediated DNA supercoiling. This is why, despite having different fluorescent intensities, tandem *bfm* has more fully red cells, because its specific orientation is able to produce a multi-modal state that favors single RFP expression (among dominant GFP expression). On the other hand, the convergent orientation for *bfm* creates multi-modal states that not only show a single fluorescence (GFP) but also co-expression of two fluorescent genes (GFP-RFP).

Compiling our microscopy images and histogram data, we can establish a trend of behavior between each orientation. Given the high expression of CFP-RFP in the divergent *tat* and the high GFP expression in the divergent *bfm*, we can assert that for divergently oriented genes, the expression of one adjacent gene is more likely to occur over the other. On the other hand, the tandem orientation is more likely to have cells that exhibit single expression of both adjacent genes, as seen by the strong, single RFP/GFP expressions in *bfm*, and the weak, single CFP/GFP expressions in *tat*. In contrast, the convergent orientation generates the most heterogeneity, with both *bfm* and *tat* producing varying levels of GFP-RFP and GFP-CFP co-expression, respectively.

While it is interesting to recognize this difference in behavior, it is equally important to study the overall gene expression trends based on the fluorescent intensities alone. As we compare the fluorescent intensity values among both the *bfm* and *tat* strains, it is evident that the convergent orientation produces the highest expression of all three fluorescent proteins, except for *tat* RFP. However, a discrepancy occurs between the divergent and tandem

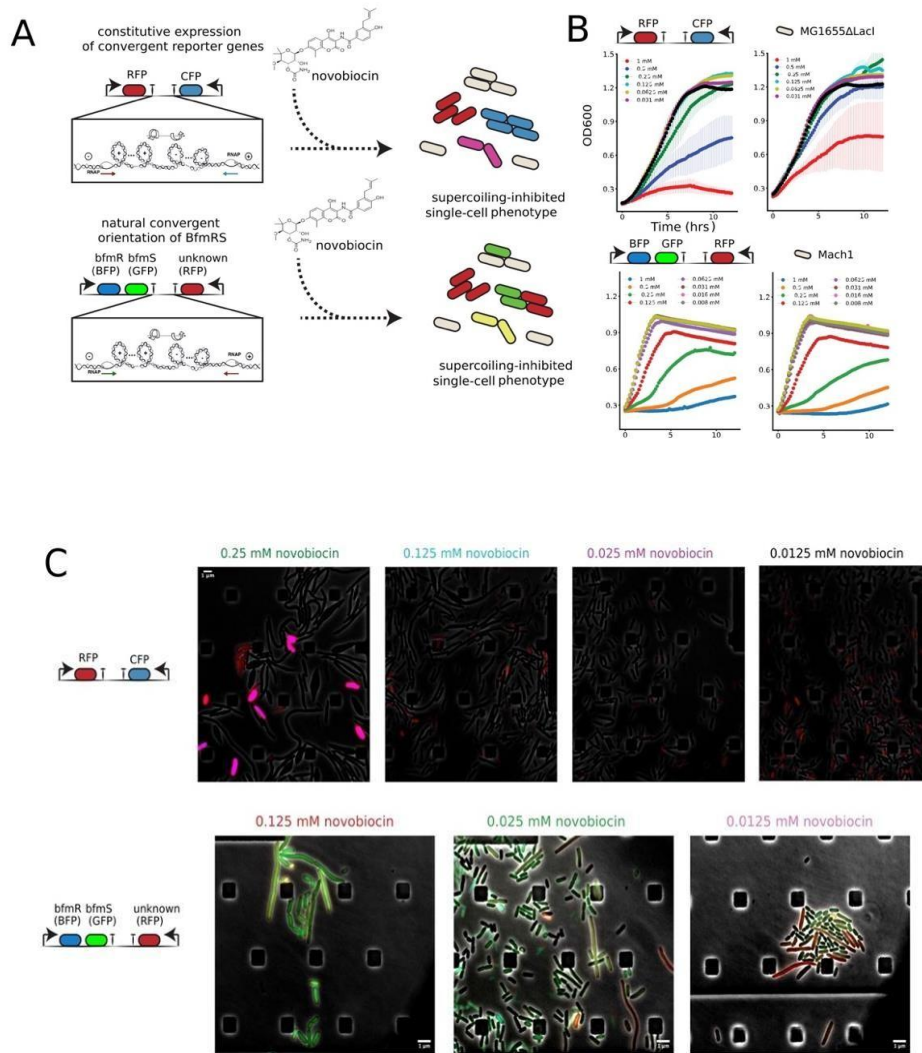
orientations. When comparing the effects of the divergent and tandem orientations between the upstream and downstream genes of both *tat* and *bfm*, multiple outcomes arise. For example, in an ideal trend, we expect that if the divergent orientation of *bfm* produces a higher expression of its downstream gene (RFP) compared to the downstream gene of the tandem orientation, then we can also expect the downstream gene in the divergent *tat* (GFP) to result in a higher expression compared to its tandem orientation. Unfortunately, this trend is not observed in our data. Instead, we see a fluctuation between divergent and tandem regarding fluorescence intensity patterns.

