

Predicting Persisters: Investigating Gene Regulation in Persister Cells

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April 8th, 2019

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Abstract: Bacterial populations produce a small number of persister cells that remain tolerant to multidrug exposure by becoming metabolically dormant. This inquiry focuses on investigating metabolic gene expression levels in persisters to determine potential predictors of bacterial persistence. Strains from the E. Coli Promoter library were imaged using single cell microscopy, and gene expression levels were measured with the integrated reporter GFP. Persister cells were isolated using ofloxacin. Results indicate that bacterial cells that survive antibiotic exposure exhibit filamentous growth before dividing. Resumption of normal growth morphology is accompanied by increased proteolytic activity in daughter cells, however more investigation is required to ascertain the identity of the persister cells.

1 Introduction

Persister cells are cells that are formed randomly within bacterial populations that are highly tolerant to antibiotics. Upon antibiotic exposure, few cells in a bacterial population are observed to persist, in which they survive by entering a metabolically dormant state. Upon removal of the antibiotic these cells vigorously repopulate. Despite their low number, persisters are seen to be responsible for many recurring infections. When a person who has a bacterial infection takes the first dose of oral antibiotics, the concentration is high enough to kill the vast majority of bacteria within the human gut. Despite this high killing rate, and the typical course of antibiotics being 10 days, a small fraction of persisters are known to survive. Ultimately when the patient stops taking antibiotics, the small fraction of persisters that survived, ultimately repopulate allowing the infection to grow again, and the cycle with antibiotics continues.

Persisters have been documented in patients with chronic recalcitrant infections and have been shown to have multidrug tolerance.

Persisters have been documented to have increased levels of typical stress responses and decreased levels of metabolic activity. However, techniques documenting the expression of levels of various metabolic, regulatory, and toxin/antitoxin genes have not been documented in persister cells over long periods of time. The aim of this thesis is to determine predictors of persister cells by documenting the activity of certain regulatory, and toxin/antitoxin and metabolic genes in the persister state. While little is known about persister cells to properly document the cellular pathway that is necessary to achieve this state, the expression levels of various genes in the persister state can be documented to understand this phenomenon further.

Persister cells were first noticed by Bigger in 1944 when he noticed that a small proportion of *Staphylococcus* cells survived after treatment with penicillin. One of the first genes that was said to induce this pathway was the toxin gene HipA documented by Harris Moyed when he noticed that persistent cells in a population of *E. Coli* cells repeatedly exposed to antibiotics were most commonly HipA mutants. HipA is known phosphorylator of tRNA. Its activation induces amino acid starvation, which inhibits replication, transcription, translation and cell wall synthesis, reducing growth and leading to persistence. Further investigations have concluded that several genes may be involved in this pathway not only because the pathway involving HipA gene involves several steps but also because there is a redundancy in the path to achieving the persister state. Lewis (2010) found that strains wherein singular Toxin/Antitoxin (TA) loci deleted did not exhibit a persister phenotype suggesting a possible redundancy in the pathway, suggesting the persister phenotype could possibly exhibit several genotypes and thus, phenotypes.

Recent findings within the clinical community have also led to increased interest in persister cells. Fauvart, M., De Groote, V. N., & Michiels, J. (2011), discovered that in patients who display chronic recalcitrant infections, namely patients of cystic fibrosis, Candidiasis and Tuberculosis showed an increase in the number of persister cells in bacterial cultures in longitudinal studies by nearly 100 fold after 40 years. Also, across many patients who had cystic fibrosis, bacterial cultures most commonly exhibited a *hipA* mutation leading to significantly higher persister cells in most patients. Overall, for cystic fibrosis and candidiasis diseases, patients displayed a significantly higher number of persister cells over time than when they were first diagnosed with the infection.

The aim of this experiment was to record the expression of levels of genes in a persister state using fluorescence microscopy. Using the promoter library engineered by Uri Alon's group (Zaslaver et al., 2006), key genes involved in the metabolic and stress response activity of *E.Coli* were targeted. The promoter library consisted of the insertion of a GFP gene downstream of a promoter corresponding to the gene being studied. In the strains of the promoter library, the GFP is transcribed at the same rate as the promoter corresponding the gene of interest. Increased fluorescence indicates that the gene is being transcribed at higher rate. This allowed for the measurement of transcription levels in real time, thereby gene expression levels for the genes downstream of that promoter through GFP fluorescence.

Ofloxacin was determined to be a suitable isolator of persister cells. Strains within the promoter library were resistant to kanamycin. Kanamycin belongs to the class of tetracycline antibiotics that inhibit protein synthesis by interfering with ribosomal assembly. Leaving tetracycline related antibiotics aside it was determined that a suitable isolator for the persister state would be ofloxacin. Ofloxacin belonging to the quinolone group of antibiotics, was the only

group that specifically targeted and killed stationary phase and non-growing cells too, along with rapidly growing cells. Fluoroquinolones are known to inhibit and prevent antibiotic growth by preventing the function of DNA gyrase, a type of topoisomerase, which reduce torsional strain during the unwinding of DNA. Topoisomerase is essential for the replication of DNA and bacterial replication. In addition, using a broad-spectrum antibiotic such as ofloxacin which is widely used in a clinical setting would be produce more clinically relevant results. Using ofloxacin would increase the confidence that the cells that survived were persistent cells.

Genes to be analyzed were selected from a wide array, including genes involved in stress responses, fatty acid metabolism, energy production, and efflux pumps. Transcriptional activity of these genes can indicate the metabolic activity of the cell and shed light on the level of dormancy a cell experiences during the persister state. More importantly, this inquiry will help recognize any predictor genes that can be used to develop specific targets for bacterial infections in the future.

2 Background:

Persisters are formed in the stationary phase of bacterial populations, isolated with the help of antibiotics. Keren, I., Kaldalu, N., Spoering, A., Wang, Y., & Lewis, K. (2004) had found that persister cells were formed mostly in the stationary phase of growth, and that they were not induced in the presence of specific antibiotics but rather that they naturally existed within this state (Figure 1). Antibiotics only helped isolate these cells. This shows that persister cells exist naturally within bacterial populations and are physiologically prepared for antibiotics before their exposure. This led me to believe that persisters must have some distinctive aberrant gene expression that allows them to survive. While Shah et al. (2006) conducted transcriptome studies

of gene regulation of persisters during antibiotic exposure, I was specifically interested in recognizing a persister cell before antibiotic exposure.

Figure 1: Persisters are isolated from a stationary state population of Bacteria.

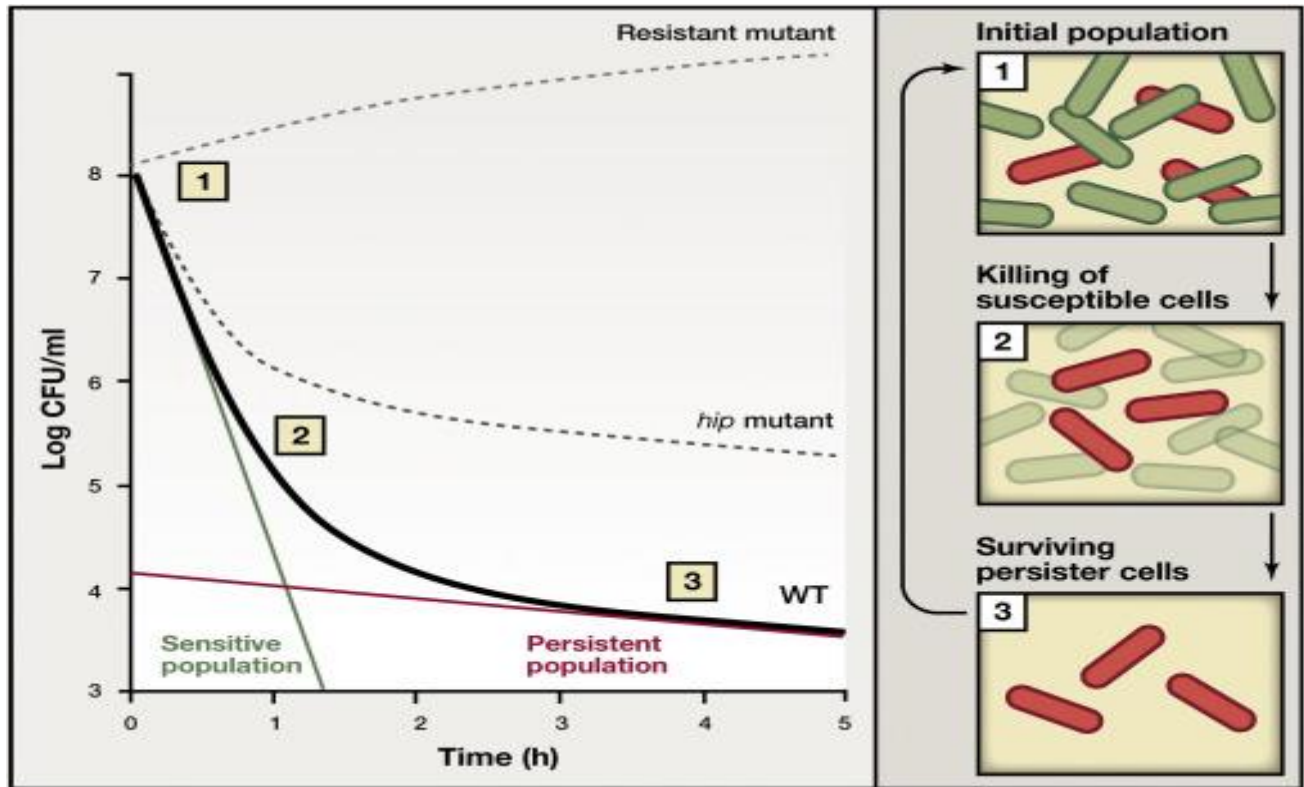


Figure caption: Taken from Maisonneuve and Gerdes (2014). (1) Lethal dose of a bactericidal antibiotic is added at time zero to a growing population of sensitive, genetically identical bacteria. The experiment reveals a characteristic biphasic killing curve. (2) The slope of the initial phase reveals the susceptibility of the bulk of the population. The initial log-linear relationship reveals an exponential killing kinetics (green line). (3) The slope of the second inactivation phase (red line) reveals the existence of a persister subpopulation that is killed with a much slower kinetics. Killing kinetics for a high persister mutant (*hip*) strain producing a highly elevated number of persisters is also shown (dark dashed line). After removal of the antibiotic (pointed by the arrow flanking the right panels), persister cells resume growth and give rise to progeny cells that are genetically identical to the cells of the original population and, therefore, as drug-sensitive as the original cells. The gray dashed line indicates how a drug-resistant mutant strain would support growth under these conditions. Adapted from Lewis (2010).

Persister cells can resume normal growth after antibiotic exposure. From Kerner et al.

(2015) it is known that persister cells can be clearly distinguished before antibiotic exposure, it is

also known that persister cells can be resuscitated from their persister state and enter the normal state (Maisonneuve and Gerdes, 2014). Bigger in 1944 had first proposed a simple solution to sterilize bacterial infections, by following high doses of antibiotics and decreasing them over time, effectively resuscitating persister cells. If this is preceded quickly a second dose of high antibiotics then this might effectively greatly reduce or even obliterate remaining persister cells from the population. This approach has been confirmed by K Lewis (2007) in *P. aeruginosa* biofilms. Sugars like mannitol, glucose in combination with aminoglycoside antibiotics like gentamycin decreased the number of persisters (Allison et al., 2011). In addition Lewis also found that dilution leads to resuscitation (2007).

