Effect of antimicrobial agents on MinD protein oscillations in *Escherichia coli*

by

Corey Kelly

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EFFECT OF ANTIMICROBIAL AGENTS ON MIND PROTEIN OSCILLATIONS IN \textit{ESCHERICHIA COLI}

Corey Kelly  
University of Guelph, 2011  
Advisor: John Dutcher

The Min protein system regulates cell division in the bacterium \textit{Escherichia coli}. The protein MinD undergoes a pole-to-pole oscillation, antagonizing formation of the division septum at the cell poles, thereby confining the septum formation to the mid-cell. The MinD oscillation period is 40 s at room temperature in healthy cells, but has been shown to be sensitive to stress on the cell. By fluorescently labeling MinD with green fluorescent protein (GFP), we are able to measure the MinD oscillation period as an \textit{in situ} metric of cell viability using high resolution total internal reflection fluorescence (TIRF) microscopy.

We have made several improvements to the method by which we measure and analyse the MinD oscillation period. A microscopy flow cell was designed and constructed and it provides temperature control and stability to a precision of ±0.05 °C in addition to allowing controlled addition of bacterial cells and reagents of interest to the imaging region of the flow cell. This flow cell enabled us to make a precise measurement of the temperature dependence of the MinD oscillation pe-
period, for which we observed an Arrhenius dependence with an activation energy of 11.8 kcal/mol. We developed a centroid-tracking method, performed in a custom MATLAB program, to extract the values of the MinD oscillation periods from our time series of TIRF microscopy images.

We measured the effect on the MinD oscillation period of exposure to the cationic antimicrobial peptide polymyxin B (PMB) and the related compound polymyxin B nonapeptide (PMBN), which does not exhibit antimicrobial activity. Exposure to PMB resulted in a 50% increase in the average MinD oscillation period $\tau$, while exposure to PMBN resulted in an 20% decrease in $\tau$. After exposure to PMB and PMBN, we measured the Arrhenius temperature dependence of the MinD temperature dependence, and calculated the associated activation energy $E_A$. We found that exposure to PMB resulted in a 45% increase in $E_A$, whereas exposure to PMBN did not significantly change the value of $E_A$. These results indicate that careful measurements of the MinD oscillation can yield information that can be helpful in evaluating the mechanism of action of antimicrobial compounds.
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Chapter 1

Introduction and Background

Biological systems have many intricate structures and mechanisms that are truly fascinating examples of Nature’s nanotechnology. A casual look through the classic textbook “Molecular Biology of the Cell” [1] provides many examples that inspire much of the current efforts in biological physics or quantitative biology. One striking example is the self-assembly of a variety of proteins within bacterial cells that control cell division, both spatially and temporally. In the rod-shaped bacterium *Escherichia coli* (*E. coli*), an important part of the cell division process is the oscillation of Min proteins along the major axis of the cell [2]. In this study, we have measured the Min protein oscillations using high resolution fluorescent microscopy, and examined the effect of antimicrobial compounds on the oscillation period.

In this chapter, I will first introduce the Min protein system and discuss the genetically modified bacterial strain that is used in our study. I will then describe the antimicrobial agents that are used in this study. Finally, I will outline the theory
of the specialized fluorescence microscopy technique used in this study.

1.1 The Min Protein System

The Min protein system is essential for proper cell division in *E. coli*. Consisting of three proteins, MinC, MinD, and MinE, the Min protein system ensures equal separation of genetic material to the daughter cells by confining the formation of the division septum to mid-cell [2]. Early studies identified a region of the bacterial chromosome which, when mutated, would often result in minicelling (asymmetric cell division resulting in small, spherical, chromosomally deficient cells) [3]. This region, which codes for the three Min proteins, became known as the *minB* locus (*min*, for minicell) and was mapped in 1983 [4], and subsequently isolated and characterized in 1988 [5].

The Min system functions by antagonizing local polymerization of FtsZ, the constituent protein of the Z ring, which serves as a scaffold for the cell division machinery [2]. A schematic diagram of the Min system is shown in Figure 1.1. MinD complexes with ATP in the cytoplasm, then binds to the cytoplasmic membrane at one pole of the cell, undergoing a surface-assisted dimerization. Dimers of MinC are recruited to the membrane by MinD, and are responsible for locally inhibiting FtsZ polymerization. Early in the division process, MinE binds to the membrane in a circumferential ring at the edge of the MinD polar zone, near mid-cell. At the edge of the polar zone, MinE displaces MinC and stimulates hydrolysis of the MinD-bound ATP, causing the release of MinD from the membrane. This process continues until the entire polar concentration of MinD has been released into the
Figure 1.1: Schematic diagram of the Min system. MinD is shown in green, MinE in red, and FtsZ in blue. Lettered circles correspond to proteins which are diffusing through the cytoplasm, while the rings and “horseshoe” shaped regions indicate proteins that are bound to the cytoplasmic membrane (adapted from [6]).
cytoplasm. MinD then reassembles at the opposite pole of the cell, where the process repeats. This oscillatory behaviour occurs with a period of approximately 40 s in healthy cells at room temperature. The mid-cell concentration of MinE prevents local membrane association of MinD, thereby allowing the local formation of the Z ring, and ultimately, the division septum [2].

A significant body of computational studies have attempted to reproduce the experimentally observed behaviour of the Min system. These models all involve reaction-diffusion equations, with reaction rates representing the association of the Min proteins with each other, and the cytoplasmic membrane. These models typically used known diffusion constants from other proteins, such as maltose-binding protein [7]. Several first-generation models reproduced the oscillatory behaviour of MinD, but required ad hoc assumptions such as the constant synthesis and degradation of MinD and MinE proteins [8]; MinE being driven onto the membrane by cytoplasmic MinD [7]; or non-physical membrane diffusion rates of MinD [9]. First generation models also largely failed to produce the mid-cell MinE ring without ad hoc modifications, such as enhancing MinD interaction with a specific concentration of MinE [8].

A second generation model by Huang et al. that used only reactions which have been directly observed in vitro resolved many of these issues [10]. The Huang model was also the first to calculate so-called “zebra-striped” multiple-wavelength patterns of MinD which had been previously observed experimentally [11].

Figure 1.2 illustrates the model proposed by Huang et al. [10] for the interactions resulting in the MinD oscillation. The rate constants used in this model indicate that the cycle is rate-limited by the hydrolysis of MinD-bound ATP on the
Figure 1.2: The model of Huang et al. for the MinD protein oscillation. 1) MinD:ATP binds to the cytoplasmic membrane. 2) MinE binds to the MinD:ATP complex. 3) MinE activates the hydrolysis of the MinD-bound ATP, releasing a phosphate ion, the MinE, and the resultant MinD:ADP complex into the cytoplasm. 4) MinD:ADP undergoes nucleotide exchange resulting in MinD:ATP, which is then able to re-bind to the cytoplasmic membrane (adapted from [10]).
cytoplasmic membrane, and the subsequent nucleotide exchange required to convert cytoplasmic MinD:ADP to MinD:ATP.

An important outstanding question regarding the Min protein system is the origin of the topological specificity of MinD (binding preferentially to areas of high negative membrane curvature [12]). There are two prevailing theories which attempt to explain this specificity. First, certain phospholipids, such as cardiolipin are known to localise to regions of high membrane curvature, and may provide preferential membrane binding sites for MinD [13]. Another theory, which has been explored both computationally [14, 15] and experimentally [16, 17], suggests that MinD polymerizes to form large, spiral-shaped structures which would be sufficiently large to directly sense and respond to membrane curvature. Experimental evidence supporting either of these theories has, thus far, been largely indirect or obtained using in vitro studies [12, 13, 18, 16].

1.2 Bacterial Strain

To study the Min system, the laboratory of Piet de Boer has developed many strains of *E. coli* with mutations to the *minB* locus [5, 19]. In 1999, Raskin and de Boer described several strains which incorporated the expression of green fluorescent protein (GFP), attached to either MinD or MinE, to allow visualization of the proteins using fluorescence microscopy [11]. One strain from the study by Raskin and de Boer, PB103/λDR122, was used in the present study.