In *bfm*, the divergent orientation generates a higher downstream RFP expression, whereas in *tat*, the tandem orientation generates a higher downstream GFP expression, although by a very small margin (tandem = 180-305 AFU, divergent = 170-300 AFU). For the upstream GFP-CFP in *bfm*, the outcome is mixed, with the tandem orientation producing higher singular GFP expression than the divergent orientation, and the divergent and tandem orientations having similar fluorescent intensities for CFP expression. The upstream CFPRFP in *tat* shows even more complex dynamics as the divergent orientation displays the highest RFP expression, followed by the convergent and tandem orientations producing comparable fluorescent intensities. On the other hand, for CFP expression, the convergent and divergent orientations tie with generating the highest expression, while the tandem orientation follows behind with a lower fluorescence intensity. Given these distinct trends, we can infer that while genes in the convergent orientation typically result in the highest genetic expression, the divergent and tandem orientations show inconsistent expression trends, requiring further experimentation to deduce a final verdict on how genetic orientation influences upstream and downstream gene expression due to supercoiling.

C. Population-Level Responses Tuned by Non-Lethal Volumes of Novobiocin

In an effort to enhance our knowledge on DNA supercoiling and its regulation on gene expression, we move our efforts to altering supercoiling not through genetic syntax but by the introduction of chemical topoisomerase inhibitors. To do so, we employ the antibiotic, novobiocin, which acts as a competitive inhibitor of DNA gyrase, a type II topoisomerase that relieves positive DNA supercoiling during transcription. In research, the use of novobiocin is useful for studying DNA supercoiling since inhibition of DNA gyrase ultimately leads to an increase in positive supercoiling during transcription. As a result, this prevents the accessibility of transcription factors and RNAP to the promoter site, eventually reducing gene expression and prohibiting the production of necessary proteins required to sustain cellular activity.

To ensure that we can observe the effects of novobiocin over time, we first determine the range of non-lethal novobiocin concentrations appropriate for our target cells. Given the high variability observed in the convergent strains, we will be using the convergent *bfm* strain alongside the convergent RFP-CFP cells (**Figure 3A**) to compare how novobiocin changes cells of established (RFP-CFP) versus undefined (*bfm*) promoter elements. Confirming optimal, non-lethal novobiocin concentrations was carried out by assembling a titration experiment in which each convergent cell strain was treated with 20 different concentrations of novobiocin followed by measurements of their corresponding OD₆₀₀ values in the plate reader (t = 12 hr). The results reveal an array of different OD₆₀₀ curves according to the amount of novobiocin added (**Figure 3B**). Due to the formation of identical OD₆₀₀ curves at certain novobiocin concentrations, not all OD₆₀₀ curves are depicted to decrease high redundancy. Assessing each plot, we can use novobiocin concentrations at ≤ 0.25 mM