3 Hypothesis

This inquiry will be conducted such that gene expression will be measured before, during and after antibiotic exposure (Figure 2), by flowing Partial Minimal Media (PMM) before antibiotic exposure and after antibiotic exposure. PMM contains sugar (glucose) and salts which will allow for regrowth after antibiotic exposure has been completed. The second flow of PMM will allow for resuscitation. I do not expect persister cells that do resuscitate after antibiotic exposure to grow during the first three hours of PMM. From Keren et al. (2004) I know that persister cells are not induced and hence I expect them to be metabolically dormant during both the initial PMM exposure and antibiotic exposure.

Figure 2: Experimental Setup

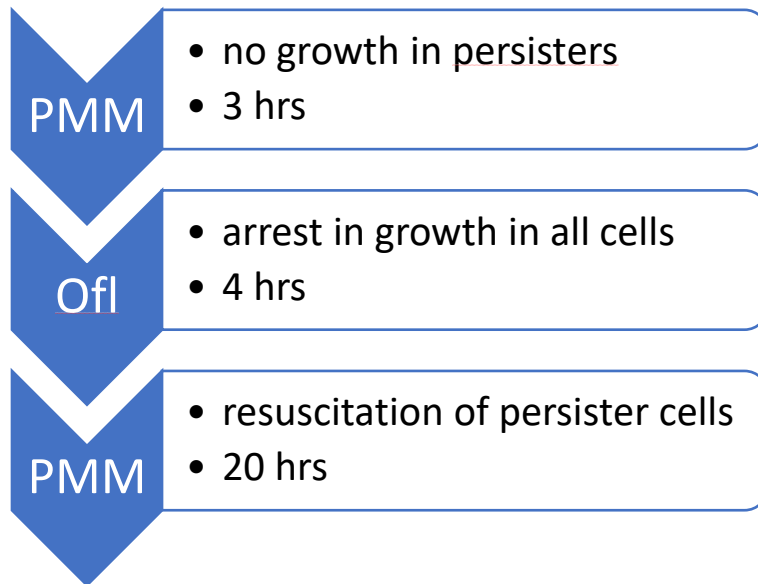


Figure Caption: Describes the time period of exposure and experimental format.

3.1 Hypothesis

The following are expectations from individual strains within the promoter library. Important to note is that all strains are genetically similar except for the placement of the GFP gene, and thus the specific gene of which transcriptional activity it is measuring. In all strains the GFP fluorescence indicates the transcription of the promoter associated with the gene specified. In addition, all expectations are for persister cells within the strain, and compared to non persister cells within the strain.

3.1.1 PlsB

The PlsB gene is a key metabolic gene active during cell division. The PlsB gene is responsible for making the enzyme Glycerol-3-phosphate Acetyl-Transferase (can be seen as GPAT in Figure 3) which utilizes Glycerol-3-phosphate (G3P) from glycolysis to synthesize fatty acids, which are constitutive parts of membranes, which is an essential metabolic function. Phospholipids, the end product of this pathway can be used to manufacture glycolipids, glycerol. Many of these products are main constituents of cell walls and membranes which are in high demand during cell division.

Shah et al. (2006) suggested that PlsB may not be required to induce the persister state but is required for the maintenance of persister state. Decreased function of PlsB gene (Spoering et al., 2006) has shown to result in fewer persister cells, rather than inducing dormancy, showing that it is necessary for the persister state. Wahl also found that PlsB is involved in stress responses, by upregulating its function. Based on past inquiries I hypothesize that PlsB gene to be upregulated before exposure compared other non-persister cells, and expect it to be expressed at the same level during ofloxacin exposure and decrease during regrowth (Table 1).

Figure 3: Role of GPAT in Fatty Acid Synthesis

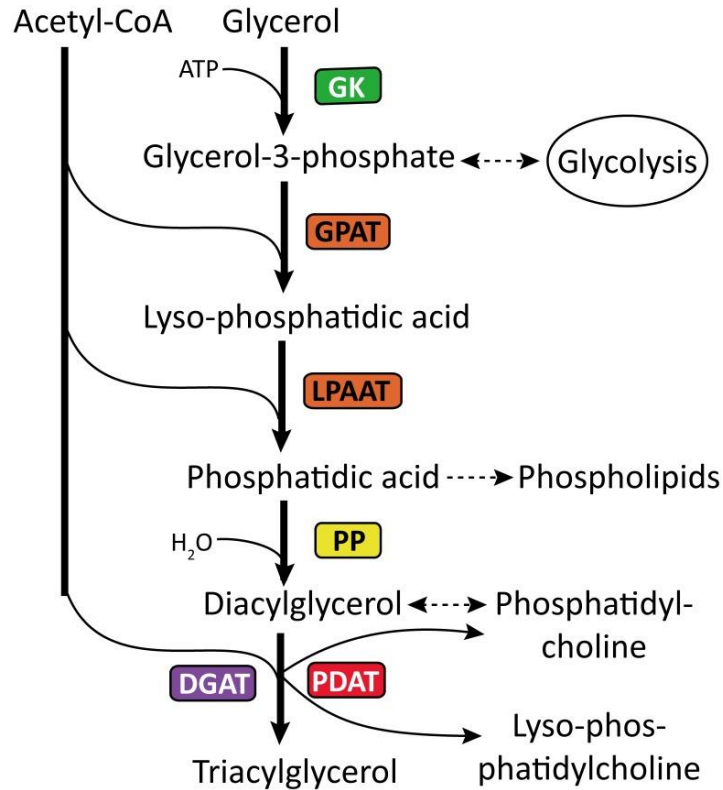


Figure Caption: Role of GPAT in the synthesis of fatty acids (Spoering et al., 2006).

3.1.2 GlpD

The GlpD gene is a key metabolic gene, responsible for producing the enzyme glycerol-3-phosphate dehydrogenase. This is a key step during glycolysis pathway, the primary pathway via which the cell produces energy by consuming sugar i.e. glucose. This is the only pathway by which the cell produces ATP to fuel all other cellular processes necessary at every stage of growth for a bacterial cell. Spoering et al. (2006) found that a strain of GlpD knockout eliminated the bulk of stationary state persisters indicating that it is necessary for persisters. In addition Lewis found that the formation of persisters was dependent on the G3P dehydrogenase activity of GlpD and not the enzyme protein itself. In addition Lewis also found that cells

overexpressing GlpD produced more persisters. Based on this inquiry I hypothesize that persister cells will upregulate GlpD during the first PMM exposure compared to other non-persister cells and continue at the same expression level during ofloxacin exposure, and decrease when resuscitated upon antibiotic removal and PMM exposure (Table 1).

Figure 4:

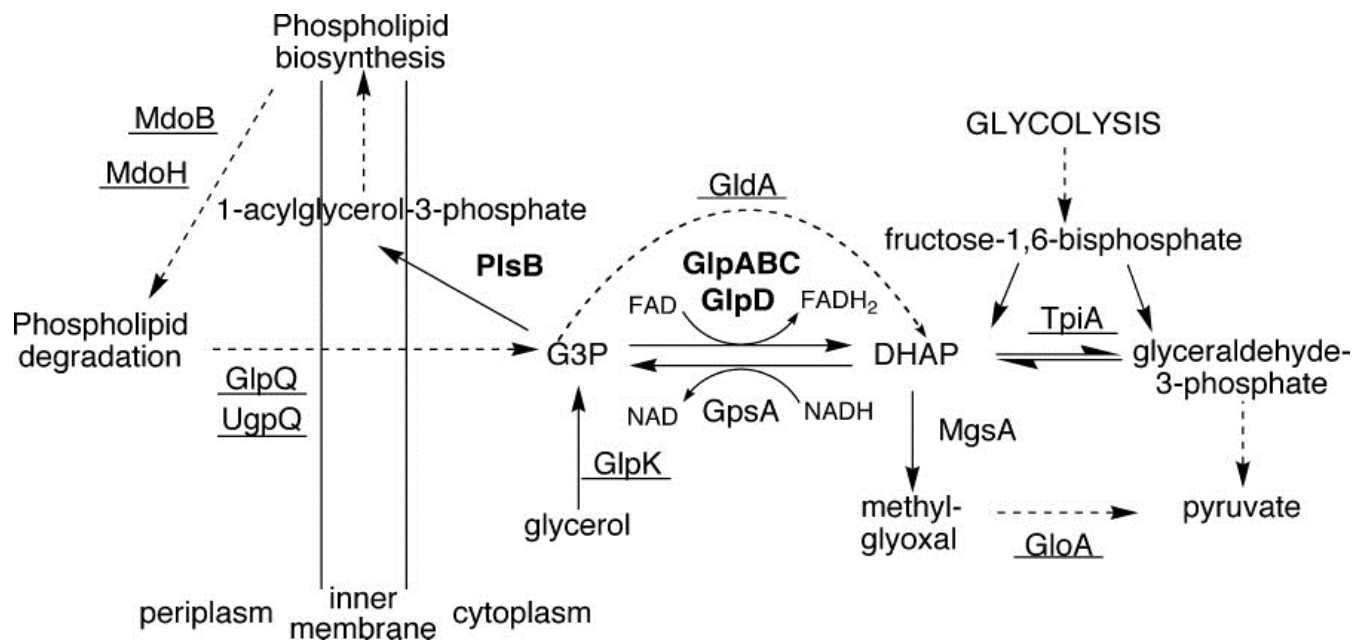


Figure: From Spoering et al. (2006). Role of glycerol-3-phosphate in *E. coli* metabolism. Enzyme names in bold indicate involvement in persistence. Underlined enzyme names indicate there was no detectable change in persistence in a deletion mutant.

3.1.3 DinJ

DinJ is part of a Toxin/Antitoxin (TA) module. A popular belief has been that TA modules are upregulated. Moyed first characterized that majority of persister cells found had mutated HipA, and subsequent gene expression studies have revealed that upregulating HipA, part of a TA module does lead to an increased number of persisters.

Typically, the toxin is a protein that inhibits an important cellular function such as translation or replication, and forms an inactive complex with the antitoxin. The toxin is stable, whereas the antitoxin is degradable. If a daughter cell does not receive a plasmid after segregation, the antitoxin level decreases owing to proteolysis, leaving a toxin that either kills the cell or inhibits propagation. (Lewis 2007). Vasquez-Laslop et al. (2006) found that increased expression of toxins from TA modules (DnaJ, MazF, HipA, ThrB) increased the frequency of persister cells. DinJ is a transcriptional repressor, and is an antitoxin. I hypothesize DinJ to be downregulated before antibiotic exposure compared to other non-persister cells and continue at the same expression level during antibiotic exposure in persister cells and increase after regrowth (Table 1).

3.1.4 DnaJ

DnaJ is a chaperone protein that participates actively in the response to hyperosmotic and heat shock by preventing the aggregation of stress-denatured proteins and by disaggregating proteins. DnaJ is also known as heat shock protein Hsp40 (uniprot). Vasquez-Laslop et al. (2006) investigated DnaJ from the TA module of DnaJ/DnaK/GrpE and found that cells that overexpressed DnaJ formed more persister cells on average than without the overexpression.

DnaJ is a toxin from the TA module and I expect it to be upregulated in times of stress, as in the persister state. I hypothesize that this gene will be upregulated before antibiotic exposure compared to other non-persister cells and continue at the same expression level during antibiotic exposure in persister cells and decrease upon antibiotic removal and exposure to media (Table 1).