The λDR122 phage, *imm*\(^{21}\) *bla*\(^{+}\) *lacI*\(^{q+}\) P\(_{lac}\)::*gfpmut2-minD minE*, contains the gene *bla*\(^{+}\), which confers ampicillin resistance upon the cells (the importance of
which is discussed in Section 2.1.1. More importantly, \( P_{\text{lac}}::gfpmut2-minD\ \text{minE} \)
codes for the protein GFPmut2 (an especially bright mutant of GFP [20]) fused to
the complete MinD protein, as well as the entire MinE protein. The inclusion of this
recombinant MinE in addition to the native MinE is necessary due to the sensitiv-
ity of the MinD oscillation period to the ratio of MinD to MinE [21]. Since the GFP
fusion protein and the recombinant MinE are downstream of the \( lac \) promoter, \( P_{\text{lac}} \),
the proteins are only synthesized in the presence of lactose (or lactose analogs, see
Section 2.1.1), allowing for precise control of when and to what extent the proteins
are expressed.

1.3 Polymyxin B

Polymyxin B (PMB) is a cyclic cationic (+5e) peptide obtained from the bacterium
\( Bacillus polymyxa \). PMB exhibits antimicrobial activity against most Gram-negative
bacteria at relatively low concentrations [22]. The structure of PMB is illustrated in
Figure 1.3. The mechanism of action of PMB is a two-stage process that has been
extensively studied [23, 24, 25]. First, PMB binds to the negatively charged lipid A
headgroup of the lipopolysaccharides in the outer membrane of the Gram-negative
bacterium, and increases the permeability of the membrane. The fatty acid tail of
PMB is then able to insert into the outer membrane, allowing the PMB molecule to
pass through it. The PMB molecule can then bind with the cytoplasmic membrane,
lysing the cell.

A related compound, polymyxin B nonapeptide (PMBN) is produced via en-
zymatic processing in which the fatty acid portion of the molecule is cleaved [26]
Figure 1.3: The structures of polymyxin B (top, charge +5e) and polymyxin B nonapeptide (bottom, charge +4e). The fatty acid chain which differentiates the compounds is shown in red.
This modification of the PMB molecule removes the ability of the compound to pass through the outer cell membrane, and as a result, PMBN cannot lyse the cell. Recently, Sahalan et al. [27] studied the release of periplasmic and cytoplasmic proteins in the presence of PMB and PMBN, showing that PMBN permeabilized the outer cell membrane, but did not compromise the cytoplasmic membrane.

1.4 Total Internal Reflection Fluorescence (TIRF) Microscopy

1.4.1 Total Internal Reflection

The change in direction of an electromagnetic wave as it propagates across a boundary between two media with different indices of refraction is described by Snell’s law. The law is a consequence of the difference of the speed of light in the two different media. Mathematically, Snell’s law is written as

\[ n_i \sin \theta_i = n_t \sin \theta_t \]  \hspace{1cm} (1.1)

where \( \theta_i \) is the angle of incidence, \( \theta_t \) is the angle of refraction and \( n_i \) and \( n_t \) are the indices of refraction of the incident and transmitted media, respectively. Figure 1.4 illustrates this relationship. When a wave passes from a medium of higher refractive index to one of lower refractive index \( (n_i > n_t) \), there will be an angle of incidence \( \theta_c \) for which the angle of refraction is \( 90^\circ \), and no light propagates into the refractive medium. This phenomenon is known as total internal reflection. By
Figure 1.4: Illustration of Snell’s law (Equation 1.1). The light, propagating in a medium with index of refraction $n_i$, is incident on the interface at angle $\theta_i$ (measured with respect to the normal to the interface.) The light, which is transmitted into the second medium with index of refraction $n_t$, is refracted such that it travels at an angle $\theta_t$ with respect to the normal to the interface.
setting $\theta_i = 90^\circ$ in Equation 1.1, the condition for total internal reflection is given by

$$\theta_c = \arcsin \left( \frac{n_t}{n_i} \right).$$

(1.2)

For all angles of incidence $\theta_i \geq \theta_c$, the wave is totally internally reflected. While none of the incident light propagates within the transmitted medium, the electric and magnetic fields associated with the wave cannot be discontinuous at the interface, so an evanescent wave must exist at the boundary, decaying away from the interface into the second medium. The evanescent wave can be described by its electric field, $E_t$, which has the form

$$E_t = E_0 \exp (-\kappa z) \exp \left[ i \left( kx - \frac{2\pi ct}{\lambda} \right) \right]$$

(1.3)

where

$$k = \frac{2\pi n_i}{\lambda} \sin \theta_i,$$

(1.4)

$$\kappa = \frac{2\pi}{\lambda} \sqrt{(n_i \sin \theta_i)^2 - n_t^2},$$

(1.5)

$\lambda$ is the wavelength of the light, $c$ is the speed of light in a vacuum, $z$ is the perpendicular distance from the interface, and $x$ is the distance measured along the interface. The intensity of the evanescent wave decays exponentially with $z$ as

$$I(z) = E_t^2 \sim I(0) e^{-z/d}$$

(1.6)

where $d$ is the penetration depth of the evanescent wave, given by

$$d(\theta_i, \lambda) = \frac{\lambda}{4\pi n_t \sqrt{\left( \frac{\sin \theta_i}{\sin \theta_c} \right)^2 - 1}}.$$  

(1.7)

A calculation of the penetration depth for the instrument used in the present study is given in Section 2.2.3.
1.4.2 Fluorescence

Certain molecular structures can be excited to higher quantum states by the absorption of electromagnetic radiation (light). The change in the energy of the system is quantized and equal to the energy of the absorbed photon $h\frac{c}{\lambda_i}$, where $h$ is Planck’s constant, and $\lambda_i$ is the photon wavelength. The system can then relax to the initial (ground) state by releasing a photon of energy

$$E_f = h\frac{c}{\lambda_i} - \Delta E$$

where $\Delta E$ is the energy lost to the system as heat. This energy loss results in the emitted photon being of lower energy (and longer wavelength) than the absorbed photon. This absorption and re-emission at a longer wavelength is known as fluorescence, and the region of a molecule that exhibits fluorescence is known as a fluorophore.

Fluorescence is the basis of many experimental techniques. In particular it serves as the basis of fluorescence microscopy, which describes a variety of microscopy techniques in which a sample containing fluorophores is illuminated with a (typically monochromatic) light source that can excite the fluorophores, and the outgoing light is filtered to measure only the fluorescent emission. Fluorescence microscopy is useful for two main reasons. First, since the measured outgoing light is emitted by fluorophores within the sample, precise attachment of fluorescent particles (or molecules) within the sample can allow the determination of the location of moieties of interest. This can be accomplished on the micron-scale using fluorescent colloidal particles, and on the nanoscale using fluorescent molecules or quantum dots. Second, since fluorophore emission is highly sensitive
to the surrounding environment, specialized fluorescence techniques can directly probe the molecular environment on the nanoscale [28].

The introduction of fluorophores into a sample can be achieved either by chemically modifying the molecule to attach it to a specific site in the system of interest, or by genetically modifying a living specimen to produce fluorescent molecules, as is the case with the expression of GFP. GFP is a 27 kDa fluorescent protein with an excitation peak at $\lambda = 400 \text{ nm}$ and an emission peak at $\lambda = 500 \text{ nm}$ [29].

