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by

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ABSTRACT

DIVIVA LOCALIZATION DURING SEPIVA DEPLETION IN MYCOBACTERIUM SMEGMATIS

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Mycobacterium is a genus of bacteria best known for *Mycobacterium tuberculosis (Mtb*), which causes tuberculosis. Proteins that are distinctive to mycobacteria can be studied in closely related species of *Mtb* in order to find potential new tuberculosis drugs based on unique mycobacterial processes. In this study, the model organism *Mycobacterium smegmatis* (*Msmeg*) was used to study the protein, SepIVA. SepIVA is a protein found in Mycobacteria, which is thought to be similar to a group of proteins that function in assisting with cell wall synthesis during cell division and cell elongation. To study the possible functions of SepIVA, the localization of the protein DivIVA was observed via microscopy. DivIVA is a better studied protein with known localization patterns in *Msmeg*. The results suggest that SepIVA is not involved in regulating the localization of DivIVA during cell division.

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CHAPTER 1

INTRODUCTION TO MYCOBACTERIA

1.1 Disease Causing Species

Mycobacterium tuberculosis (*Mtb*) is the cause of tuberculosis, the leading infectious disease worldwide (Anderson, 2018). Bacterial infection requires growth and division of the bacteria; therefore, it is important to understand bacterial cell division (Peterson, 1996). A critical part of cell division is based on synthesizing new cell wall structures (Wu, 2018). Mycobacteria are rod shaped acid fast bacteria, with one cell membrane, a superficial peptidoglycan layer, and a distinctive outer coating of mycolic acid and arabinogalactan. The pathogenic species, *Mtb*, is similar to the non-pathogenic species, *Mycobacterium smegmatis* (*Msmeg*) in the way it builds its cell wall. The study of a newly found and crucial *Msmeg* division protein, SepIVA, may provide insight into the division process that is distinctive to mycobacteria (Wu, 2018).

1.2 Peptidoglycan

Peptidoglycan is a structural component of most bacteria that provides strength and shape for the cell. It is made up of glycan strands (sugars) crosslinked by peptides. This peptide-sugar structure surrounds both gram positive and gram negative bacteria outside of the cytoplasmic membrane. Since peptidoglycan is a single molecule that surrounds the whole cell, new peptidoglycan subunits have to be carefully inserted into the existing structure to allow for cell growth and division without lysing the cell (Vollmer, 2015). In Mycobacteria, the peptidoglycan layer is further covered by a layer of arabinogalactan, and

an outermost layer of mycolic acids (Zuber, 2008). During cell elongation, Mycobacteria build new peptidoglycan by incorporating peptidoglycan cell wall precursors at the poles of the cell (Meniche, 2014).

1.3 DivIVA Protein

The DivIVA protein is a fairly well understood protein in M*smeg* (Kaval, 2017). This protein is hypothesized to be involved in the recruitment of early cell wall synthesis proteins, and to be a determining factor in sites of growth (Meniche, 2014). However, it has been proven that DivIVA localizes to the poles of M*smeg* (Meniche, 2014). This would make sense, if the current hypothesis that DivIVA has a function in cell wall precursor enzyme recruitment is true, since mycobacteria have polar growth (Meniche, 2014).

1.4 SepIVA Protein

According to previous research, Mycobacterium contain a particular protein that has previously not been studied, SepIVA. This protein is a homolog to DivIVA, and is necessary for cell division (Wu, 2018). Drawing from information known about DivIVA proteins, informed guesses can be made as to the function of SepIVA due to their structural similarities. DivIVA homologs are involved in the recruitment and placement of other cell wall building proteins, and so we hypothesize that SepIVA may have similar recruitment functions for cell wall precursor enzymes (Kaval, 2017: Wu, 2018). It is also important to keep in mind while studying SepIVA that it is a necessary protein for cell division, without which the cells are not able to divide (Wu, 2018).