3.1.5 TolC

TolC is an efflux pump, known for the export of antibiotics and other toxic compounds from the cell. Sun et al. (2014) found that bacterial multi-drug efflux systems actively pump antibiotics out to reduce cellular, thus facilitating bacterial survival.

This was further investigated using single cell microscopy for TolC efflux pump by Pu et al. (2016). The TolC pump was found to be upregulated in bacterial persister cells that survived treatment under carbenicillin, furthering questions about the dormant state of bacterial persister cells. I hypothesize that this gene will be upregulated before antibiotic exposure compared to other non-persister cells and continue at the same expression level during antibiotic exposure in persister cells and decrease upon antibiotic removal and exposure to media (Table 1).

Table 1: Summary of Gene Hypothesis

Gene Observed	Gene Function	Media Exposure (3 hrs)	Ofloxacin (4 hrs)	Media (20 hrs)
DnaJ	Heat shock protein	↑	↑	↓
DinJ	Transcriptional Repressor	↓	↓	↑
PlsB	Fatty Acid Metabolism	↑	↑	↓
GlpD	Glycolysis Metabolism	↑	↑	↓
TolC	Efflux Pump	↑	↑	↓

Figure Caption: Summary of individual gene regulation expectations listed in persister cell. Overall function was also noted.

4 Procedure

4.1 Pilot Study

I conducted the experiment initially in 96 well plates This allowed for high efficiency, allowing to screen multiple strains from the promoter library at once. Dr. Kralj from previous experiments with the flow cell technique had exposed cells to both partial min media and kanamycin and again to min media, the second time thoroughly washing out all of the antibiotic. This allowed for the repopulation of any persister cells. Dr. Kralj had noticed that all cells that repopulated after antibiotic exposure also grew normally during it, leading us to believe that any cells that divide normally during antibiotic exposure had a high chance of being persister cells. 96 well plates did not allow to expose the cells to both partial min media and ofloxacin because there was no outlet. I only observed the TolC strain initially to determine the situations under which a persister cell could be seen verifying my results from Pu et. al., (2016).

However, owing to the fact that long movies approx. 18 hours were taken, and agarose pads are 90% water, this was difficult. The pad shrunk constantly changing the location of the cells. The same cells were not observed over 18 hrs.

I switched to the flow cell method which allowed to measure accurately over a longer period of time affects of antibiotics, without any loss of water from the pads. This allowed for an airtight apparatus, eliminating any concerns about evaporation. Additionally, this allowed for me to see if the same cells would repopulate after exposure to ofloxacin.

Using TolC as my troubleshooting strain and cross referencing known results from Pu et. al., (2016), I varied the amount of ofloxacin used in the experiment. Lewis had used a

concentration of 5 ug/ml and had observed significant effects at that concentration so I started with that, however Lewis had not used single cell imaging techniques, but rather measured optical density of liquid cellular solutions over time allowing for a larger sample size. Under an exposure of 5 ug/ml of 12 hours, no growth or persister cells were observed. However, with loss of water from pads, maintaining continuity of the experimental image was difficult.

I switched to the flow cell method, after which I confirmed that cells would only proliferate only after the removal of the antibiotic which high throughput screening would not have allowed for. In addition I decreased the antibiotic exposure from 7-4 hours and from a concentration of 5 to 2.5 ug/ml. Previous time exposure and concentrations did not allow for regrowth of any kind.

With 5 $\mu\text{g/ml}$ we saw almost no change, with no repopulation and no regrowth after ofloxacin exposure and media flow. We reduced it to 2.5 $\mu\text{g/ml}$ and noticed that cells still grew, but abnormally. I also varied the time of exposure to ofloxacin testing at 2 ½ and 4 hours. At 2 ½ hours cells elongated continually under ofloxacin exposure and did not stop after media flow. With 4 hours of exposure, I saw an arrest of the abnormal growth. I used this time exposure as it would make it easier to visually detect persister cells. I continued my inquiry at this concentration. I noticed several observations common for all the 8 strains I had imaged.

4.2 Methods

The cells were grown up in overnight cultures in a shaking incubator at 37 degrees Celsius. Strains were from the Promoter library developed by Dharmacon. All the strains were resistant to Kanamycin and therefore were grown up in 30 ug/ml Kanamycin LB (from manual).

Before imaging, liquid cultures were diluted by a factor of 16.6 times with Kanamycin LB media.

1% Agarose pads were made using partial minimal media (PMM). Pads were very small to allow at least 6 to into a flow cell chamber. 0.8ul of the diluted liquid cell culture was placed on each pad and allowed to dry for approximately 3 minutes before flipping over onto the coverslip. This apparatus was allowed to dry for another 3 minutes before sandwiching to form the flow cell chamber. Agarose pads were placed such that there were visible gaps between the pads, such that media would be able to surround the pads on all 4 sides.

Ofloxacin stocks were made at 1mg/ml with DI sterile autoclaved water, and frozen at -20 degrees Celsius. Ofloxacin was subsequently diluted to 2.5µg/ml with PMM and filled in 20ml syringes. One more 20ml syringe was used for PMM.

Cells were imaged using a flow cell chamber (Figure 5). The inlet port was attached to a T junction, receiving media from two syringes: one containing PMM, and one containing ofloxacin at a concentration of 2.5µg/ml. The syringes were connected to timed pumps which regulated the rate of entry of media into the flow cell chamber. This setup allowed for the complete washing out of antibiotic, by stopping antibiotic flow and subsequently pumping media in.

Once all tubes and syringes were attached, PMM was carefully pumped into the flow cell chamber to check for leaks and to ensure that media would cover all spaces around the cells. Images were taken with Nikon with NIS Elements software, every 3 minutes for a total of approximately 27 hours. Each syringe when being pumped, was flowing in media at the rate of 20µl/minute. The experimental setup consisted of 3 hours of PMM, 4 hours of ofloxacin

exposure and 12 hours of PMM, following 8 hours of no media being pumped in. It is important to note that it takes approximately 5 minutes for the media to diffuse through the entire pad, and 10 minutes for media to be completely pumped out of the chamber.

Persister cells were visually identified and characterized based on the presence of regrowth during the second PMM exposure. Code was written in MATLAB to analyze the level of fluorescence over the time course of the experiments in persister cells. Filamentation which was seen as the elongation without dividing of cells, was also visually characterized.

Figure 5

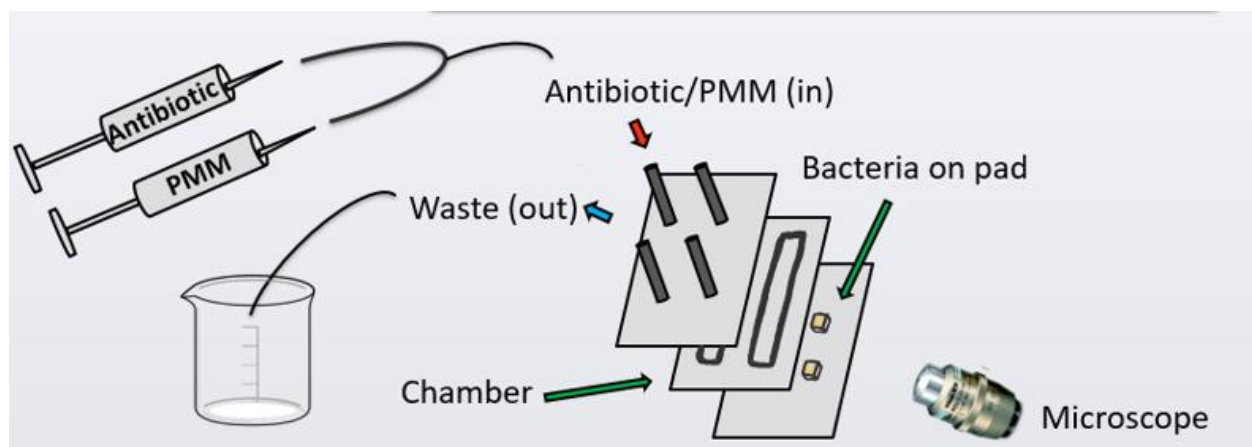


Figure caption: Experimental setup.

5 Preliminary Results

All strains showed cells that survived antibiotic exposure and successfully regrew. However Table 1, the expression of the genes seen during exposures was not as expected (Table 2). Henceforth, all cells that survived antibiotic exposure and showed regrowth will be referred

to as “survivor cells” and will reserve discussion on their identity as a persister cell later in this inquiry

5.1 Plsb

During the movie, it was observed that survivor cells did grow during the first three hours of PMM flow, and subsequently stopped growth during antibiotic exposure. Cells showed no sign of growth for approximately 5-15 hours after antibiotic was flowed out of flow chamber, after which they started filamenting and rapidly dividing, wherein the whole of the parent cell gave rise to daughter cells that also divided successfully. However, during regrowth cells that survived and regrew, were comparatively bright in their filamentous stage, and later after dividing, completely dimmed down until the microscope could not capture distinct boundaries of each cell. After dimming down, the cells continued to grow as indicated by the surrounding brighter dead cells getting continually pushed out by the dim colony of cells (Figure 1a-e).

Figure 6.1a

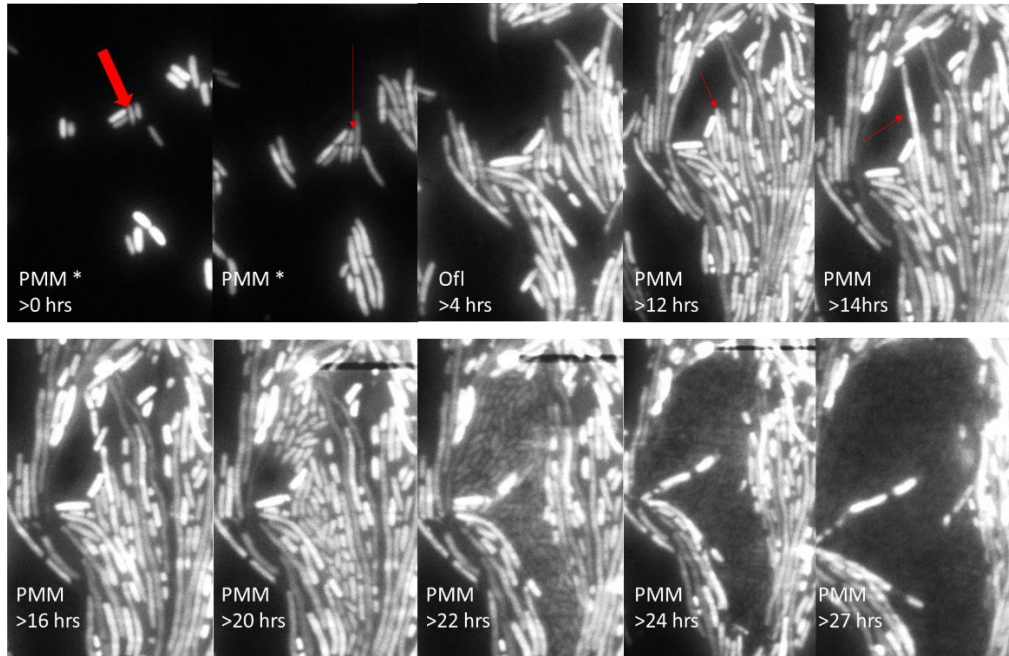


Figure caption: Red arrows point to persister cells. PMM* indicates first exposure to PMM. First line indicates chemical exposed to and the second line indicates time point of the movie. During second PMM exposure it can be seen that cells filament and subsequently divide while GFP expression decreases over time.