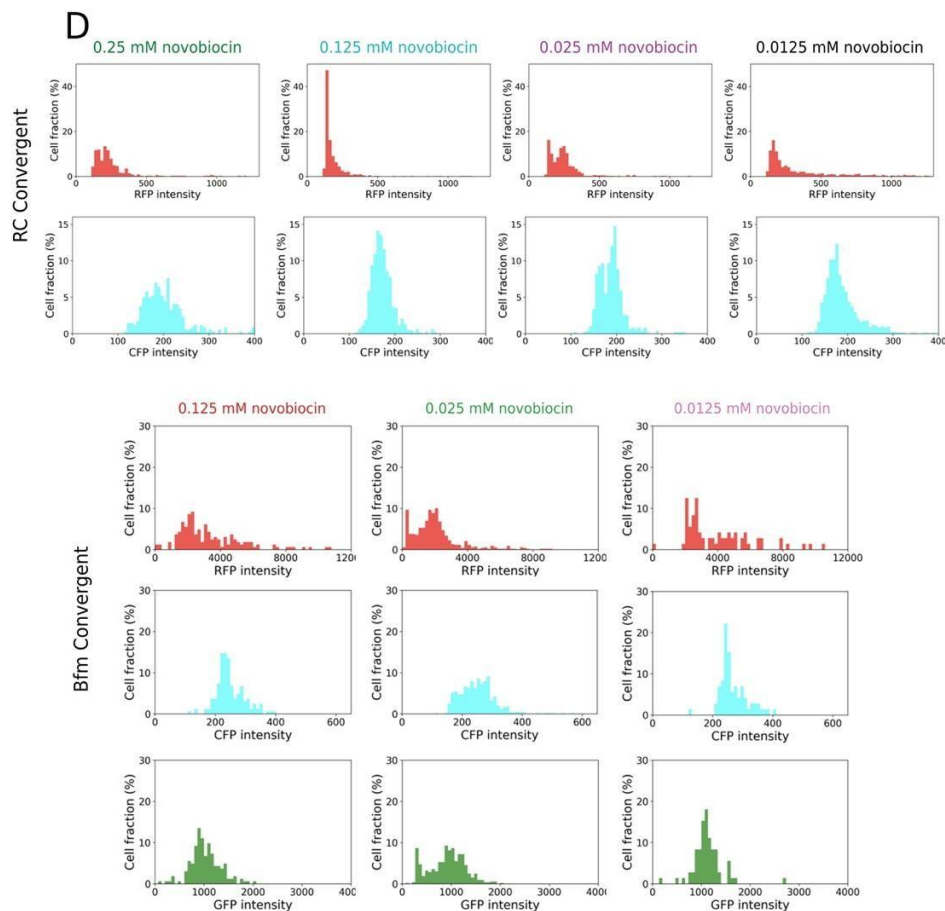


Figure 3. Tuning convergently oriented cells with varying levels of novobiocin, a gyrase inhibitor. A) Schematic of theoretical single cell effects prompted by inhibiting the introduction of negative supercoils due to *gyrB* inhibition by novobiocin. **B)** Plots presenting respective OD₆₀₀ curves over time to visualize the highest, non-lethal novobiocin concentrations for *E. coli* MG1655 Δ LacI (top) and Mach1 (bottom) cells. **C)** Snapshot of a 12-hour time-lapse microscopy experiment of cells exposed to novobiocin at decreasing levels of novobiocin. For RFP-CFP cells (top), snapshot was taken at $t = 7$ hr of experiment, using 0.250 mM, 0.125 mM, 0.025 mM, and 0.0125 mM novobiocin. For *bfm* cells (bottom), snapshot was taken at $t = 4$ hr of experiment, using 0.125 mM, 0.025 mM, and 0.0125 mM novobiocin. Concentration at 0.250 mM for *bfm* resulted in poor cell viability. Scale bars represent 1 μ m. **D)** Histogram plots presenting the fraction of cells vs. specific fluorescence intensities at a determined time point. The time point for each group of cells correspond to their microscopy images (RFP-CFP: = 7hr, *bfm*: $t = 4$ hr).

for the convergent RFP-CFP expressing cells and ≤ 0.125 mM for the convergent *bfm* cells.

Unlike the previous microscopy experiments, the cells are imaged in media suspension using a microfluidic single-cell trapping plate (Millipore Sigma CellASIC B04A-03 Plate). This was intentionally done to ensure that consistent novobiocin treatment was being exposed

to the cells since the plate provides a constant flow of fresh media over time. As such, to see how novobiocin can potentially tune cellular expression due to increased positive supercoiling, we use varying concentrations of novobiocin, starting with the highest, nonlethal concentration of each strain. For the convergent RFP-CFP expressing strain, we used four concentrations: 0.25 *mM*, 0.125 *mM*, 0.025 *mM*, and 0.0125 *mM*. Similarly, we also used the same four concentrations for the convergent *bfm* cells; however, due to poor cell viability at 0.25 *mM*, only 0.125 *mM*, 0.025 *mM*, and 0.0125 *mM* are visualized.

Since we intend to see how supercoiling induces certain cellular responses because of novobiocin, we can use the same time points in Figures 1 and 2 to accurately picture changes from pre-existing data. As a result, the convergent RFP-CFP cells are visualized after 7 hours of imaging, while the convergent *bfm* cells are visualized after 4 hours, replicating the analysis conditions of Figures 1 and 2, respectively (**Figure 3C**). Due to the different bacterial hosts between the two convergent strains, the imaging times differ due to their unique cellular kinetics that bring peak expression profiles at varied times. Therefore, as we observe the microscopy results for both convergent strains at these time points, distinct phenotypes arise.