1.4.3 TIRF

To measure the MinD oscillations in *E. coli* cells, we use a technique known as total internal reflection fluorescence (TIRF) microscopy. In the TIRF experiment, the excitation light is incident on the sample at a sufficiently large angle such that the light is totally internally reflected, creating an exponentially decaying evanescent wave at the sample-coverslip interface, as illustrated schematically in Figure 1.5. The evanescent wave excites fluorophores only within a small distance ($\sim 100 \text{ nm}$) of the coverslip surface, which significantly increases the contrast relative to that in epifluorescence techniques, in which all fluorophores within the sample are excited. In the present study, we excite the fluorescence by using a microscope objective that is designed to achieve total internal reflection of the excitation light. This results in the excitation of fluorophores only in the lower surface of bacterial cells that are adhered to the coverslip surface. In Figure 1.6 is shown the same region of a bacterial sample imaged using TIRF and epifluorescence illustrating the improvement in contrast in the TIRF images.
Figure 1.5: A schematic (not to scale) diagram of TIRF microscopy. The angle of incidence $\theta$ is chosen to be larger than $\theta_c$ (see Equation 1.2) such that the incident light (blue) is totally internally reflected. This gives rise to an exponentially decaying evanescent wave at the sample-coverslip interface, which excites fluorescence only in fluorophores that are within approximately 100 nm of the coverslip surface. The fluorescent light (green) is collected and sent to a high sensitivity CCD camera.
Figure 1.6: Fluorescence microscopy images of the same region of the flow cell in which many fluorescently-tagged *E. coli* cells are visible. The images were collected using epifluorescence (top) and TIRF (bottom). One can see that there is a significant increase in the contrast between the cells and the background in the TIRF image. The scale bar corresponds to 5 µm.
1.5 Scope of Thesis

This thesis has two main goals. First, we seek to refine the process by which the MinD oscillation period is measured and analysed. We use high resolution TIRF microscopy to improve the spatial resolution of our fluorescence images. To make use of these improved images, we develop a MATLAB program that can be used to perform high throughput image analysis on large populations of cells simultaneously. To streamline the experimental procedure and gain precise control over the introduction of reagents to the experiment, we design and build a microscopy flow cell with high precision temperature control. In addition to stabilizing the temperature at which an experiment is performed, precise temperature control allows us to perform temperature-dependence studies.

The second goal of this thesis is to study the effect on the MinD oscillation period of exposure to the antimicrobial agent polymyxin B (PMB) and the related compound polymyxin B nonapeptide (PMBN). This experiment serves not only to study the mechanism of action of these compounds, but also to gain insight into outstanding issues regarding the mechanism of the Min protein system itself.
Chapter 2

Materials and Methods

2.1 Biological Protocols

2.1.1 Bacterial Growth Procedure

Agar plates were prepared from powdered LB agar (Difco #240110) with 100 µg ml\(^{-1}\) of the antibiotic ampicillin (ampicillin sodium salt, Sigma #A9518) added after autoclaving and before pouring. The ampicillin resistance of strain PB103/λDR122 (see Section 1.2) allows it to grow normally in the presence of ampicillin, while other (contaminating) strains cannot. A single colony from the plate was transferred to lysogeny broth (LB, Difco #244620) and grown overnight to late exponential phase. 1 ml from the overnight culture was then transferred to fresh LB containing 50 µmol l\(^{-1}\) of the lactose analog isopropyl β-D-1-thiogalactopyranoside (IPTG, Sigma #I6758) and grown for 5 h. The presence of IPTG triggers the expression of the recombinant (GFP-tagged) MinD proteins. Because of this, the duration of the second growth phase must be carefully monitored to yield a reproducible
amount of protein expression in each experiment.

Before performing the MinD oscillation measurement, the cells were resuspended in phosphate buffered saline (PBS) such that a pH value of 7 was maintained. The resuspension was accomplished by centrifuging 1 ml of the final culture to create a pellet of the bacteria, pipetting off the supernatant and adding 800 µl PBS. The resuspension was then repeated such that upwards of 96% of the growth medium was removed.

2.2 Imaging and Flow Cell

One of the main goals of this thesis was to improve the instrumentation and methods used to measure the MinD oscillation period in *E. coli* bacteria. This has resulted in several improvements, including the design and construction of a specialized flow cell (Section 2.2.1), the use of high-resolution total internal reflection fluorescence (TIRF) microscopy (Section 1.4.3) using a high magnification (150×) TIRF objective lens, and the development of a novel, high-throughput method for analyzing the microscopy images (Section 3.1).

2.2.1 Custom Flow Cell

Our MinD oscillation experiments were performed using a specialized flow cell which was designed in collaboration with Mr. Steve Wilson of the Department of Physics machine shop at the University of Guelph. The flow cell was then machined by Mr. Wilson.

The flow cell is designed to fit onto the translation stage of our Olympus IX71
inverted optical microscope, and is compatible with the short working distance (0.1 mm) of our 150× TIRF lens. Photographs of the flow cell are shown in Figure 2.1.

The flow cell consists of three metal blocks B1, B2 and B3. The flow path begins at the inlet port (i), then passes through block B2 to the region between block B2 and gasket G1, where a serpentine flow path (ii to iii, visible in Figure 2.1 frame 2, with block B1 and gasket G1 removed) allows the fluid to equilibrate with the temperature of the flow cell. The length of the serpentine flow path was chosen to be large enough such that the temperature of the fluid as it reaches the imaging region of the cell was within 0.1 °C of the set point of temperature controller, based on heat transfer and fluid flow equations. After passing through block B2, the fluid enters the imaging region of the cell (iv to v) which is defined by block B2, gasket G2, and the microscope coverslip. The coverslip fits into the rectangular region visible in frame 4 of Figure 2.1, and is pressed against gasket G2 by block B3, forming a seal. After passing through the imaging region of the cell, the fluid passes through block B2 to the outlet port (vi).

The flow cell shown in Figure 2.1 was designed specifically to be compatible with experiments on biological samples. The main block of the cell (B2) is made of titanium, which discourages bacterial adhesion and allows it to be autoclaved between experiments. Blocks B1 and B3 do not contact the fluid at any point, and are made of aluminium. The two gaskets G1 and G2 that define the flow regions of the flow cell are made of Viton (Goodfellow, #FV313100), a synthetic rubber that is chemically resistant and does not leech into the fluid within the flow cell. To this end, we also use tubing and connectors made of polyether ether ketone.
Figure 2.1: Images of the flow cell. Image 1 shows the fully-assembled flow cell on the microscope sample stage. Frames 1 and 2 show top-down views of the cell, while frames 3 and 4 show bottom-up views. The flow path is indicated by sequential Roman numerals, i through vi, with a red arrow indicating the direction of flow in the imaging region of the cell. An explanation of the remaining labels is given in the text.
(PEEK, Idex Health and Science products 1533L, P-200Nx, and XP-235x). Between experiments, all gaskets and connectors were cleaned by soaking in anhydrous ethanol, and the tubing was discarded.

The flow cell is temperature-stabilized using two 100 W cartridge heaters (C) (Chromalox, #CIR-1031/120V) and a type-J thermocouple (T), that are connected to a Eurotherm 808 proportional-integral-derivative (PID) controller. The PID parameters, which were determined using a tuning algorithm within the controller, are $P = 8.4$, $I = 54$ and $D = 0$. These parameter values resulted in the temperature control of the flow cell to within $\pm 0.05 \degree C$, with no measurable overshoot with respect to the set point temperature.

### 2.2.2 Adhesion Layers

For surface sensitive techniques such as those employed in this study, measurements can only be made on bacterial cells which are located at the coverslip interface. In particular, for measurements of a dynamical system such as the MinD protein oscillation, it is necessary that the bacterium of interest be completely immobilized on the coverslip for the duration of the experiment.

To facilitate bacterial adhesion, we coated our microscope coverslips with a layer of the cationic polymer poly-L-lysine (PLL, Sigma, #P4707). Since the coverslip glass and the bacterial cell membrane are negatively charged, the positively charged polymer adheres strongly to both.

Prior to applying the adhesion polymer, the coverslips were cleaned by acid washing for 2 h, then thoroughly rinsed with deionized water. Several drops of
0.01 % PLL solution were placed on the coverslip using a pipette. After 20 minutes, the PLL solution was rinsed with deionized water, and the coverslips were dried under a dry nitrogen stream. New coverslips were prepared for each experiment, and used within several hours of applying the adhesion layer.

### 2.2.3 Total Internal Reflection Fluorescence (TIRF) Microscopy

Microscopy images were acquired using an Olympus IX71 inverted optical microscope with a 150× magnification TIRF objective lens. This lens has greatly improved our imaging resolution over the 60× lens that we have previously used, as can be seen by comparing the images shown in Figure 2.2. Fluorescence of the GFP in the GFP-tagged MinD molecules was excited with a 20 mW Spectra Physics Cyan (λ = 488 nm) Laser System, and images were captured with a high sensitivity, 1.4 megapixel CCD camera (Retiga EXi) using Image Pro Plus (version 7.0.1.658) software. We measured the laser power at the microscope objective to be 1.25 ± 0.05 mW. To reduce photobleaching of the GFP fluorophores, we limited the exposure time to 200 ms and attenuated the laser light intensity by a factor of four using neutral density filters such that the laser power at the sample is ∼0.3 mW. Almost all time sequences of images used in this study contained 50 images, taken at 4 s intervals. This density of data points minimizes photobleaching while still spanning several MinD oscillation periods allowing an accurate measure of the oscillation period. This places a lower limit of several minutes on the time between consecutive measurements of the oscillation period.