Figure 6.1b

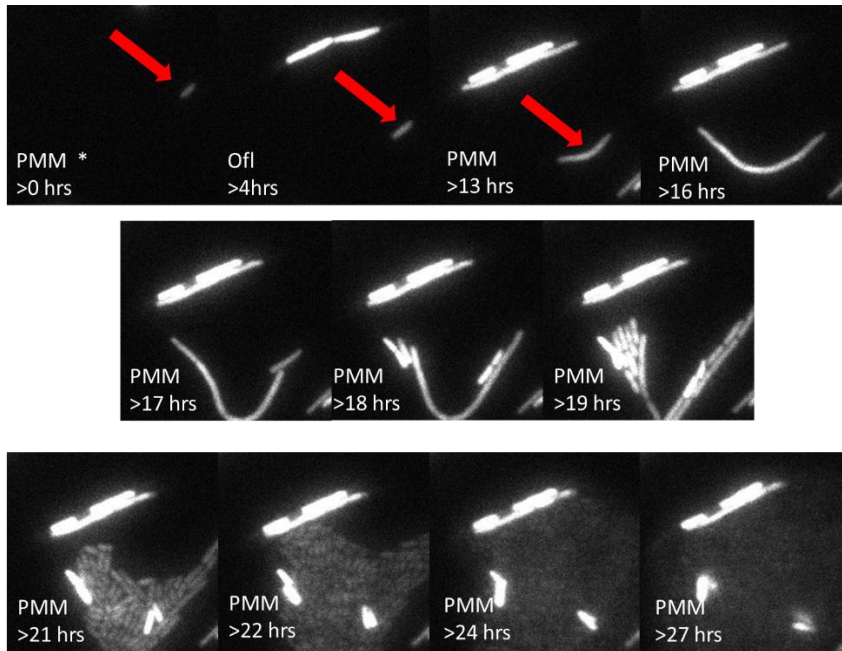


Figure caption: Red arrows point to persister cells. PMM* indicates first exposure to PMM. First line indicates chemical exposed to and the second line indicates time point of the movie. During second PMM exposure cells filament and subsequently divide while GFP expression decreases over time. In last four panels, cells that divide towards the end of the filament grow bright and subsequently do not divide, similar to other dead cells.

Figure 6.1c

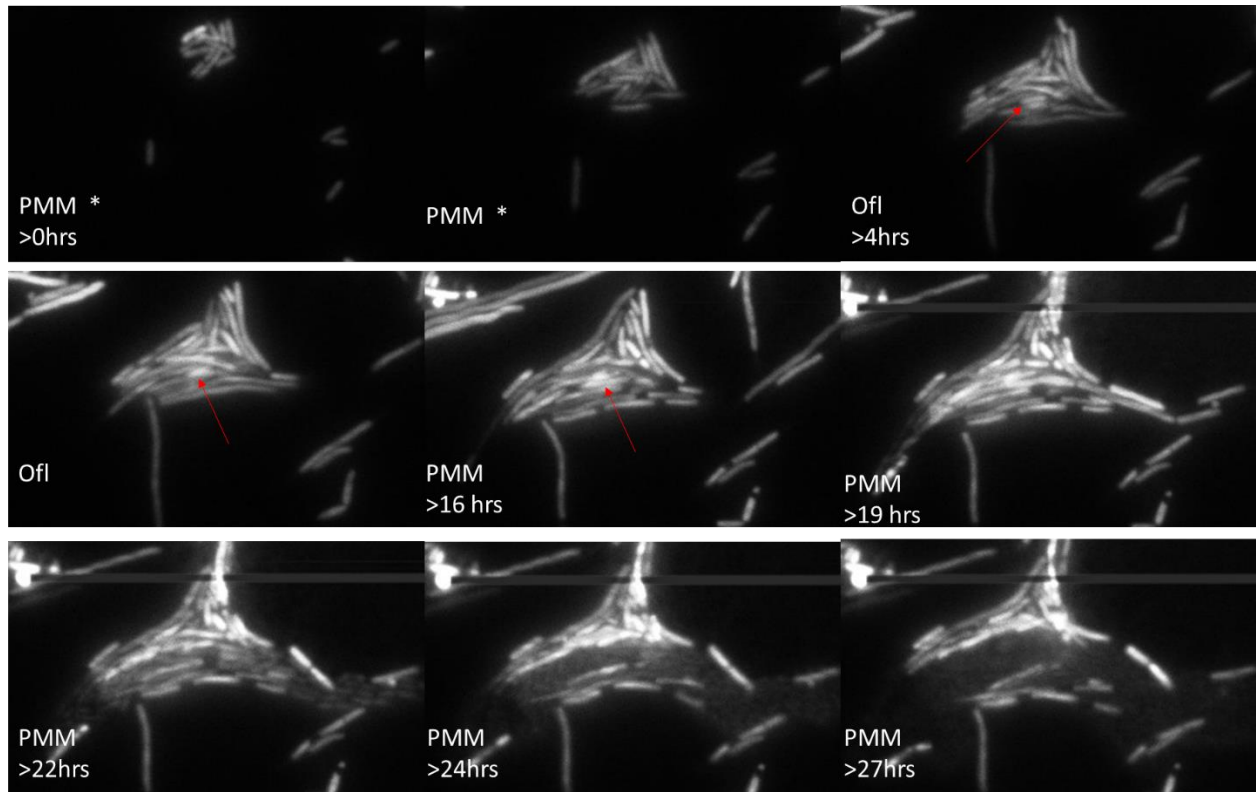


Figure caption: Red arrows point to persister cells. PMM* indicates first exposure to PMM. First line indicates chemical exposed to and the second line indicates time point of the movie. During second PMM exposure cells filament and subsequently divide while GFP expression decreases over time.

Figure 6.1d

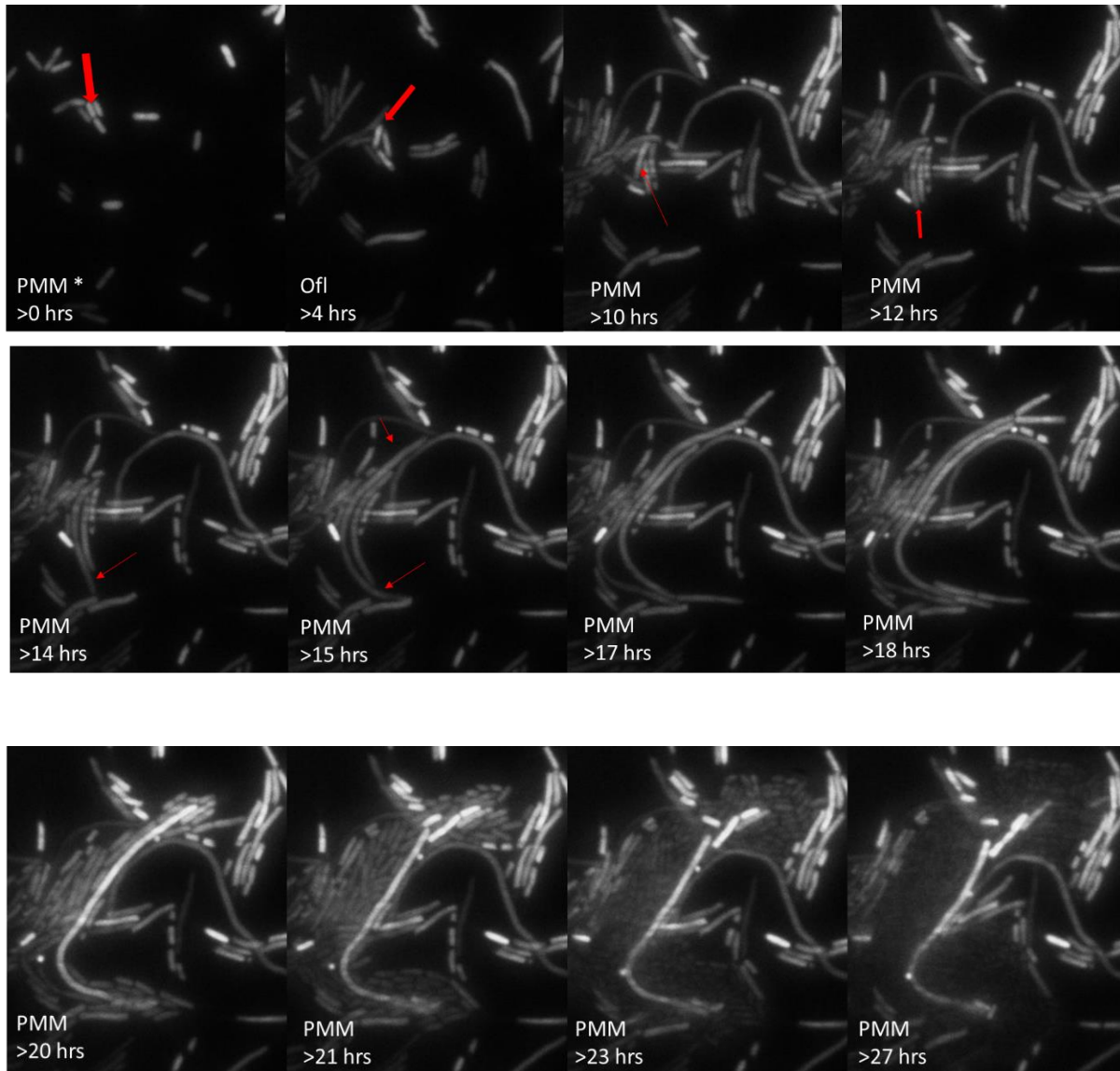


Figure caption: Red arrows point to persister cells. PMM* indicates first exposure to PMM. First line indicates chemical exposed to and the second line indicates time point of the movie. During second PMM exposure cells filament and subsequently divide while GFP expression decreases over time.