In the highest novobiocin concentration sample for the convergent RFP-CFP cells, we see noticeable co-expression of both RFP and CFP in a few cells. However, it is important to note that despite seeing co-expression with CFP, we believe this result to be insignificant, as CFP expression began at the time of imaging and persisted until the end; the addition of novobiocin did not seem to produce such CFP co-expression over time. As such, the remaining population of cells reveals little to no expression of either gene, with only a few cells expressing RFP nearby. The same low RFP expression profile can also be seen in the three other novobiocin concentrations, suggesting a limited range of effects between concentrations of 0.0125 *mM*

0.125 *mM*. Based on the microscopy results alone, we can argue that even with a ten-fold reduction from 0.125 *mM* to 0.0125 *mM*, no major changes to cellular responses transpire. On the other hand, when we observe the effects of decreasing novobiocin in the convergent *bfm* strain, a slight trend emerges.

By pure observation of the microscopy images, the convergent *bfm* cells experience an increase in RFP expression as the novobiocin concentration decreases. On the other hand, any significant alterations to the state of GFP and CFP fluorescence are hard to detect by itself, requiring the histogram plots (**Figure 3D**) to help describe how the behavior of the cells' fluorescence shifts by the presence of novobiocin. Comparison of the convergent *bfm*'s plots reveals the same trend identified with its RFP expression, as novobiocin increases, the fluorescent intensities of each fluorescent gene decrease, as seen by the shift of all the histograms to the left. As we analyze the plots for the convergent RFP-CFP cells, we also see fewer CFP-expressing cells, though its fluorescence intensity remains mostly constant. However, in the corresponding RFP plots, it is evident that despite having fewer cells fluoresce, higher novobiocin concentration results in the RFP histogram shifting to the right, indicating an increase in fluorescence intensity. Although some discrepancy exists at the 0.125 *mM* concentration, we see the same behavior occur at the 0.025 *mM* concentration, therefore suggesting that increased novobiocin may contribute to enhanced RFP expression.

While this outcome contradicts the expectation of novobiocin's effect (decreased gene expression), we can deduce that novobiocin can fine-tune population-level responses, though some results may vary based on either cell type, genetic design, or both. From decreased fluorescing cells in *bfm* and increased RFP intensity in the RFP-CFP expressing cells,

novobiocin can be used to effectively describe how alterations in DNA supercoiling dynamics can influence cellular responses, although the mechanism of how must be further studied.

III. Discussion

The propagation of DNA supercoils during transcription has an immense influence on cellular regulation. As a key component of “topological epigenetics”, the role of supercoiling controls gene expression not only through environmental factors such as carbon sources, osmotic shock, oxygen levels, and thermal stress^{17,18,19}, but also on the degree of physical alterations (folding, twisting, etc.) based on local genetic composition. As a result, understanding the extent of gene regulation by supercoiling is especially complicated, as new research is constantly providing new information about the range of its effects in transcription. By studying refactored virulence-regulating genes from the pathogen *A. baumannii*, our results show how complex supercoiling dynamics are, not only between different cells, but also due to genetic syntax.

Using RFP-CFP cells, our initial pursuit in gauging how gene expression varies by syntax-mediated supercoiling displayed clear phenotypic disparities between each orientation.

Whereas the divergent and tandem orientations displayed bright co-expression of the RFP and CFP genes, the convergent orientation resulted in fluorescent intensities comparable to the negative control. At the same time, we also observed distinct phenotypic variation among the fluorescently refactored *tat* and *bfm* gene clusters, in which each orientation also brought about notable trends in expression. Specifically, we saw that in the convergent orientation, high co-expression of both adjacent genes was produced, while in divergent and tandem orientations, expression of one adjacent gene and singular expression of each adjacent gene was generated,

respectively. When we compare the fluorescent intensities between the *tat* and *bfm* constructs, the convergent orientation generated the highest overall fluorescent intensity while the RFP-CFP cells saw more intense expression in the divergent orientation. Therefore, when we compare the expression profiles between each orientation of the three constructs (*tat*, *bfm*, and RFP-CFP), we see a diverse range of gene expression that illustrates the significance of syntax on supercoiling.

As the expression differences of the *tat/bfm* cells and RFP-CFP cells are studied, a major distinction exists. Unlike the simple two-gene design for the RFP-CFP expressing cells, the *tat/bfm* cells use three genes, with two being under the same promoter. When we compare our microscopy results to their respective designs, we see that in all cases, the two genes linked together dominate the phenotypic profile of the cells to some capacity. As a result, we can attest that this unique outcome could be a result of the gene/transcript length. Previous research implies a relationship between gene length and supercoiling²⁰, and our data reinforces this sentiment, given the clear increase in expression of longer transcripts. Therefore, this opens another question: how does the length of a genetic transcript contribute to supercoiling dynamics in conjunction with its syntax? First, we need to understand the foundational premise of how supercoiling regulates gene expression.