In Figure 2.3 we show a plot of the theoretical penetration depth for the TIRF
Figure 2.2: TIRF microscopy images of a single cell collected using a 60× (left) and 150× (right) TIRF objective lens. The image collected using the 150× lens shows the presence of a “horseshoe”-shaped localization of fluorescent intensity at the cell poles that was not seen in the images collected using the 60× lens. The height of each image corresponds to 2.6 µm.
setup in the present study, based on Equation 1.7. Following the adjustment of the microscope to achieve the TIRF condition, we estimate that the penetration depth is approximately 150 nm to 200 nm.

2.3 General Experimental Procedure

The use of a flow cell allowed us to simplify our experimental procedure since it allowed the successive introduction of reagents and bacterial cells. Experiments in the current study were carried out using the following procedure.

After the flow cell was assembled and placed on the microscope stage, the input flow connector was attached to a syringe pump (KD Scientific, #KDS100) and the flow cell was filled with PBS. During this initial flow, the time for the solution to reach the centre of the imaging region was measured. When subsequently introducing solutions, this transit time was used to precisely identify the time at which the solutions reached the imaging region. The temperature controller was activated and after several minutes the temperature of the flow cell was stable: 25.00 ± 0.05 °C. A syringe containing bacteria in PBS (see Section 2.1.1) was placed on the syringe pump, and the solution was pumped into the cell. Once the bacterial solution reached the imaging region of the cell, the flow was turned off, and several minutes were allowed for the bacteria to adhere to the coverslip. During this time, we acquired TIRF microscopy images to monitor the surface density of bacteria adhered to the coverslip. Our image analysis method requires the bacteria to be isolated from neighbouring cells (see Section 3.1 for details) so when the coverage of bacterial cells became sufficiently large so that they began to aggregate,
Figure 2.3: The theoretical penetration depth $d$ as a function of angle of incidence for the TIRF setup in the present study. The blue curve was calculated using Equations 1.2 and 1.7 with $n_i = 1.52$ (coverslip glass), $n_t = 1.33$ (water) and $\lambda = 488$ nm. The vertical line corresponds to $\theta_i = \theta_c$, and the horizontal line is $d(\theta_i = 90^\circ) = 52.8$ nm.
we flushed the bacterial solution with fresh PBS. We then took several TIRF microscopy image sequences, which provided a measure of the MinD oscillation period of “healthy” cells.

At this point, the individual experiments diverged. For temperature dependence studies, we simply increased the temperature in fixed increments, allowing several minutes for stabilization at each temperature. For studies of exposure to antimicrobial compounds, we flowed solutions of either PMB (Polymyxin B sulfate salt, Sigma #P4932) or PMBN (Polymyxin B nonapeptide hydrochloride, Sigma #P2076) dissolved in PBS into the flow cell. We began acquiring images when the solution reached the centre of the imaging region, but continued flowing solution for several minutes, ensuring that the entire flow region was filled.
Chapter 3

Image Analysis

The TIRF microscopy images of *E. coli* cells undergoing MinD protein oscillations typically contain many cells oscillating independently. To calculate the average value of the oscillation period, it is necessary to analyze the time series of images for individual cells and then to calculate the average value and associated experimental uncertainty. In this Chapter, we describe the techniques that we have developed to efficiently analyze the data.

In previous studies [30, 31], image analysis has been performed to extract MinD oscillation periods. They tracked the intensity averaged over a circular region centred about one of the bacterial cell poles and fitted the resulting curve to a sinusoidal function to obtain the oscillation period. This analysis procedure provides a good starting point for the methods developed in the present study. Motivated in part by our need for a more high-throughput method for analysing our images, and inspired by the analysis methods of Unai *et al.* [32], we have developed a novel MATLAB-based interface for analysing MinD protein oscillations.
3.1 MATLAB Analysis Code: *Min Analyse*

We selected MATLAB as the programming language because it is straightforward to use MATLAB to process large data sets such as our time series of TIRF microscopy images and also because MATLAB has extensive mathematical libraries and user interface (UI) capabilities. We used MATLAB 7.10.0 (R2010a), along with the Image Processing and Optimization toolboxes.

Our images are stored as 16-bit multipage TIFFs (consisting of three-dimensional matrices of intensity values.) In *Min Analyse*, a time series of images is loaded into memory, and the user can scroll sequentially through all of the individual frames, or display them sequentially in a loop at a user-selected speed. An intensity threshold option is included, which can be used to increase the contrast in the displayed images. We note that enhancing the contrast only affects the display of the images on the screen, and does not change the intensity values that are used in the image analysis procedure. The user is prompted to input the time step (the time between subsequent exposures used when acquiring the microscopy images) and to specify the microscope objective lens magnification, such that the spatial and temporal resolution of the image stack can be determined. A screenshot of the UI displaying this information is shown in Figure 3.1.

To analyze the images, the user then clicks on the image to define regions of interest (ROIs) which contain the cells to be analysed. This allows the user to exclude cells which are not well adhered, or which are irregularly sized or shaped. The criteria that we use to select cells are as follows:

1. The bacterium must lie in the imaging plane. Occasionally, only one bacterial
Figure 3.1: A screenshot of the Min Analyse user interface after an image stack has been loaded into the program. Controls related to the loading and display of the image stack are located above and below the image frame, and all analysis-related controls are located on the right side.
pole adheres to the coverslip, and the cell extends out of the plane of focus.

2. The bacterium must be approximately 2 \( \mu \text{m} \) in length, as is characteristic of \textit{E. coli} in the late exponential phase.

3. The bacterium must be well-isolated from other cells. This ensures that the intensity information obtained from the analysis can be associated with a single cell.

4. The bacterium must not be undergoing division. Cells in which a division septum has begun to form are easily identified and excluded.

5. The bacterium must be rod-shaped. In addition to spherical “mini-cells” which result from asymmetric division, physically damaged cells are often characterized by deviations from the rod shape.

In Figure 3.2 we show several bacteria that illustrate the above criteria. In Figure 3.3 is shown a screenshot of an image with several selected ROIs. ROIs can be translated after their placement, and their size can be modified to accommodate different magnifications of images, or to study cells of different sizes. Each ROI is numbered, and its coordinates are provided in the table at the right of the UI, as shown in Figure 3.1.

In our analysis of the MinD oscillation for each cell, we calculate an intensity-weighted centroid position \( \mathbf{r}_c \) (henceforth referred to simply as the centroid), which is defined as

\[
\mathbf{r}_c = \frac{\sum I_i \mathbf{r}_i}{\sum I_i}
\]
Figure 3.2: TIRF microscopy images illustrating the criteria used for selecting E. coli bacteria for analysis. 1) An appropriate bacterium for analysis: rod-shaped, lying in the imaging plane, and approximately 2 μm in length. 2) An elongated bacterium. This often results from failed division. 3) A bacterium that is adhered to the coverslip only at one pole. Since the MinD proteins oscillate from pole-to-pole, the entire oscillation would not be visible in this image. 4) A cluster of bacterial cells. Using the current analysis method, it is not possible to distinguish fluorescent intensity from cells which touch or overlap. 5) A bacterium which is dividing. The division septum is visible at the mid-point of the cell. 6) A bacterium which is slightly crescent-shaped. Healthy E. coli cells are symmetric and rod-shaped. The height of each image corresponds to 5.8 μm.
where $\mathbf{r}_i$ is the vector pointing to the $i^{th}$ pixel in the image, $I_i$ is the corresponding intensity value, and the summations run over all pixels in the image. This is a common image processing technique that is used to characterize the shape of objects in images [33]. For the analysis of our images, the centroid can be used to track the oscillation of fluorescence intensity corresponding to MinD-GFP. In Figure 3.4, we show a cell with the corresponding centroid indicated at three different times. Within each ROI corresponding to a single cell, the centroid is calculated for each image frame, which allows the tracking of its trajectory as a function of time. Figure 3.5 contains a plot of one such trajectory in which the trajectory is given in terms of the x- and y- coordinates of the image.