Figure 7.1e

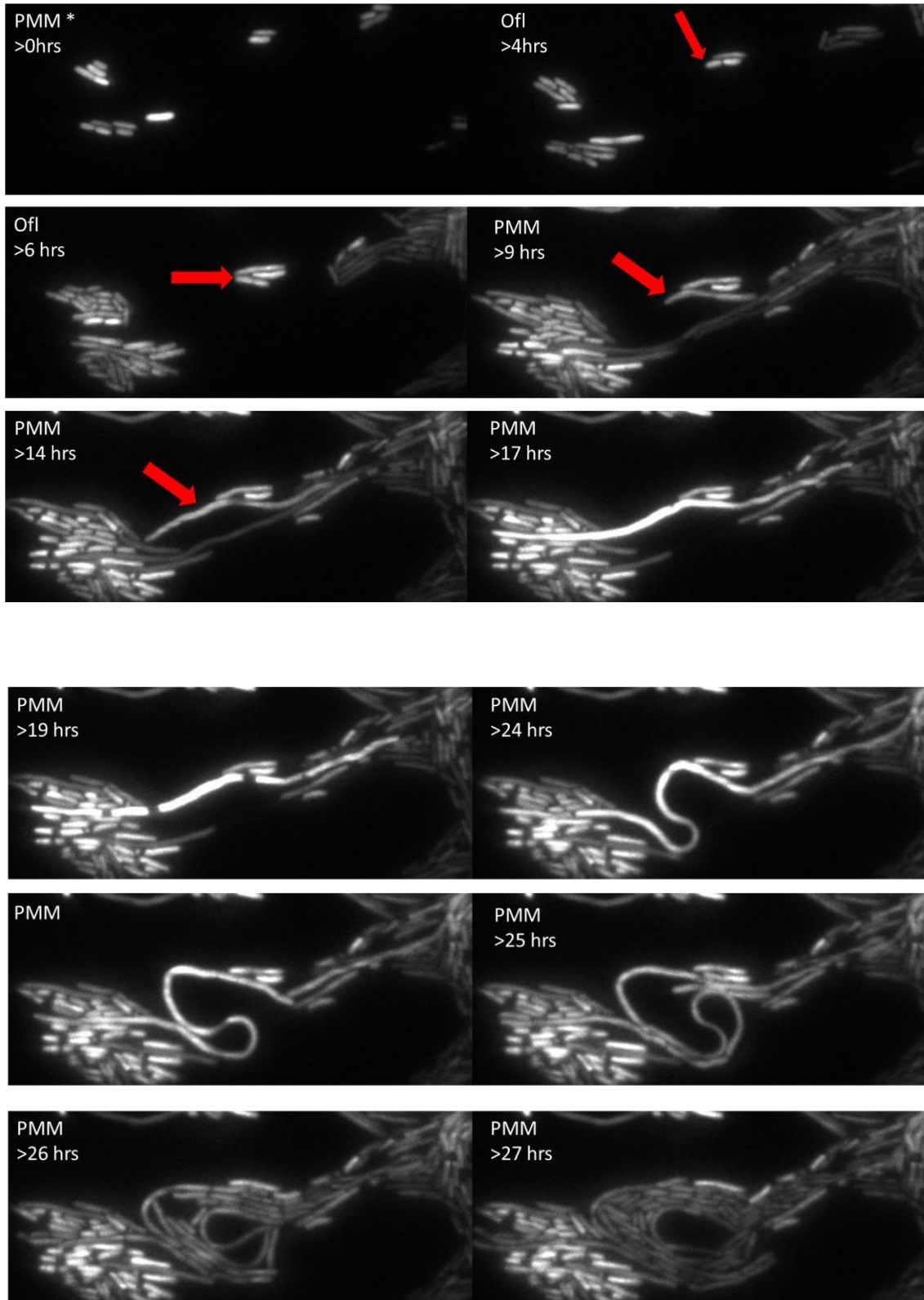
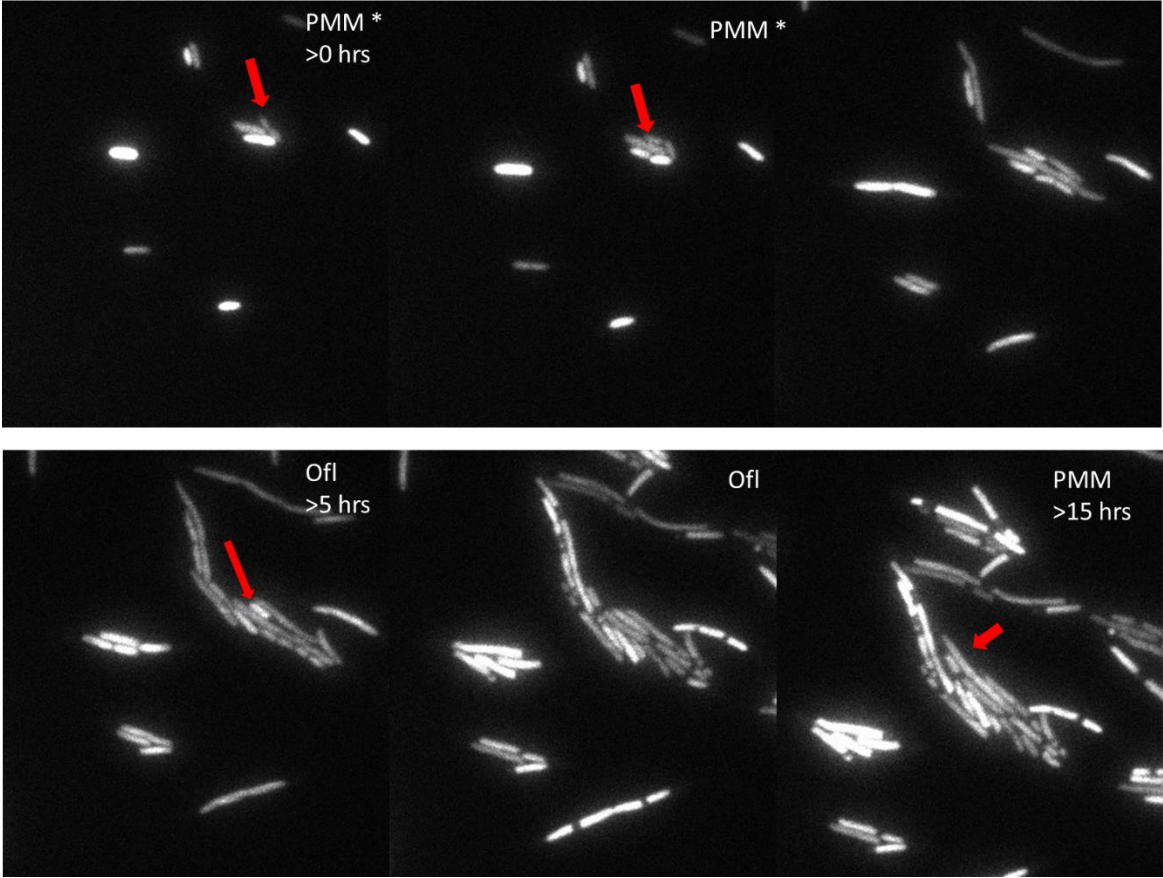


Figure Caption: Red arrows point to persister cells. PMM* indicates first exposure to PMM. First line indicates chemical exposed to and the second line indicates time point of the movie. During second PMM exposure cells filament and subsequently divide while GFP expression decreases over time.

5.2 GlpD

One survivor cell was seen within the GlpD strain. The cell did not grow during the first 3 hours of media exposure and antibiotic exposure. Approximately 8 hours after antibiotic exposure stopped and media exposure began, the survivor cell began filamenting and started rapidly dividing. Similar to the PlsB strain, the cells dimmed during regrowth until distinct cell edges could not be seen under the microscope.

Figure 7.2



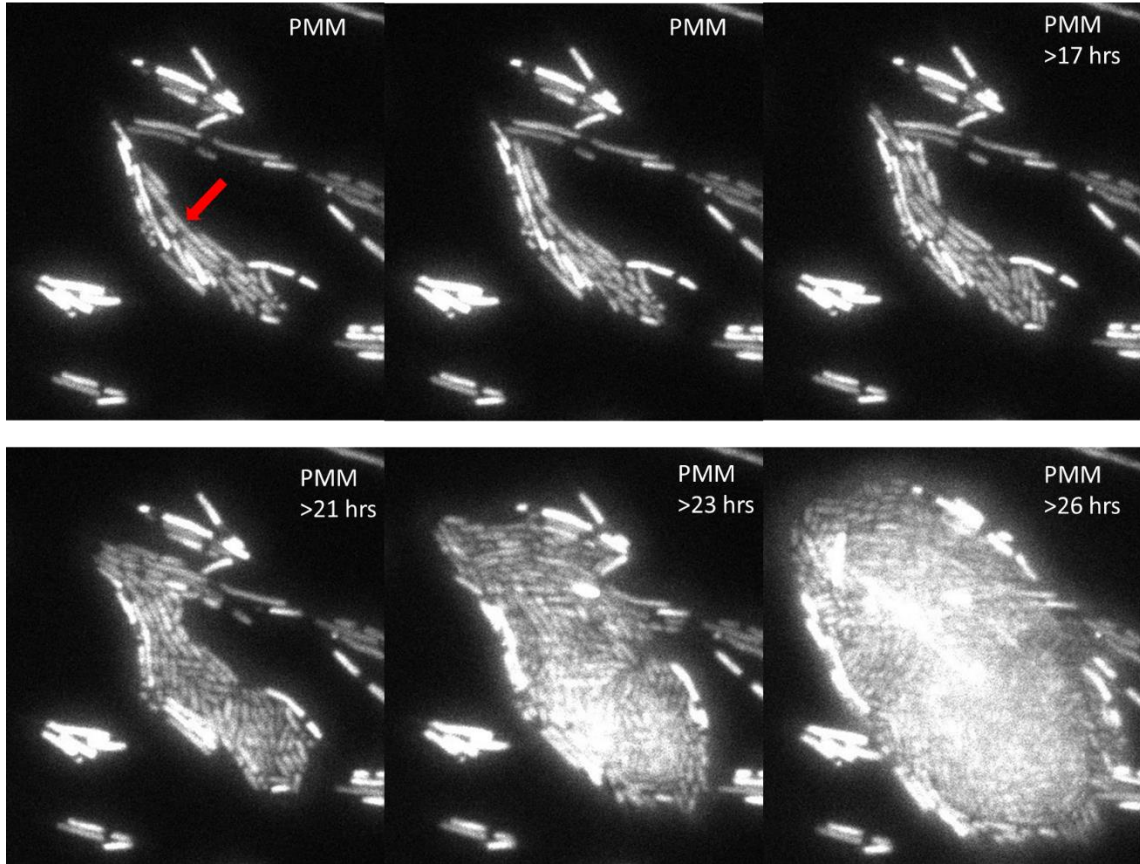
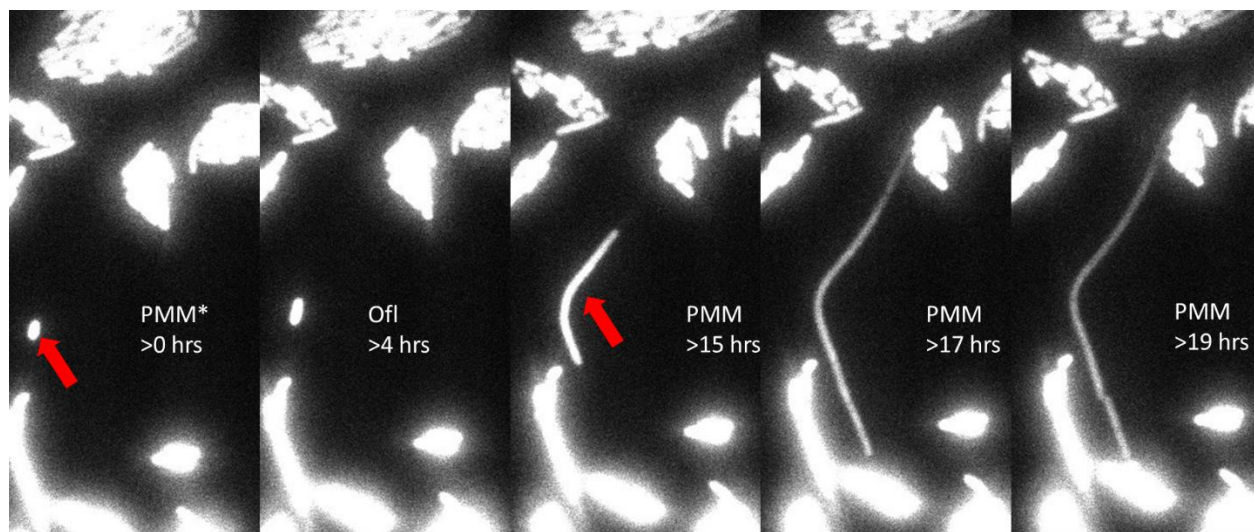


Figure caption: Red arrows point to persister cells. PMM* indicates first exposure to PMM. First line indicates chemical exposed to and the second line indicates time point of the movie. During second PMM exposure cells filament and subsequently divide while GFP expression does not decrease over time.