Transcription begins when negatively supercoiled DNA separates the double-stranded DNA helix, allowing for the subsequent binding of the RNAP to the promoter site. As a result of RNAP binding, the strands open further to form the transcription bubble, which creates positive supercoiling (right-handed twists) downstream and negative supercoiling (lefthanded twists) upstream of the bubble as RNAP moves²⁰. As we fit this model into the context of multiple genes, we see constant interactions between negative and positive supercoiling, as shown in

Figure 1A. In relation to orientation, we propose that the degree of superhelicity of each supercoil relies on the direction in which the gene is transcribed. Specifically, as a gene is transcribed in the direction downstream of the promoter, it induces increased positive supercoiling following the transcription bubble. If the gene is transcribed in the direction upstream of the promoter, it induces increased negative supercoiling before the transcription bubble. Therefore, we postulate that the extent to which gene expression varies is heavily reliant on the interactions between varying degrees of positive and negative supercoiling.

For the convergent orientation, since both genes face towards one another, we get increased positive supercoiling from the upstream gene and increased negative supercoiling from the downstream gene. Rather than cancel each other out, the confrontation of these two supercoils reaches a state of equilibrium, in which the forces act upon each other balance out²¹. The result should be moderate expression of both genes, which is seen in both the convergent *tat/bfm* cells but not in the RFP-CFP expressing cells. We hypothesize this to be a consequence of transcript lengths. It can be speculated that the longer the sequence of DNA the RNAP is transcribing, the more torsional stress builds up in the direction of transcription, creating higher levels of supercoiling. If we apply this to our convergent model of the *tat/bfm* cells, the outcome would be stronger positive supercoiling facing the negative supercoiling of the downstream gene, since the upstream promoter controls two genes. Consequently, lower expression levels of the downstream gene should be expected as accumulated positive supercoiling stalls progressing transcription. As such, the consequence of this is the emergence of multi-modal states in the population. Notably, this is what we see in our *tat/bfm* results.

Since the RFP-CFP expressing cells have transcript lengths of roughly the same size, we expect to see moderate expression of both genes. Surprisingly, there is little to no

perceivable expression of the convergent RFP-CFP expressing cells, likely due to the strong equalization of supercoiling that neutralizes the expression. Given that some CFP is seen minimally, we can confirm the presence of negative supercoils left of the transcription bubble, as this relaxed form supports the movement of RNAP to continue transcription and maintain some gene expression.

Understanding where positive and negative supercoils propagate and how the direction of transcription either strengthens or weakens its superhelicity, we apply this model to our divergent and tandem orientations. In the divergent orientation, as the genes transcribe in opposite directions, their promoters end up facing each other in the center. Using the same logic as the convergent orientation, we believe that although the positive supercoiling of the *tat/bfm* upstream genes is weaker due to the RNAP moving in the opposite direction, its longer length is still able to produce positive supercoils strong enough to hinder the promoter of the downstream gene. We attest this by comparing the relative number of turns created by each gene. For *bfm*, the upstream GFP and RFP genes create a total length of 1392 base pairs (bp) (GFP = 714 bp, RFP = 678 bp) while the downstream BFP gene is 702 bp. A single DNA turn is 10.5 bp, resulting in roughly 123.5 turns in the upstream genes and roughly 67 turns in the downstream gene. As a result, we can imagine that due to this significant difference, the downstream promoter becomes saturated by superhelical twists with turns double its own amount. This would effectively impede on the downstream gene's promoter function, leading to a favoritism of expression for the upstream genes and no co-expression as we see in convergent. Again, we see this result in our *tat/bfm* cells.

When we compare these results to the RFP-CFP cells, the divergent model proves to be true with some slight discrepancy. In the presence of similarly sized genes, the weakened

positive and negative supercoiling between the two genes is still able to express both, but at varying degrees. As we analyze our microscopy images and histogram plots, we see coexpression of both genes due to the lack of high positive supercoiling. However, if we consider our supercoiling model, there should be much more CFP fluorescence than RFP, given the presence of relaxed negative supercoiling ahead of its promoter. Instead, we see much higher RFP intensities, which should be considerably lower in expression.