Since MinD oscillates from pole to pole and not necessarily along the x- or y-axes, it is better to define the centroid in the frame of reference of the cell, for which the major and minor axes of the bacterium define the axes of the “cell” coordinate system. To transform from “image” coordinates (in which the boundaries of the image define the coordinate axes) to “cell” coordinates, we must first calculate the position of the centre of the bacterium, and the angle between the major axis of the bacterium and the x-axis of the “image” coordinates. These values are calculated using the intensity within the ROI averaged over several periods of the oscillation (typically 4 to 5 periods). By calculating this time-averaged image, we obtain a direct measure of the shape of the cell. The centroid (Equation 3.1) of this time-averaged image corresponds to the geometric centre of the bacterium $\{X_C, Y_C\}$. The angle between the major axis of the bacterium and the x-axis of the “image” coordinates is determined by performing an intensity-weighted least squares fit of the ROI pixels to a straight line. Since the bacteria are rod-shaped, this line of slope
Figure 3.3: A snapshot from *Min Analyse* showing many *E. coli* cells in the TIRF microscopy image and the selection of several ROIs centred on individual *E. coli* cells. The height of the image corresponds to 40 µm.

Figure 3.4: TIRF microscopy images of the same *E. coli* cell at three different times, with the position of the centroid (Equation 3.1) indicated as a white dot. Image height corresponds to 2.6 µm.
Figure 3.5: The intensity-weighted centroid position for a single *E. coli* cell as a function of time. The x and y axes refer to “image” coordinates that are shown on the inset TIRF microscopy image, which is an image that is the average of 50 images during the 200 s measurement time. The white dots indicate the centroid positions at each time.
$m$ will lie along the major axis of the cell, such that the angle of the bacterium $\theta$ is

$$\theta = \tan^{-1} m. \quad (3.2)$$

After the geometric centre of the bacterium $\{X_C, Y_C\}$ and the angle $\theta$ are determined, a point $\{x, y\}$ in image coordinates can be transformed to cell coordinates $\{x', y'\}$ by performing a translation and then a rotation

$$\begin{bmatrix} x' \\ y' \end{bmatrix} = \begin{bmatrix} \cos \theta & \sin \theta \\ -\sin \theta & \cos \theta \end{bmatrix} \left( \begin{bmatrix} x \\ y \end{bmatrix} - \begin{bmatrix} X_C \\ Y_C \end{bmatrix} \right). \quad (3.3)$$

Equation 3.3 is applied to all coordinate pairs in the centroid trajectory, resulting in a trajectory which highlights the pole-to-pole nature of the MinD oscillation. The data shown in Figure 3.5 in “image” coordinates is shown in Figure 3.6 after the transformation to “cell” coordinates.

Since the transformation highlights the pole-to-pole oscillation of intensity within the bacterial cell, it is straightforward to calculate the oscillation period $\tau$. We obtain $\tau$ by fitting the major axis component of the centroid trajectory $y'(t)$ to a general sinusoidal function

$$y'(t) = A \cos \left( \frac{2\pi}{\tau} t - \phi \right) \quad (3.4)$$

where $A$ is the amplitude of the oscillation and $\phi$ is a phase shift. Fits of the data to Equation 3.4 are performed using the MATLAB Optimization Toolbox function fslqnonlin(). This function is used to perform a nonlinear least squares fit using a trust-region-reflective algorithm [34]. The fitting function returns the best fit parameter values for $A$, $\tau$ and $\phi$ together with statistics related to the quality of the fit (such as the coefficient of determination, $R^2$). The centroid trajectory associated
Figure 3.6: The intensity-weighted centroid position for a single *E. coli* cell as a function of time. This is the same dataset as shown in Figure 3.5, replotted after calculating the transformation described in Equation 3.3. The pole-to-pole nature of the MinD oscillation is evident in the major axis component of the trajectory. The inset TIRF microscopy image is a time-averaged image of the cell being measured (averaged over 200 s.) The white dots indicate the centroid positions at each time, and the “cell” coordinate axes are shown in red.
with each ROI can be plotted along with the best fit curve within the Min Analyse program, and the $\tau$ values are output to the table on the right side of the UI. The user can then save the contents of the table (ROI coordinates, $\tau$ values, and the associated $R^2$ values) to a text file for further analysis. Figure 3.7 shows the major-axis data from Figure 3.6, replotted along with the corresponding best fit curve.

### 3.2 Statistics

For an objective lens magnification of $150 \times$, the number of bacterial cells in one image, $N$, is usually of the order of 100. A typical distribution of measured $\tau$ values is shown in Figure 3.8. Although the distributions are often broad, as indicated by standard deviation values $\sigma$ that can be as large as 20% of the mean value, the average value of each distribution is well-defined. To properly estimate the error in a given measurement, we characterize it by the average value of $\tau$, $\tau_{AVG}$, and the standard error $\sigma_{\tau}$ which is defined as

$$\sigma_{\tau} = \frac{\sigma}{\sqrt{N}}.$$  

(3.5)

By using the standard error as a measure of the uncertainty in the average value, we obtain uncertainties which take into account the large number, $N$, of measurements. For the data plotted in Figure 3.8, the calculated value of $\tau_{AVG}$ is $42 \pm 1$ s.

Since the MinD oscillation period is highly sensitive to environmental factors [31, 30, 35], small differences in experimental conditions can cause measurable differences in the best fit $\tau$ values. To facilitate comparisons between different experi-
Figure 3.7: The intensity-weighted centroid position for a single *E. coli* cell as a function of time. These are the major-axis data shown in Figure 3.6, plotted along with the best fit curve, as determined by the *Min Analyse* program. The fit parameters are $A = 0.27 \, \mu m$, $\tau = 38.8 \, s$, $\phi = 0.37 \, \text{rad}$ and $R^2 = 0.89$. 
Figure 3.8: A histogram of 88 measured $\tau$ values from a time series of TIRF microscopy images of *E. coli* cells at 30°C, using a bin width of 2 s. For this distribution, $\tau_{AVG} = 42$ s and $\sigma_\tau = 1$ s.
ments, we consider *relative* changes in the oscillation period. For example, at the beginning of an experiment in which the environmental conditions are changed, an image series of the cells is acquired and the best fit $\tau$ value is used as a reference value for all subsequently measured $\tau$ values.
Chapter 4

Results and Discussion

4.1 Initial Tests and Measurements

4.1.1 Temperature Dependence of MinD Oscillation Period

As an initial test of our temperature-controlled flow cell, we have measured the temperature dependence of the MinD oscillation period in the range of 25°C to 45°C. In Figure 4.1 is shown a plot of the normalized MinD oscillation period as a function of temperature. We note that the images acquired above 35°C required the time between images to be decreased from 5 s to 1 s to properly fit the MinD oscillation to Equation 3.4, since the period value is much smaller at these temperatures. Touhami et al. [35] measured the temperature dependence of the MinD oscillation period in the same strain of *E. coli* and analysed the data in terms of an Arrhenius relationship

\[ k = k_0 \exp \left( -\frac{E_A}{k_B T} \right) \]  

(4.1)
Figure 4.1: The normalized MinD oscillation period as a function of temperature. Initially, the value of the oscillation period was measured at 25 °C ($\tau_{AVG} = 49 \text{ s}$) and then the temperature was increased in increments of 3 °C, allowing several minutes between measurements to ensure stabilization of the temperature. Temperature values were stable to within ±0.05 °C over the course of each measurement. Each data point corresponds to at least 40 bacterial cells.
where \( k \) is a reaction rate constant, \( k_0 \) is a constant, \( E_A \) is an activation energy, \( k_B \) is Boltzmann’s constant and \( T \) is the absolute temperature. By defining a rate constant \( k \) that is proportional to the inverse of the MinD oscillation period

\[
k = \frac{B}{\tau}
\]

(4.2)

where \( B \) is a constant of proportionality, and then taking the natural logarithm of both sides of Equation 4.1, Equation 4.1 can be rewritten as

\[
\ln \left( \frac{1}{\tau} \right) = -\frac{E_A}{k_B} \cdot \frac{1}{T} + C
\]