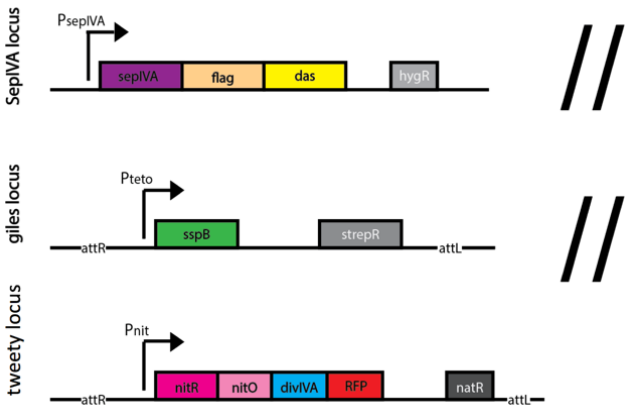
CHAPTER 2

PLASMID CREATION

2.1 Overview

DNA plasmids are useful tools to force the expression of a desired gene or gene fusion. Plasmids often contain a specific sequence that allows for integration into the chromosome. In order to run experiments to observe DivIVA localization during SepIVA depletion, a plasmid was built to induce the expression of a fluorescently tagged DivIVA protein.

Figure 2.1: Genetic Modifications of CB1332



2.2 Methodology

Polymerase chain reaction (PCR) was used for gene amplification. After amplification and purification was complete, Gibson assembly was used to fuse together the new genes with the vector plasmid.

2.2.1 Gene Abstraction

The promoter gene, PNit, and the gene encoding a nitrile inducible repressor, NitR, were amplified from plasmid CT106, where the genes were already joined. This was done using forward (1403) and reverse (1404) primers and a KODXtreme 25µL PCR reaction mixture. This consisted of 0.75 µL of primer 1403, 0.75 µL1404 primer, 1.25 µL dimethyl sulfoxide (DMSO), 1 µL of CT106 template mixture, 2.25 µLnuclease free water, 5 µL KOD deoxyribonucleotide triphosphates (dNTPs), 12.5 µL 2X KOD buffer, and 0.4 µLKODXtreme enzyme.

The nitrile promoter that the repressor binds to, ONit1, was amplified out of plasmid CT106 using forward (1399) and reverse (1400) primers and a Q5 25µL PCR reaction mixture. This consisted of 1.25 µL of primer 1403, 1.25 µL1404 primer, 5 µL 5X GC enhancer, 1 µL of CT106 template mixture, 10.75 µLnuclease free water, 0.5 µL 10mM dNTPs, 5 µL 5X Q5 buffer, and 0.25 µLQ5 enzyme.

The protein acting as the dependent variable, DivIVA, and the red fluorescent protein (RFP), were amplified out of plasmid CB1261, where the genes were already joined, using forward (1410) and reverse (1411) primers and a KODXtreme 25μL PCR reaction mixture. This consisted of 0.75 μL of primer 1410, 0.75 μL1411 primer, 1.25 μL DMSO, 0.5 μL of CB1261 template mixture, 2.75 μLnuclease free water, 5 μL KOD (dNTPs), 12.5 μL 2X KOD buffer, and 0.4 μLKODXtreme enzyme.

2.2.2 Stitching

In order to stitch the DNA together into a new plasmid creation, vector plasmid CT250 was digested with restriction enzymes XbaI and HindIII. This was made into a circular plasmid by performing a three part Gibson 10 μL reaction involving DivIVA-RFP,

ONit1, and the digested CT250. The ratio was 1:3 for vector to insertion DNA fragments where 2μL CT250 vector, 2μL DivIVA-RFP, and 1μL ONit1 were added together in a Gibson tube containing pre-mixed Gibson mixture. A control was also done with 2μL CT250 vector and 3μL nuclease free water in a Gibson tube. These tubes were placed in the PCR machine on the Gibson setting for 1 hour at 50°C. The Gibson reactions were transformed into Top10 *E.coli* via heat shock, and then plated onto nourseothricin (nuo) 40 µg/mL lysogeny broth (LB) plates, and set in 37°C overnight. Small samples of each individual colony were used to perform a GoTaq PCR amplification of the transformed plasmids, in order to check if the correct size of the insert is present in the plasmid through gel electrophoresis. A 20 μL GoTaq reaction was performed using 1 μL of forward primer 1399, 1 μL reverse primer 1411, 1 μL DMSO, 7 μL of nuclease free water, 10 μL GoTaq, and a sample of the colony. Colony samples were added by touching sterile toothpicks to the colony, and placing the tip into the reaction tube. After the DNA was amplified in the PCR machine, 10 μL of each tube, and 6 μL of DNA ladder was loaded into a TAE 1% gel, and run on 120 V. Colonies 35 and 36 were chosen to be inoculated and grown based on having DNA bands of the expected insert size, 1706 bp. Each colony was inoculated in 5mL of LB media with 5 μL of nat, and set in the 37°C overnight. The cultures were each miniprepped to isolate the plasmid contents. Both samples of purified plasmid were sent for sequencing, and the colony 35 plasmid was used to continue the project.