Unfortunately, more conflict emerges as we apply the same logic to the tandem orientation. When the genes are in tandem orientation, the upstream gene develops strong positive supercoiling that meets with the downstream gene's weak negative supercoiling. If we use the same logic from the convergent model, we expect that, in addition to the length and direction of transcription, the downstream gene of the *tat/bfm* cells would be effectively silenced due to high positive supercoiling interference on its promoter. While we can argue that case for the *tat* cells, the *bfm* cells still display a considerable amount of RFP expression that should be significantly smaller. Despite the RFP-CFP cells aligning with this model by producing greater RFP intensities than CFP, it is interesting to note that the RFP intensities of the tandem orientation are substantially lower than those of the divergent orientation. However, something to consider regarding these results is how the emergence of multi-modal states could be contributing to these outliers. Of course, while these models are useful guides of what to expect based on genetic syntax, they are not rigid frameworks to consult. As a result, the inconsistency in expression profiles can be due to the orientation's natural tendency to produce multi-modal states. While transcript length is shown to be a major contributor to how multi-modality occurs according to orientation, it is evident that further research is needed to identify all the complexities that helps conduct such phenotypes.

The strength of positive supercoiling demonstrates clear advantages of diversifying the rates of gene expression of surrounding genes. In our novobiocin experiments, we show a variation of genetic responses as decreasing concentrations are added to the convergent *bfm* and RFP-CFP cells. Since novobiocin inhibits gyrase function, we expect an accumulation of positive supercoiling in regions of overwound, positive DNA. As we reference our convergent supercoiling model once again, having accumulated positive supercoiling should lead to the diminished expression of the downstream gene. Interestingly, we observe this trend in both our RFP-CFP and *bfm* cells in different ways.

In increasing concentrations of novobiocin, the convergent *bfm* histograms show shifts of each fluorescent gene's intensities to the left, indicating lowered expression. For the RFPCFP cells, higher novobiocin concentrations decrease the overall number of cells that express CFP while its fluorescence intensity remains constant. However, the most interesting outcome is the *increase* in fluorescence intensity seen in the RFP-CFP cells' RFP gene. While the mechanism of this outcome is rather elusive, we believe that the increase of novobiocin may be reducing the formation of negatively supercoiled-induced R-loops. The formation of these R-loops has many regulatory features, one of which includes the stalling of up- and downstream transcription due to the increased torsional stress that occurs from upstream RNAP movement²². Thus, as novobiocin is added and fewer negative supercoils are introduced, we decrease the presence of downstream R-loops that consequently heightens the transcription and expression of the upstream RFP gene. Though more information is needed to validate these mechanisms, it is evident that novobiocin can tune the statistics of cellular responses by modifying supercoiling strengths.

Overall, our data emphasizes the dynamic relationship between genetic orientation and supercoiling, revealing how the composition of a group of genes is intimately connected to the degree of supercoiling and its effects. Our results also highlight the significance of transcript length, revealing that depending on the orientation, the distance required for the RNAP to move could alter the strength of superhelicity. As a result, this helps determine transcription efficiency and whether gene expression is improved or restricted. Among the outcomes of supercoiling-mediated gene regulation is the production of multi-modal states, which we know can bring about numerous advantages, especially in pathogenic microbes. Whether it is a subgroup with robust resistance capabilities or a group that differentially expresses high virulence, the rise of multi-modal expression is useful in procuring novel phenotypic variations that could improve bacterial survival.

In the future, we plan on observing how supercoiling-induced expression of the refactored *tat* and *bfm* gene clusters may change in the presence of their native environment. Specifically, while these are prevalent genes of the pathogenic *A. baumannii* strain, they are also naturally present in the non-pathogenic, opportunistic *A. baylyi* strain, a strain of bacteria also under the *Acinetobacter* genus. Doing so will allow us to garner more thorough information on the behavior of these genes, prompting better foundational understanding of the relationship between supercoiling and virulence. Therefore, by observing the degree to which gene orientation and subsequently, DNA supercoiling, can induce different multimodal states in virulence-regulating genes, we can understand how the virulence of a pathogen is initiated and sustained over time, as our knowledge on supercoiling patterns due to the direction and length of genes can help in predicting cell fate and behavior.

IV. Methods

A. Genes of Interest, Plasmid Assembly, and Bacterial Strains

Gene clusters *bfmRS* and *tatABC* from the pathogenic bacterial strain, *Acinetobacter baumannii*, were chosen as reference models given their roles as regulators in host cell adhesion, biofilm formation, and antibiotic resistance. The two gene clusters were modified in the Geneious Prime software by replacing the native genes (including adjacent genes) with the coding sequences of the three fluorescent proteins, sfGFP, mRFP, and tagBFP. To ensure that the natural gene expression profiles of each cluster were preserved, the promoter regions of each gene cluster were maintained with no additional alterations. Each fluorescently modified gene cluster was then edited to display convergent, divergent, and tandem gene orientations.