(4.3)

where \( C \) is a constant defined as

\[
C = \ln \left( \frac{k_0}{B} \right).
\]

(4.4)

The form of Equation 4.3 allows one to plot \( \ln(1/\tau) \) against \( 1/T \) to obtain a straight line with a slope equal to \( E_A/k_B \). This type of plot is commonly referred to as an Arrhenius plot. Figure 4.2 shows an Arrhenius plot of the data from Figure 4.1 together with the best fit straight line. The slope of the best fit straight line is \(-5960 \text{ K}\) which corresponds to an activation energy of 11.8 kcal/mol (0.51 eV), based on Equation 4.3. This is consistent, to within a factor of two, with the value of 20 kcal/mol obtained by Touhami et al. [35] under less well-controlled conditions, and is also equal to the free energy of a single ATP hydrolysis event, 11.5 kcal/mol [36]. This supports the conclusion of Touhami et al. that the MinD temperature dependence is due to temperature-dependent hydrolysis of ATP, and is also consistent with the model of Huang et al., which suggested that the hydrolysis of ATP and subsequent nucleotide exchange are the rate-limiting steps in the MinD oscillation [10].
Figure 4.2: An Arrhenius plot of the data from Figure 4.1 along with the best fit straight line. The best fit line has an intercept of 16.1, a slope of $-5960\,\text{K}$ and $R^2 = 0.99$. The slope of the best fit line can be used to calculate the activation energy of the process associated with the temperature dependence, as determined by Equation 4.3.
4.1.2 Dependence of MinD Oscillation Period on PMB Concentration

After measuring the MinD oscillation period in PBS, we introduced the minimum inhibitory concentration (MIC, the lowest concentration known to inhibit bacterial growth) of PMB, 50 mg L\(^{-1}\) in PBS, to the extracellular environment using our flow cell, and we measured the effect on the MinD oscillation period. In Figure 4.3 are shown histograms of the measured \(\tau\) values before and after exposure to PMB, together with fits to general Gaussian functions

\[
C = a \exp \left( -\frac{(\tau - b)^2}{2c^2} \right)
\]

(4.5)

where \(C\) is the histogram count, \(a\) is the height of the Gaussian peak, \(b\) is the centre position of the peak, and \(c\) is related to the width of the peak (\(1.17c\) is the half-width at half-maximum of the peak). In Table 4.1 we summarize the parameter values associated with the distributions and their respective fits. Qualitatively, we observe an increase in the peak position, along with a broadening of the distribution, after exposure to PMB. This is reflected in the increased values of \(\tau_{AVG}\) and \(b\) (measures of the peak centre position), the increased values of \(\sigma\) and \(c\) (measures of the distribution width), and the decreased value of \(a\) (the peak height) after PMB exposure. Using the same \textit{E. coli} strain as the present study (PB103/\(\lambda\)DR122), Downing \textit{et al.} showed that there was an increase in the MinD oscillation period after exposure to the antibiotic gentamicin, the cationic antimicrobial peptide protamine, and polyvalent cations [30]. Although the authors do not mention a broadening in the distribution of measured values, their sample sizes (20 to 30 bacterial cells per measurement) may be too small to allow the calculation of a statistically
Figure 4.3: Histograms of measured \( \tau \) values before (blue) and after (red) exposure to 50 mg L\(^{-1}\) PMB, along with best fit Gaussian curves (Equation 4.5). Fit parameters and statistics are shown in Table 4.1. Both histograms consist of 88 measured \( \tau \) values and have bin widths of 2 s.

<table>
<thead>
<tr>
<th></th>
<th>( \tau_{AVG} ) (s)</th>
<th>( \sigma ) (s)</th>
<th>( \sigma_\tau ) (s)</th>
<th>( a )</th>
<th>( b ) (s)</th>
<th>( c ) (s)</th>
<th>( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before PMB Exposure</td>
<td>42</td>
<td>10</td>
<td>1.0</td>
<td>16</td>
<td>39</td>
<td>4.9</td>
<td>0.88</td>
</tr>
<tr>
<td>After PMB Exposure</td>
<td>62</td>
<td>21</td>
<td>2.2</td>
<td>5.5</td>
<td>55</td>
<td>17</td>
<td>0.72</td>
</tr>
</tbody>
</table>

Table 4.1: Statistics and fit parameters for data shown in Figure 4.3. Values \( a, b, c \) and \( R^2 \) are obtained from performing a least-squares fit to Equation 4.5 in MATLAB. The values \( \tau_{AVG}, \sigma, \) and \( \sigma_\tau \) are discussed in Section 3.2.
meaningful measure of the broadening of the measured distributions.

In Figure 4.4 we show the effect of changing the concentration of PMB to which the cells are exposed. For the range of concentrations that was studied, there is a consistent 40% to 70% increase in the MinD oscillation period relative to the value measured before PMB exposure, but no significant concentration dependence. Given that the majority of the concentrations studied were larger than the MIC value for PMB (50 mg L$^{-1}$) and that the charge on PMB is large and positive (+5e), it is possible that ionic effects dominate any structure-specific mechanisms for PMB at these large PMB concentrations. For this reason, all subsequent experiments in this study, with the exception of the bacterial growth studies in Section 4.2.1 were performed using a concentration of 2.5 mg L$^{-1}$, which is only 5% of the MIC value of PMB.

At high PMB concentrations, in addition to the increase in the oscillation period, we observed qualitative changes in the nature of the MinD oscillation. Figure 4.5 compares TIRF microscopy images of cells before and after exposure to 600 mg L$^{-1}$ PMB (12 times the MIC value). After exposure, many cells contained (sometimes multiple) localized regions of high fluorescent intensity. These regions of fluorescence are non-dynamic, i.e. they do not participate in the pole-to-pole oscillation which is still apparent in some of the cells. While these regions clearly indicate some effect of PMB on the distribution on MinD-GFP in the cell, they cannot be characterized within the scope of our current analysis method. This highlights our need for more sophisticated methods of image analysis for certain experimental conditions.
Figure 4.4: Plot of the MinD oscillation period relative to that measured before exposure to PMB, as a function of PMB concentration at 25°C. The first data point indicates the MinD oscillation period for healthy cells, prior to PMB exposure. All subsequent points indicate the relative change in the MinD oscillation period in a single experiment in which the given concentration of PMB was introduced to the extracellular environment. Each data point corresponds to at least 90 bacterial cells, with the exception of the 600 mg L$^{-1}$ value, for which 12 cells were measured (discussed in text). The black dotted line indicates the MinD oscillation period prior to PMB exposure, and the blue dotted line indicates the average of all data points other than the first, corresponding to a 61% increase in the oscillation period.
Figure 4.5: TIRF microscopy images comparing *E. coli* cells before and after exposure to 600 mg L$^{-1}$ PMB. The red arrow indicates an example of the non-dynamic, localized fluorescence which is apparent in many cells after exposure. The height of each image corresponds to 11.3 µm.
4.1.3 Time Dependence of PMB Exposure

In an attempt to determine the time scales associated with the action of PMB on *E. coli*, we have measured the MinD oscillation period as a function of time of exposure of the bacterial cells to 2.5 mg L$^{-1}$ PMB. In Figure 4.6 is shown a plot of the MinD oscillation period relative to that measured before exposure to PMB as a function of PMB exposure time. We observe an approximately 50% increase in the MinD oscillation period within 10 minutes of introducing PMB to the flow cell. As discussed in Section 2.2.3, the minimum time between consecutive measurements of the MinD oscillation period in this study is several minutes. This time between data points is too large to accurately determine the time scale associated with the increase in the oscillation period. However, we do observe that the increase occurs over a time of approximately 10 min, which is consistent with the time of action for PMB measured in protein release assays [27], which have a similar time resolution.