5.3 DinJ

Only one survivor cell was seen within the DinJ strain. Cells that survived did not grow during the first 3 hours of antibiotic exposure and did not grow during antibiotic exposure, and subsequently filamented and rapidly grew 8 hours after antibiotic exposure. Resultant daughter cells subsequently dimmed down until they were not visible.

Figure 7.3



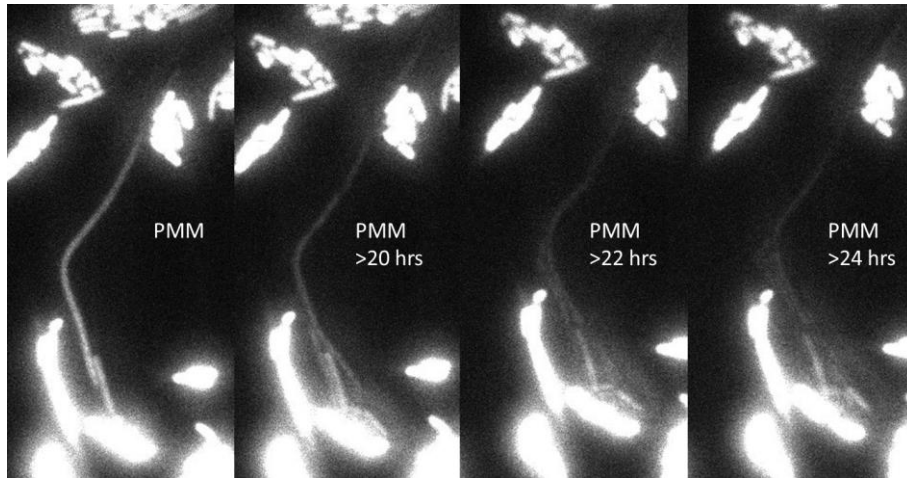


Figure caption: Red arrows point to persister cells. PMM* indicates first exposure to PMM. First line indicates chemical exposed to and the second line indicates time point of the movie. During second PMM exposure cells filament and subsequently divide while GFP expression decreases over time.

5.4 DnaJ

Only one survivor cell was seen within the DnaJ strain. Cells that survived did not grow during the first 3 hours of antibiotic exposure and did not grow during antibiotic exposure. Survivor cells subsequently filamented and rapidly grew approximately 8 hours after antibiotic exposure stopped. Cells subsequently dimmed down until they were not visible by the microscope but could still see cell growth occurring as surrounding bright dead cells were pushed outwards.

Figure 7.4a

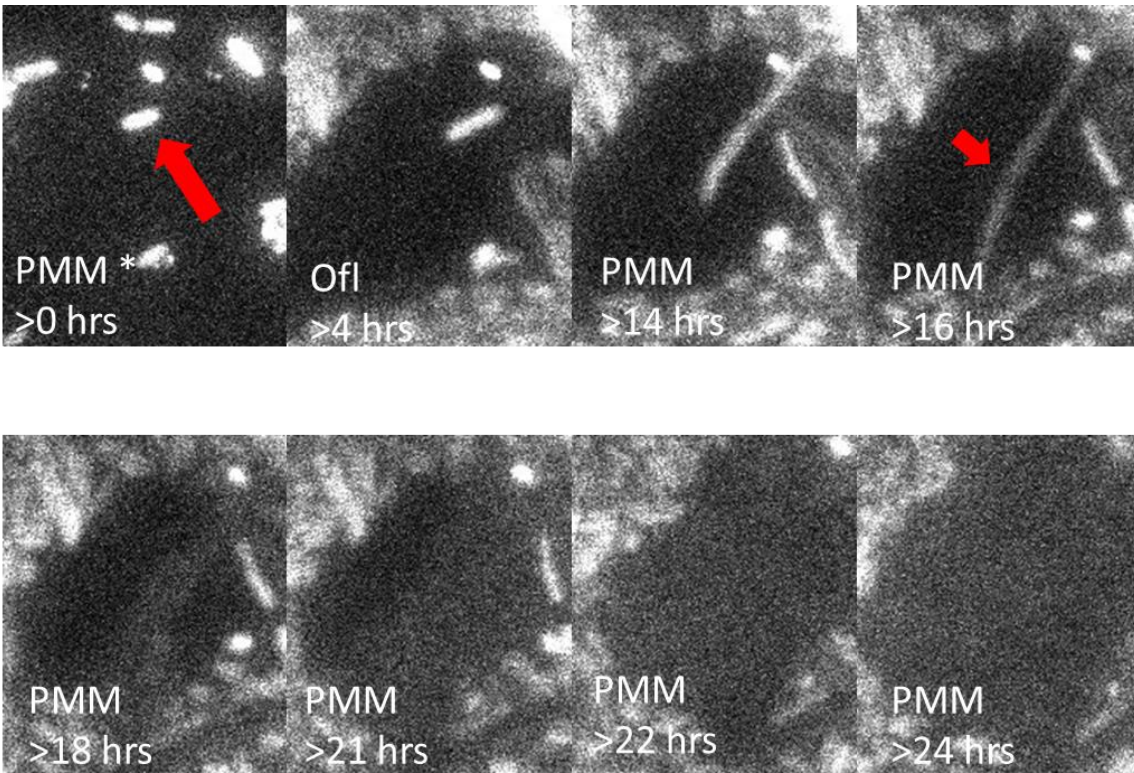


Figure caption: Red arrows point to persister cells. PMM* indicates first exposure to PMM. First line indicates chemical exposed to and the second line indicates time point of the movie. During second PMM exposure cells filament and subsequently divide while GFP expression decreases over time.

Figure 7.4b

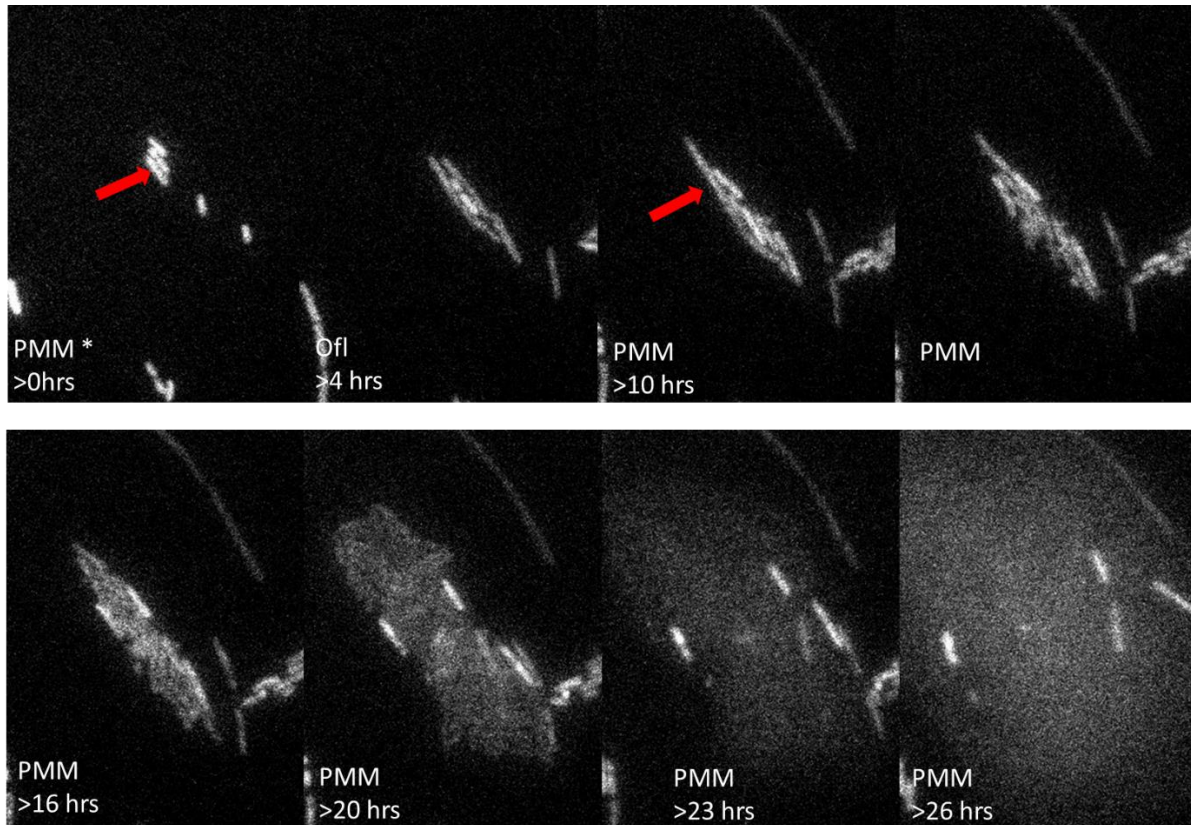
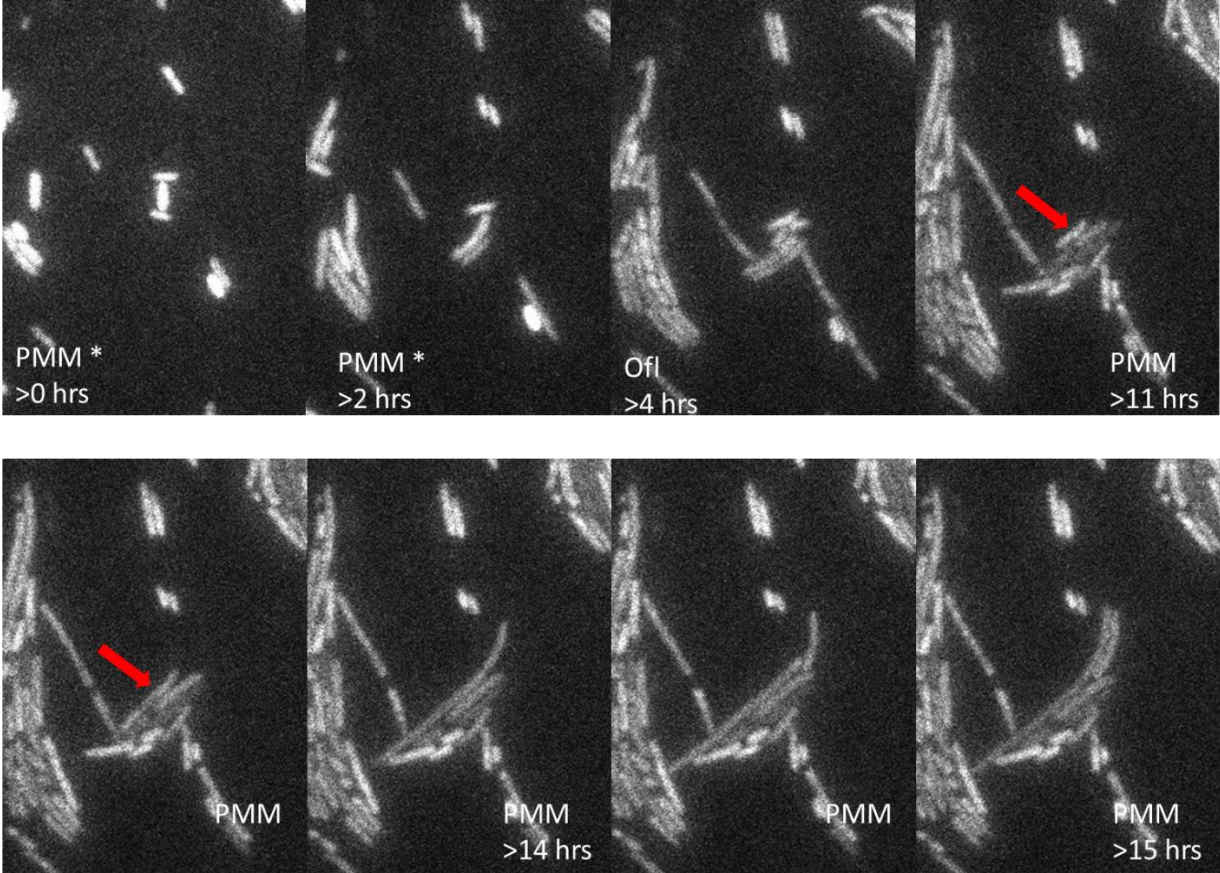


Figure caption: Red arrows point to persister cells. PMM* indicates first exposure to PMM. First line indicates chemical exposed to and the second line indicates time point of the movie. During second PMM exposure cells filament and subsequently divide while GFP expression decreases over time.