To construct our plasmids, the modified gene constructs and their corresponding primers with added BsaI cut sites were ordered as gBlocks from Integrated DNA Technologies. A plasmid including a pBBR1 oriV site and kanamycin resistance gene (*kanR*) was used as our backbone. Polymerase chain reactions to amplify the inserts and backbone were performed using the Vazyme 2x Phanta Flash Master Mix and were later followed by Golden Gate assembly using the NEBridge Golden Gate Assembly Kit - BsaI-HF v2 (NEB E1601). For bacterial transformation, the Golden Gate product was inserted into Invitrogen MultiShot FlexPlate Mach1 T1^R Competent Cells (Invitrogen C8681201) using the transformation protocol as provided by the cell product's manual. The cells were subsequently plated on LB agar plates with 50 $\mu\text{g}/\mu\text{L}$ kanamycin and left to incubate at 37° C overnight. Colonies grown were screened using colony PCR and were then inoculated in selective LB media overnight to extract plasmids via Qiagen's QIAprep Spin Miniprep Kit (Qiagen 27104). For plasmid

verification, miniprep products were sent to Eurofins Genomics and sequence-verified by whole plasmid sequencing. Colonies with the best sequenced plasmids were preserved as glycerol stocks and used for future experiments.

B. Preparation of RFP-CFP Expressing Cells

To model gene orientation dynamics, RFP-CFP expressing cells were already available in the lab as plasmids designed and built in house. The genetic design of the fluorescent genes consists of an upstream RFP gene, controlled by the Tet promoter, and a downstream CFP gene controlled by the Lac promoter. Different orientations of the fluorescent genes (convergent, divergent, and tandem) were constructed into a plasmid consisting of a ColE1 origin of replication and an ampicillin resistance marker. The plasmids were then transformed into the *E. coli* MG1655 Δ LacI strain, a MG1655 strain that includes a deletion of the lactose repressor gene *lacI*. For downstream use, glycerol stocks of the MG1655 Δ LacI cells containing the different orientations of each RFP-CFP strain was made and stored in the -80° C freezer.

C. Single-Cell Fluorescence Microscopy

1. Cell Culture Conditions

To prepare cells for microscopy, cultures were made by scrapping glycerol stocks from the -80° C freezer and inoculating them into 3 mL of selective LB media in 15 mL culture tubes. For the *tat* and *bfm* strains, kanamycin at 50 μ g/ μ L is used for selection while the RFPCFP strains require 100 μ g/ μ L ampicillin. The tubes are then placed into a shaking incubator set to 37° C at 250 rpm and left to incubate overnight for 12 hours. The next day, the OD₆₀₀ values of each culture tube was recorded to prepare for passaging. Based on the measured OD₆₀₀ value, each culture is passaged into new selective media at a final OD₆₀₀ of 0.01. For the *tat* and *bfm*

strains, the passaged cultures were placed back into the shaking incubator at 37° C, 250 rpm, and left to incubate until an OD₆₀₀ of 0.06-0.08 is reached, roughly 1.5-2 hours. The RFP-CFP strains also follow the same protocol with a minor change of 37° C to 29° C for passaging due to previous research noting better expression profiles at 29° C. Once at the target OD₆₀₀, the cells are ready to use for further microscopy use.

2. Agarose Pad and Microfluidic Cell Trap Plate Preparation

Given the different observation requirements in this project, two different microscopy techniques are used. To observe normal gene expression of the different genetically oriented genes, agarose pad microscopy is used as a simple and low-cost method to acquire time-lapse imaging of cells. Rather than standard agarose used in normal DNA gel electrophoresis, a low-melting point agarose (Invitrogen™ UltraPure™ Low Melting Point Agarose, 16520050) was used to create a 1.5% agarose gel with LB media due to its better cell embedding capabilities. Once heated and slightly cooled, selection (50 $\mu\text{g}/\mu\text{L}$ kanamycin or 100 $\mu\text{g}/\mu\text{L}$) was added to the gel before being poured onto a glass slide-cover glass setup. To achieve a thin layer of agarose gel, a 22 x 22 mm coverslip is first placed onto a 76 x 26 mm glass slide. By doing so, this allows the gel to take on a specific shape rather than spontaneously spreading on the glass slide. From here, roughly 300 μL of the slightly cooled agarose gel spiked with the selection is carefully spread on the cover glass (use of entire volume is not required). Quickly after evenly spreading the agarose onto the cover glass, another cover glass is placed on top of the agarose, effectively sandwiching it to become a thin layer. When the agarose is completely cooled and solidified (1-3 minutes), the top cover glass is removed and a blade is used to cut the gel into even square pads. Next, 1 μL of prepared culture is pipetted and spread onto each agar pad and