4.2 Comparison of Exposure to PMB and PMBN

In Figure 4.7 we compare the time dependence of the MinD oscillation period after exposure to 2.5 mg L$^{-1}$ PMB and PMBN. Although an accurate estimate of the time of action of the two compounds is difficult to determine due to the small density of data points on the plot, the action of both PMB and PMBN occurs over approximately 10 min. This similarity aside, the effect of PMBN on the MinD oscillation period is qualitatively different from that of PMB. We observe an approximately 20% decrease in the MinD oscillation period after exposure to 2.5 mg L$^{-1}$ PMBN. This effect is unexpected for a number of reasons.
Figure 4.6: Plot of the MinD oscillation period relative to that measured before exposure to PMB as a function of time of exposure to 2.5 mg L$^{-1}$ of PMB, at 25$^\circ$C. The black dotted line indicates the MinD oscillation period prior to PMB exposure, and the blue dotted line indicates the average of all data points other than the first two, corresponding to a 44% increase in the oscillation period. The number of bacterial cells measured for each data point ranges from 11 to 42.
Figure 4.7: Plot of MinD oscillation period relative to that measured before exposure to PMB or PMBN as a function of exposure time, at 25 °C. The first data point indicates the MinD oscillation period for healthy cells, prior to PMB or PMBN exposure. All subsequent points indicate the relative change in the MinD oscillation period with time since 2.5 mg L\(^{-1}\) of PMB (blue points) or PMBN (red points) in PBS was introduced to the extracellular environment. The horizontal lines indicate the average of all period values measured after 10 min and offer a measure of the long-term (time-independent) effect of exposure on the oscillation period. For PMB there is a 44% increase, and for PMBN there is an 18% decrease, in the oscillation period. For the PMBN data, the number of bacterial cells measured for each data point ranges from 14 to 50.
It is not very surprising that these structurally related compounds would have qualitatively different effects on the MinD oscillation period. It is known from protein release assays [27] that PMB passes through the outer membrane to reach the periplasmic space, but PMBN does not disrupt the cytoplasmic membrane. However, because PMBN does not disrupt the cytoplasmic membrane, it is surprising that PMBN has any effect on the MinD oscillation period, as the entire Min protein system exists in the cytoplasmic space of the cell. The observation of a decrease in the MinD oscillation period upon exposure to PMBN is, in fact, very unusual: almost all other conditions which cause a change in the MinD oscillation period result in an increase in the value of the period. The sole exception to this, to the best of our knowledge, is the increase in temperature, as discussed in Section 4.1.1.

4.2.1 Effect of PMB and PMBN on Bacterial Growth

Since the MinD oscillation ensures proper cell division in *E. coli*, large deviations in the oscillation period can cause cells to divide improperly, or not at all. When the MinD oscillation is disrupted, an obvious question to ask is whether or not the cells are still able to divide. Due to photobleaching of the GFP fluorophores, we are unable to monitor the MinD oscillation through an entire cell division cycle (~20 min), but we can carry out our standard growth procedure (see Section 2.1.1 for details) in the presence of PMB and PMBN. In Figure 4.8 is shown the optical density measured at $\lambda = 600$ nm (OD$_{600}$, a standard measure of bacterial density) for cells grown in LB medium, and LB medium supplemented with 50 mg L$^{-1}$ of either PMB or PMBN. The bacterial growth measurements indicate that over the
Figure 4.8: A plot of optical density measured at $\lambda = 600$ nm ($\text{OD}_{600}$) as a function of time for cells grown in LB medium (black), and LB medium supplemented with 50 mg L$^{-1}$ PMB (blue) and PMBN (red).
7 h experiment, the presence of PMB prevents bacterial division. The presence of PMBN at the same concentration, however, does not seem to have any measurable effect on the rate of bacterial division.

Since the oscillation of MinD is essential to healthy cell division, it is unusual that PMBN changes the MinD oscillation period without preventing, or changing the rate of, successful cell division. We suggest two explanations for these apparently incompatible results. First, it is possible that a 20% decrease in the oscillation period is insufficiently large to affect the division process. Complete knockouts of each component of the Min system have resulted in minicelling [11], but no systematic study has correlated a given change in the MinD oscillation period with a change in the rate of bacterial division. It is possible that the cells can divide at the same rate for a range of values of the MinD oscillation period.

Another possible explanation is that an increase in the MinD oscillation period disrupts proper cell division, but a decrease does not. It is possible that there is a minimum frequency at which MinD must oscillate from pole to pole to sufficiently antagonize FtsZ polymerization, and that surpassing this frequency does not change the efficacy of this antagonization. An increase in temperature is also known to decrease the MinD oscillation period [35], but the temperature-dependent growth rate of *E. coli* [37] does not allow us to attribute the change in division rate directly to the change in the MinD oscillation period in this case.

The effect of PMB on the bacterial growth rate merits discussion. While it is not surprising that exposure to an antimicrobial agent would prevent bacterial growth, it is interesting that the MinD oscillation persists in the majority of the exposed cells. This indicates that the increase in the MinD oscillation period is not
necessarily the reason for the inability to divide, but likely an indirect effect of the
damage done to the cell by the PMB. This result should be kept in mind when us-
using the MinD oscillation as an \textit{in situ} metric of cell viability, as the persistence of
the oscillation in this case does not necessarily indicate that the cells are viable.

\textbf{4.2.2 Arrhenius Dependence of Cells Exposed to PMB and PMBN}

In an attempt to elucidate the mechanism by which PMB and PMBN affect the
MinD oscillation period, we measured the temperature dependence of the oscil-
lation period after exposure to either PMB or PMBN. By analysing these data in
terms of an Arrhenius process as described in Section 4.1.1, we can compare the
activation energies associated with the MinD oscillation phenomenon before and
after exposure to PMB and PMBN. Arrhenius plots of these data are shown in Fig-
ure 4.9.

Prior to exposure to either PMB or PMBN, the activation energy associated with
the temperature dependence of the MinD oscillation period was $11.8 \text{ kcal/mol}$. Af-
ter exposure to PMB, the activation energy increased to $17 \text{ kcal/mol}$. While we
cannot necessarily associate a mechanism with this change, the increase in this
value is not unexpected. It is reasonable that more energy is expended to maintain
cellular processes in a damaged cell, as the processes will be, in general, operating
at lower efficiency. The small range of temperature values for which the oscillation
period is shown in Figure 4.9 after PMB exposure is a result of the small (statisti-
cally insignificant) population of cells with measurable MinD oscillation periods
at temperatures above $35 ^\circ \text{C}$.
Figure 4.9: An Arrhenius plot of the temperature dependence of the normalized MinD oscillation period after exposure to 2.5 mg L$^{-1}$ of PMB (blue) and PMBN (red) together with the corresponding best fit straight lines. Each of the three data sets is normalized to its value at 25°C. The data and best fit straight line for cells prior to exposure to PMB and PMBN (from Figure 4.2) are shown in black. For PMB, the best fit line has an intercept of 27.8, a slope of $-8300$ K ($E_A = 17$ kcal/mol) and $R^2 = 0.99$. For PMBN, the best fit line has an intercept of 17.4, a slope of $-5200$ K ($E_A = 10$ kcal/mol) and $R^2 = 0.99$. 
After exposure to PMBN, the measured activation energy was 10 kcal/mol, which is slightly less than the value of 11.8 kcal/mol that was measured before exposure to PMBN. This result is quite reasonable, given that PMBN does not disrupt the cytoplasmic membrane (based on protein release assays [27]), and should therefore not compromise cellular processes. The decrease in the oscillation period requires an increase in the rate of ATP hydrolysis (which fuels the MinD/E binding). This increased reaction rate suggests that the overall energy consumption of the process has increased after exposure to PMBN. It is also worth noting that we see similar percent changes in the oscillation period and activation energy after exposure to either compound. Exposure to PMB results in a 50% increase in the oscillation period, and a 45% increase in the activation energy. Exposure to PMBN results in a 20% decrease in the oscillation period, and a 15% decrease in the activation energy.