Figure 7.4c



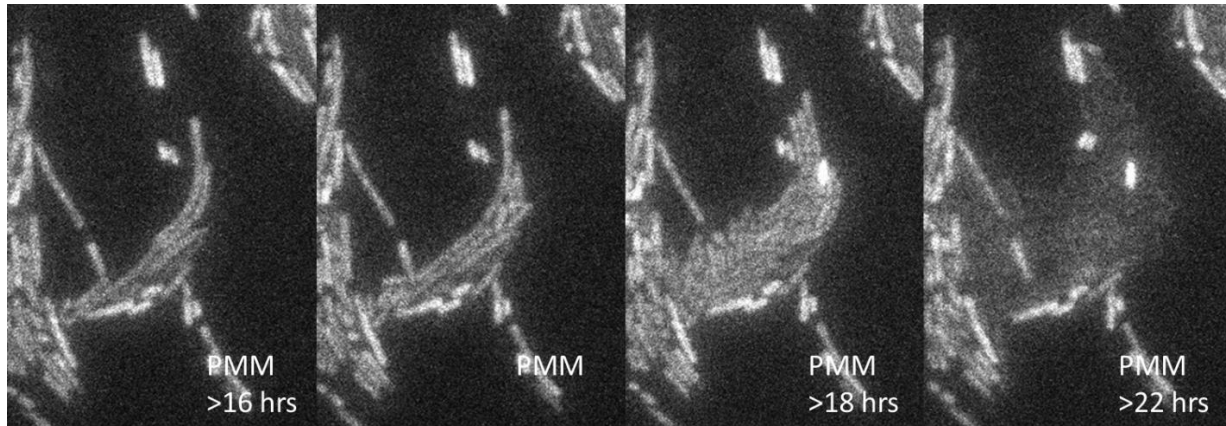
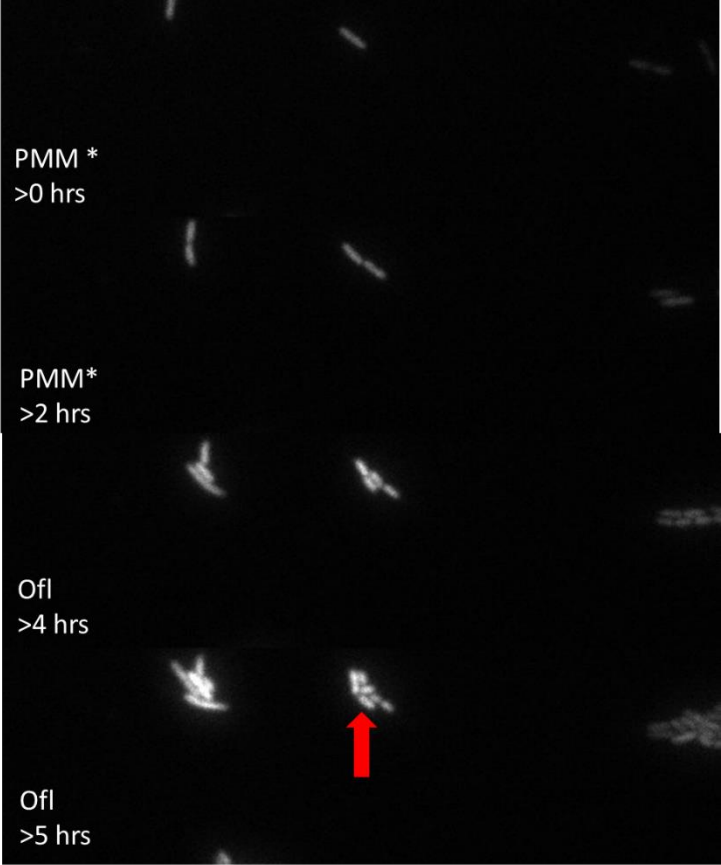


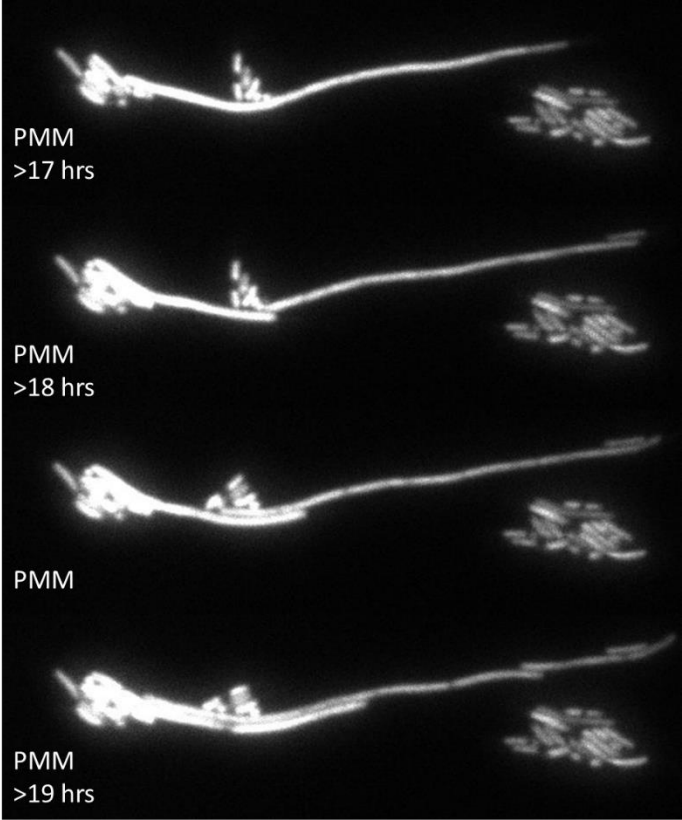
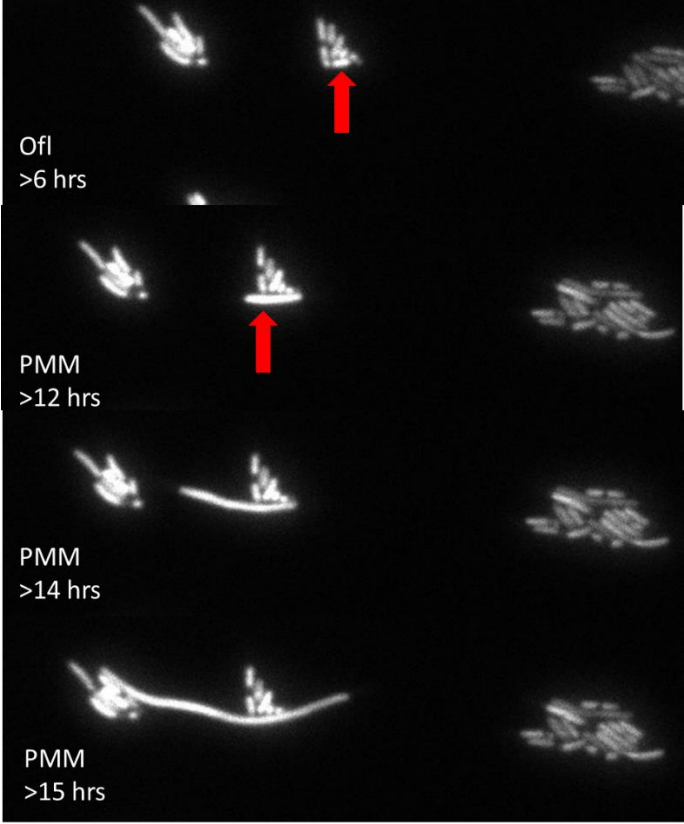
Figure caption: Red arrows point to persister cells. PMM* indicates first exposure to PMM. First line indicates chemical exposed to and the second line indicates time point of the movie. During second PMM exposure cells filament and subsequently divide while GFP expression decreases over time.

5.5 TolC

Cells that survived and regrew that were imaged from the TolC strain also showed similar characteristics to the Plsb strain survivors. They grew during the first 3 hours of pmm exposure, arrested in growth during antibiotic exposure, and subsequently after a gap of approximately 7-15 hours after antibiotic exposure stopped, rapidly filamented and divided. The resulting daughter cells eventually gradually dimmed, but were still visible by the end of the movie.

Figure 7.5





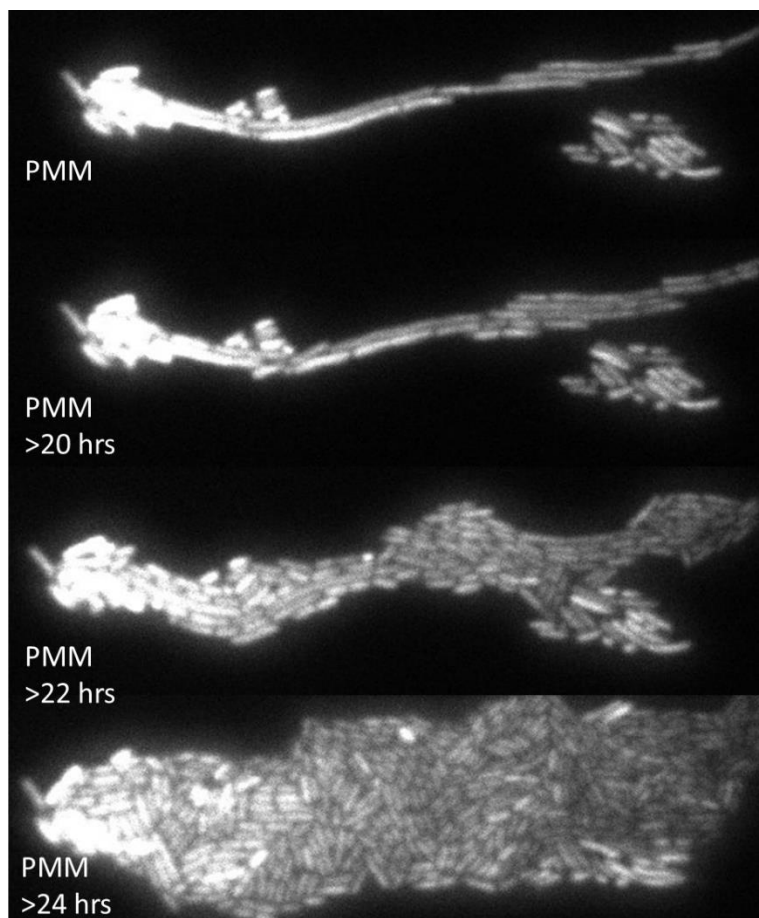


Figure caption: Red arrows point to persister cells. PMM* indicates first exposure to PMM. First line indicates chemical exposed to and the second line indicates time point of the movie. During second PMM exposure cells filament and subsequently divide while GFP expression remains relatively constant over time.