left to absorb for 20 minutes. Inadequate time for cell absorption leads to poorly trapped cells. Using a prepared WillCo Well (WillCo Wells KIT-3512) dish, the cell-absorbed agar pads are flipped and placed onto the glass bottom with the culture side facing down. Once the agar pads are placed on the dish, 10 μ L of MQ water is spread in aliquots around the edges of the dish to promote moisture and prevent the gel from drying out too quickly. To maintain humid conditions in the dish, a lid is placed on top with the sides secured by Parafilm (Millipore Sigma P7543).

Instead of an agarose pad, the novobiocin experiments require the use of a CellASIC ONIX B04A (Millipore Sigma B04A-03) plate to provide a consistent flow of treated media over a long duration of time. To set up the CellASIC plate, the plate is carefully removed from its packaging to avoid spills of pre-filled PBS buffer. To fill the plate accordingly, the culture, media, and waste columns are emptied of PBS. Since we are treating the cells with only novobiocin, the very first column is emptied, however, the number of columns can be altered depending on how many types of media is intended to be used. Once emptied, the very first column is loaded with 200 μ L of different novobiocin concentrations in LB media from ascending order starting from the top (low to high) while the cell culture column is loaded with 200 μ L of prepared cell culture. The waste column remains empty. Once the plate is accurately assembled, it is connected to the manifold of the CellASIC Onix2 Microfluidic System where it is vacuum sealed to allow proper control of culture and media flow in the central cell chamber.

3. Microscopy Protocol

All microscopy experiments were conducted using the Olympus IX83 Inverted Microscope coupled with the Tokai heating chamber to allow for time-lapse visualization of cell activity. Using the 100x objective, the Tat and *bfm* strains incubated at 37°C and were visualized using four different channels: bright field, mCherry (excitation/emission: 565-585/600-690nm), GFP (excitation/emission: 460-480/495-540nm), and CFP (excitation/emission: 436/480nm). Visualization of the RFP-CFP strains were incubated at 29°C and only had three channels: bright field, mCherry, and CFP. For the agar pad experiments, images were taken with the cellSens software with imaging intervals of 20 minutes for 22 cycles, resulting in a 7-hour run. For the CellASIC plate experiments, images were taken every 20 minutes for 37 cycles, resulting in a 12-hour run. Due to the larger surface area and constant media flow, CellASIC supports longer time-lapse imaging than agar pads. On the other hand, agar pad microscopy has stricter time limits due to eventual overgrowth of cells and risk of agar dehydration over time.

D. Plate Reader Experiment for Non-Lethal Novobiocin Concentrations

For proper observation of novobiocin treatments without risks to cell viability, a plate reader experiment was conducted to determine the highest, non-lethal novobiocin concentration required for analyzing cell expression. Cell cultures were prepared as described in the cell culture conditions but with minor changes. Rather than diluting to an OD₆₀₀ of 0.01 for cell passaging, overnight cultures were diluted to an OD₆₀₀ of 0.05 and left to incubate until they hit an OD₆₀₀ of 0.4-0.6. This is significant for novobiocin applications because an OD₆₀₀ of 0.4-0.6 commonly represents the cells in log-phase, a time in which cell division is high,

allowing novobiocin to work more effectively against gyrase. At the same time, a 96well culture plate was also prepared. In order to avoid the edge-effect phenomenon common in plate reader experiments, water was loaded in the first and last columns (1 and 12) and the first and last rows (A and H) of the plate to create a border. The remaining wells are used for our samples. Different novobiocin concentrations were tested by doing a series of 20, 1:2 dilutions starting with a working concentration of 1 *mM* novobiocin. To effectively execute this layout, 19 wells were loaded with 125 μL of prepared culture, making sure to leave the first well empty. Separately, a 1 *mM* novobiocin sample was made using the same prepared culture, which is used to start our series of dilutions. 1:2 dilutions were performed by simply taking an equal volume of the 1 *mM* novobiocin-treated cells as the loaded wells (125 μL) and mixing them together. This step is repeated by using the newly formed novobiocin treated samples and adding equal volumes to the next previously prepared culture well. Lastly, the very first well is loaded with our working 1 *mM* concentration.

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