The MinD oscillation persisted in the majority of cells exposed to 2.5 mg L$^{-1}$ PMB and PMBN, however, the effect on the exposed cells of an increase in temperature differed. As mentioned above, after exposure to PMB, very few cells had measurable MinD oscillation periods at temperatures above 35 °C. This was not the case for cells exposed to PMBN. Shown in Figure 4.10 are TIRF microscopy images of cells for which the temperature was slowly increased from 25 °C to 40 °C after being exposed to 2.5 mg L$^{-1}$ PMB (left) and PMBN (right). PMBN-exposed cells maintained the distribution of MinD-GFP observed prior to exposure to PMB or PMBN, and had oscillation periods that could be measured at and above 40 °C. PMB-exposed cells, on the other hand, exhibited a complete loss of the oscillation of MinD-GFP for temperatures greater than 35 °C, and formed regions of non-
dynamic intensity similar to those discussed in Section 4.1.2. This increased sen-
sitivity to temperature is likely due to the strong effect of PMB exposure on the
integrity of the cytoplasmic membrane.
Figure 4.10: TIRF microscopy images of PMB- (left) and PMBN-exposed (right) cells collected at 40 °C. The cells were exposed to either PMB of PMBN at a temperature of 25 °C and the temperature was slowly increased to 40 °C. The height of each image corresponds to 15.2 μm.
Chapter 5

Summary and Conclusions

5.1 Summary of Results

The results of this thesis can be grouped into two categories. First, we have made multiple improvements to the experimental procedure and methods of analysis, as summarized below in Section 5.1.1. We have also carried out experiments measuring the change in the MinD oscillation period in response to changes in various experimental conditions, which are summarized in Section 5.1.2.

5.1.1 Experimental Improvements

The design of the flow cell allows precise control of the temperature and the addition of bacterial cells and reagents (see Section 2.2.1). Experiments are straightforward and reproducible, and take place in a completely closed environment, ensuring minimal contamination. All flow cell components were chosen to be compatible with biological experiments, and are either sterilized or discarded between
experiments. Within several minutes of changing the temperature set point, the temperature within the flow cell stabilizes and remains unchanged throughout the course of an experiment to within ±0.05 °C, for temperatures in the range of 25 °C to 45 °C.

We minimized photobleaching of the GFP fluorophores to maximize the number of images we could obtain in each time series of TIRF microscopy images. We accomplished this by limiting the exposure time of each image to 200 ms and reducing the excitation laser intensity to less than 1 mW. With this configuration, we were able to gather a sufficient number of images per oscillation for several oscillations which allowed the accurate determination of the oscillation period.

We developed the MATLAB program *Min Analyse* to analyse our time series of TIRF microscopy images (see Section 3.1). This program included a full user interface (UI) which allowed the identification of regions of interest (ROIs) for each cell and provided a simple means of determining the MinD oscillation period values for a large number of cells from each time series of images. Statistical analysis of the TIRF images and fitting of the time dependence of the cell centroid position to a sinusoidal function were incorporated into the program.

### 5.1.2 Experimental Results and Conclusions

We have measured the temperature dependence of the MinD oscillation period for the range of temperatures 25 °C to 45 °C (see Section 4.1.1). We analysed the temperature dependence in terms of an Arrhenius plot, allowing the determination of the associated activation energy. For healthy cells, we determined the activa-
tion energy to be 11.8 kcal/mol. This value is of the same order as the value of 20 kcal/mol obtained by Touhami et al. [35], and is strikingly similar to the free energy of a single ATP hydrolysis event (11.5 kcal/mol [36]). This suggests that the MinD temperature dependence is due to temperature-dependent hydrolysis of ATP, and is consistent with the model of Huang et al., which suggests that the MinD oscillation is rate-limited by the hydrolysis of ATP and the subsequent nucleotide exchange required to convert cytoplasmic MinD:ADP to MinD:ATP [10].

We have performed several experiments involving the introduction of the antimicrobial agent PMB and the related compound PMBN to the extracellular environment. We have measured the dependence of the MinD oscillation period on the concentration of PMB to which the cells are exposed, over the range of 2.5 mg L$^{-1}$ to 600 mg L$^{-1}$ (see Section 4.1.2). For the exposure to PMB, we observed a consistent broadening of the distribution of measured MinD oscillation periods, and an increase of 60% in the average value of the oscillation period, with no measurable dependence on the PMB concentration. After exposure to high concentrations of PMB, we observed qualitative changes in the distribution of MinD-GFP within the cell, characterized by bright, static features.

We measured the dependence of the MinD oscillation period on the time of exposure to PMB at a concentration of 2.5 mg L$^{-1}$ (see Section 4.1.3). Over the first 10 min, we observed a 44% increase in the oscillation period. This time scale is consistent with the time of action of PMB, as measured in protein release assays [27]. After the first 10 min of exposure, we measured no change in the oscillation period.

We compared the effect of exposure to 2.5 mg L$^{-1}$ PMB and PMBN on the MinD oscillation period (see Section 4.2). The exposure to PMBN had a similar time of
action to that of PMB, affecting the MinD oscillation period within the first 10 min of exposure. Unlike the PMB-exposed cells, those exposed to PMBN exhibited an 18% decrease in the MinD oscillation period, on average. To our knowledge, the only other change in the environment of the cells that results in a decrease in the MinD oscillation period is an increase in temperature.

In an attempt to correlate the changes in the MinD oscillation period with a change in the ability of the cells to properly divide, we measured the growth rate of the cells in the presence of 50 mg L\(^{-1}\) PMB and PMBN (see Section 4.2.1). Using OD\(_{600}\) measurements, we observed that the presence of PMB inhibits bacterial growth, while the presence of PMBN has no effect on the cell division time.

We measured the temperature dependence of the MinD oscillation period in cells after exposure to 2.5 mg L\(^{-1}\) PMB and PMBN, and analyzed the results in terms of an Arrhenius process (see Section 4.2.2). After exposure to PMB, we found that the activation energy had increased from 11.5 kcal/mol to 17 kcal/mol (50% change). This is to be expected, as PMB is an antimicrobial, and processes in a damaged cell will be operating at less than optimal efficiency, requiring more energy. After exposure to PMBN, we measured the activation energy to be 10 kcal/mol: slightly decreased from the value measured in unexposed cells (15% change). Since the increased oscillation period requires an increased rate of ATP hydrolysis, it is interesting that exposure to PMBN would increase the MinD oscillation period while only slightly decreasing the activation energy, as this implies that the cell is consuming energy at an increased rate.
5.2 Future Work

The present study has led to several intriguing results that should be investigated further.

One future direction for our studies of polymyxin B (PMB) will be to augment and enhance our methods of analysis to identify and quantify the non-dynamic fluorescence intensity observed in cells exposed to high concentrations of PMB, as shown in Figure 4.5. It would also be beneficial to expand our current analysis to measure the duration of each stage of the MinD oscillation (dwell time at poles, pole-to-pole transit time). This would allow us to determine which portion of the Min mechanism responds to a given change in experimental conditions. To precisely determine these time scales would require the use of a more sensitive camera, e.g. an EMCCD camera.

Further decreasing photobleaching in our imaging setup would allow longer timescales to be examined. This would, for example, make it possible to track the MinD oscillation in a single cell through its entire life cycle, directly correlating a given change in the MinD oscillation with a change in the ability of the cell to divide properly.

Perhaps the most interesting result we have presented, and indeed that which most inspires further study, is the response of the MinD protein oscillation to the presence of polymyxin B nonapeptide. Factors which increase the MinD oscillation period are intuitively understood as a change in the efficiency of the cellular processes in response to a damaging or stressing environment. The protein release assays performed by Sahalan et al. [27] would suggest that since PMBN is
unable to disrupt the cytoplasmic membrane, the mechanism by which PMBN affects MinD is necessarily indirect. Since these assays do not preclude the possibility that PMBN somehow passes the outer (and potentially inner) membrane without releasing protein, a more direct measure of the position of PMBN molecules within the cell is desirable. Studies of the effect on the MinD oscillation of peptides which are structurally similar to PMBN (cyclic and cationic, in particular), or other compounds which are known to have the same action on *E. coli* (outer membrane permeabilizers [38]) could also prove enlightening.

Recently, the time-dependent mechanical properties of individual bacterial cells have been measured using a nanocreep experiment in which an atomic force microscopy (AFM) tip is pressed into a bacterial cell with constant force and the displacement of the AFM tip [39] is measured. This technique has been used to characterize the mechanical response of the cell to exposure to PMB and PMBN [40]. It would be very interesting to combine the TIRF measurement of the MinD protein oscillation with the AFM nanocreep measurement to simultaneously measure the effect of PMB and PMBN, as well as the effect of novel cationic antimicrobial peptides (CAPs) [41].
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