5.6 Summary of Results

From these results it can be seen that there were two kinds of survivor cells observed. One kind that showed growth during the first three hours of PMM flow, and one that did not. Hypothesis was that persister cells would not grow during the first three hours of PMM flow. In

addition it can also be seen that all surviving cells showed filamentation before dividing rapidly and then dimming down, which were all common properties. Also, from figures 1c and 3a it can be seen that cells first divided towards the end during regrowth. These cells did not divide, and over time became bright.

Table 2: Summary of Results

Image	Gene	Showed Growth during first 3 hours of PMM flow (Y/N)	Arrested in Growth during Antibiotic Exposure (Y/N)	Time period after antibiotic exposure stopped and filamentation began (hours)	Noticeable dimming of cells seen (Y/N)	Was the Dimming an expected Response after antibiotic was flowed out (Y/N)
Figure 7.1a	PlsB	Y	Y	5	Y	Y
Figure 7.1b	PlsB	N	Y	7	Y	Y
Figure 7.1c	PlsB	Y	Y	9	Y	Y
Figure 7.1d	PlsB	N	Y	3	Y	Y
Figure 7.1e	PlsB	Y	Y	3	Y	Y
Figure 7.2	GlpD	N	Y	10	Y	Y
Figure 7.3a	DnaJ	N	Y	7	Y	Y
Figure 7.3b	DnaJ	N	Y	5	Y	Y
Figure 7.4	DinJ	N	Y	7	Y	N
Figure 7.5	Tolc	Y	Y	5	Y	Y

Figure Caption: Large variability was noticed Visual assessment of time after antibiotic removal till filamentation started was noted for each persister cell noticed for each strain Florescent activity visually observed was compared to hypothesis and non persister cells over time.

6 Discussion

From the results, there are three distinct physiological aspects of *E. Coli* survivor cells that can be discussed

- Filamentation
- Explanation for the gap between the time from when ofloxacin exposure stopped and filamentation began
- Consistent Dimming of survivor cells after regrowth.

6.1 Filamentation

Filamentation can be an indicator of abnormal growth or cell scarring in bacterial cells. Filamentation, wherein a cell continually elongates, showing chromosomal duplication without cell division, was a definitive stage in all persister cells. Filamentation is usually associated with abnormal growth patterns. Aneuploidic states in mammalian cells have been extensively studied for its role in cancers. In bacteria it is usually associated with cell scarring, and non-functioning DNA repair mechanisms and inability to completely replicate. Dewachter et al. (2017) found that mutant isoforms of ObgE which regulates a major checkpoint in cell division also induces an aneuploidic state in which daughter cells are unable to separate.

Quinolone induced filamentation is only exhibited at subminimal concentrations and an indication of the stress response in bacteria. All the survivor cells in the movie that observed the plsb and tolc strains both showed filamentation characteristics that were similarly described by Bos et al., (2015). The paper describes that ciprofloxacin, induces a unique mechanism of resistance in *E.Coli* cells at low concentration levels. Since ciprofloxacin and ofloxacin have

similar mechanisms of function, they can be treated as analogous however their effectiveness is not. Considering the differences between the bactericidal activity of ofloxacin and ciprofloxacin, Wolfson and Hooper (1985) analyzed their comparative MIC's on E.Coli and overall found ciprofloxacin required a lower concentration to be effective compared to ofloxacin. Bos et al. (2015) found that *E. Coli* shows abnormal growth patterns of filamentation in the presence of .625 µg/ml of ciprofloxacin. After exposed to 4 hours of ciprofloxacin, cells showed aneuploidic conditions along with filamentous structure, which successfully segregates non ofloxacin impacted chromosomes towards the top. Bos et al. (2015), argues that exposure to ciprofloxacin induces a stress response in bacteria commonly called the SOS response, which involves many toxin/antitoxin pathways, that help the bacteria successfully survive stressful conditions. The SOS response, effectively mutates the chromosomes at the end, eventually budding and giving rise to ciprofloxacin resistant cells. Bos used single cell imaging to document the phenomenon. Bos et al. (2015), also observed that at antibiotic concentrations higher than 0.625µg/ml this was subsequently not observed. Hence, *E. Coli* only exhibit filamentation at a specific subminimal concentrations as described by Bos et al. (2015) and is an indicator of the SOS response.

However, contrary to what Bos et al. (2015) observed, in figures 6.1b and 6.1c it can be clearly seen that cells that segregated from the end, did not divide. In the movies it can be seen that they segment off first, but grow bright and do not divide further, unlike the cells that proliferated from the middle of segment. Also, Bos et al. (2015) concluded that cells that segmented towards the end were ciprofloxacin resistant because they showed an upregulation of the Sula protein which induces the SOS response in bacteria. However, these cells were not subsequently re-exposed to ciprofloxacin to confirm resistance. Although the first cells do arise from the ends of ofloxacin treated filamentous *E. Coli*, they do not divide further, and only cells

from the middle proliferate. In the paper, it is mentioned that cells first divide towards the end, but the movies also did not span any longer than 6 hours. These results could potentially refute Bos et al. (2015) results.

Many cells did show abnormal growth characteristics with no regrowth. The majority of cells, under ofloxacin exposure, performed their last cell division after approximately 2 hours after ofloxacin exposure started, wherein they filamented, and segmented multiple times, in the span of approximately 3 minutes. Cells that did divide in this manner did not regrow. Cells that did not divide, showed abnormal elongation after this point, with cells elongating without dividing. Ofloxacin targets the DNA replication fork, inhibiting the function of topoisomerase which reduces torsional strain. Possible reasons for this abnormal elongation seen under 2.5 ug/ml and not under 5 ug/ml could be that ofloxacin targeted one end of the replication fork, thus promoting abnormal cellular growth.

6.2 Waiting Period

In all of the persister cells imaged from the strains mentioned, there was a noticeable gap between the time that ofloxacin exposure stopped and media exposure began, and first sign of elongation of the persister cell. This time period showed some variance usually falling between the range of 7-15 hours after ofloxacin exposure stopped. During this time the expression of the gene being measured in that particular gene does not change, nor does the external appearance of the cell change. Cells during this time do not grow bright or dim.

6.3 Consistent Dimming of Survivor Cells after Regrowth:

All survivor cells dimmed down during regrowth until they were not visible by the camera. The GFP fluorescence considerably dimmed until there the cells were not visible. While

this was expected for DnaJ and TolC it was not expected for PlsB, GlpD and DinJ. Actively dividing and growing cells need to express PlsB and GlpD especially for active growth, and energy production and cell wall synthesis for reproduction.

Since these are essential genes and their functioning is necessary during regrowth, another possible explanation could be the GFP itself. In the promoter library GFP is transcribed at the same rate as the gene that it succeeds, lending an indirect measurement of expression. One possible explanation is that these survivor cells have increased protease activity, which could lead to rapid digestion of GFP as it is being produced. This is a more plausible theory than the gene not functioning. Coupled with the noticeable aneuploidic state, this could have provided an environment where mutations have occurred, leading to possible heritable variations in the daughter cells. In addition, cells that divided towards the end grew bright like the surrounding dead non-persister cells and did not regrow contrary to what Bos et al (2014) noted. This could potentially be a mechanism by which toxic proteins from antibiotic exposure could be pushed to the extreme ends of the filament, thus allowing the cells from the middle to grow but not from the ends.

6.4 Persistence, Tolerance or Resistance

Kerner (2015) had found that persisters are not found in the exponential phase of growth, but rather more likely to be found in the logarithmic phase. Additionally, Kerner found that antibiotics do not induce the formation of the persister cell, but rather they help isolate it. In a population of bacterial cells, randomly selected persister cells will exist in a dormant state, without dividing. This characterization of a classical persister cell was only found 5/10 times in the persister cells that I imaged.

Tolerant cells show a diminished growth rate compared to persisters, which go completely dormant (Brauner et al., 2016). Since antibiotic exposure was only 4 hours, and MIC was also not determined for this strain in this experiment, it is difficult to determine if the cells observed were tolerant or persistent. From Bos et al (2015) the cells observed could also be persistent, but one major dissimilarity with the physiology described by Bos, is the budding off of healthy daughter cells from the ends of the cells, rather than the middle, which was observed. For this reason, the cells are more likely to be tolerant or persistent, rather than resistant.

7 Conclusion:

From the results of this experiment, no definitive conclusions can be made regarding the identity of the cells, or the expression levels of key regulatory genes in predicting the persister state. My hypothesis (Table 1) could not be accurately accepted owing to the discrepancy in the GFP reporter and its rapid degradation. More inquiries are required, both in verifying the external appearance of cells during the phase of regrowth, and the identity of the persister cells.

However, based on experimental results, low doses of antibiotics could prove to be ineffective or harmful, based on the identity of the bacteria. Increasing quantities of antibiotics are finding themselves in the environments around us, giving rise to more harmful, tolerant, and resistant strains of bacteria, rendering people more susceptible for infections. Super-bugs have generated increased concerns in the past decade, but increased incidence of persistent bacteria do warrant further investigations. Different mechanisms of antibiotic resistance are also being researched extensively, and this research could provide different mechanisms by which this could happen.

8 Future Experiments

8.1 Cellular Appearance:

Survivor cells showed a consistent dimming over time, and in many cases resulted in a disappearance of cell edges which did not allow for further observation of regrowth. In order to observe this critical phase, movies can be taken under bright light for the same strains, and distinct external cellular appearance be observed properly during regrowth.

8.2 Confirmation of Identity of Persister Cells

Lewis (2007) exposed cells to repeatedly to different classes of antibiotics, including ampicillin and ofloxacin, confirming the multidrug tolerant nature of the regrowing cells. This would be a method of ensuring that all surviving cells are persister cells.

Future experiments can expose cells to ofloxacin and subsequently a β -lactam antibiotic or tetracycline, and allowing a total of 30 hour phase of regrowth which is longer than the one used in the experiment, increasing the chances of capturing a persister cell. Considering the waiting period between the time ofloxacin exposure stopped and filamentation began, and its variance (Table 2) it is important to increase regrowth time. After allowing for adequate regrowth, flowing in a final solution of ethidium bromide, which will enter only dead cells, will confirm the identity, and the state of the surviving cells. However, this method will yield a very low percentage of persisters.

A better confirmation method could be to expose cells to ofloxacin, and subsequently allow cells to grow in PMM while observing survivor cells. Consequent re- exposure to

ofloxacin should kill all cells that are not resistant, or tolerant or persistent. However if all cells that do proliferate during the intermediate PMM phase survive then they are either tolerant or persistent. If few cells survive, then they could be persisters.

8.3 Increased Protease Activity

Few strains of the promoter library have been transformed with mScarlet 118 wherein mScarlet 118 was transformed to a promoter that is constitutive (always active). Movies have been taken and analyzed, but considering the variability in the waiting period, longer movies perhaps 35 hours movies need to be taken to better the chance of capturing a persister cell. Using a double reporter strain wherein mScarlet 118 is visible under the 561 nm wavelength of light and GFP is visible under 488 nm wavelength of light, both of them can be distinctly seen. If the cells do have increased protease activity then cells expression both the GFP and mScarlet should not be fluorescent in either channel.

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