2.2.3 Final Plasmid Assembly

The colony 35 plasmid was digested with NotI-HF. This was made into a circular plasmid by performing a two part 10 μL Gibson reaction combining PNit-NitR and the digested plasmid. In a Gibson tube with 5 μL of Gibson mixture, 3 μL of vector/digested

plasmid and 2 μL of insert was added together. A control was also done with 3 μL vector/plasmid and 2 μL nuclease free water. These tubes were placed in the PCR machine on the Gibson setting for 1 hour at 50°C. About 10 μL of Gibson product was transformed into Top10 *E.coli* via heat shock, and then plated onto nuo 40 μ LB plates and set in 37°C overnight. Using the colonies that grew on the media, a 20 μL GoTaq reaction was performed using 1 μL of forward primer 1399, 1 μL reverse primer 1411, 1 μL DMSO, 7 μL of nuclease free water, 10 μL GoTaq, and a sample of the colony. After the DNA was amplified in the PCR machine, 10 μL of each tube, and 6 μL of DNA ladder was loaded into a TAE 1% gel, and run on 120 V. Only colony 20 had a DNA band of the correct size, 1271bp. This colony was inoculated in 5mL of LB media with 5 μL of nat, and set in the 37°C overnight. The culture was miniprepped to isolate the plasmid contents. The purified plasmid was sent for sequencing. The sequencing results were hard to read, but the insert was verified by transforming this plasmid into a wild type (WT) M*smeg* to see if fluorescence of wag31 was present, and inducible. This procedure was done similarly to the procedure explained in section 3.1.

2.3 Transformation

Once the plasmid was created successfully, it was transformed into a strain of M*smeg* that was genetically modified to produce SepIVA that is fused to a Flag and a DAS protein tag (Murphy, 2018). This strain was also genetically modified at the giles locus to transcribe *sspB* in the presence of anhydrotetracycline (ATC). SspB's function is to bind to the DAS tag and the protease, ClpP, to cause the degradation of SepIVA (Kim, 2011). Because the cell requires SepIVA to live we cannot build a *sepIVA* knockout strain. Therefore, we must use a strain in which we can control SepIVA levels in order to

determine the cellular functions in which SepIVA is required. Once the plasmid is transformed into the cell, it integrates in to the genome at a phage integrase site, and in this plasmid, the tweety phage integrase site was used. This culture was frozen down in -80°C for storage.

Figure 2.2: ATC Inducible Degradation of SepIVA

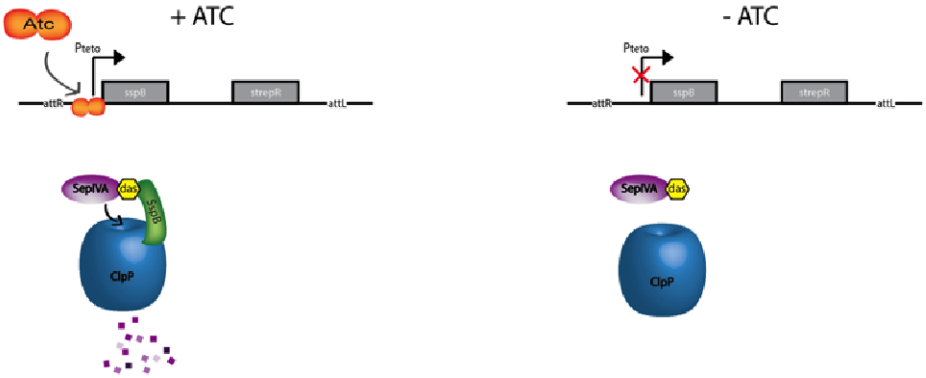
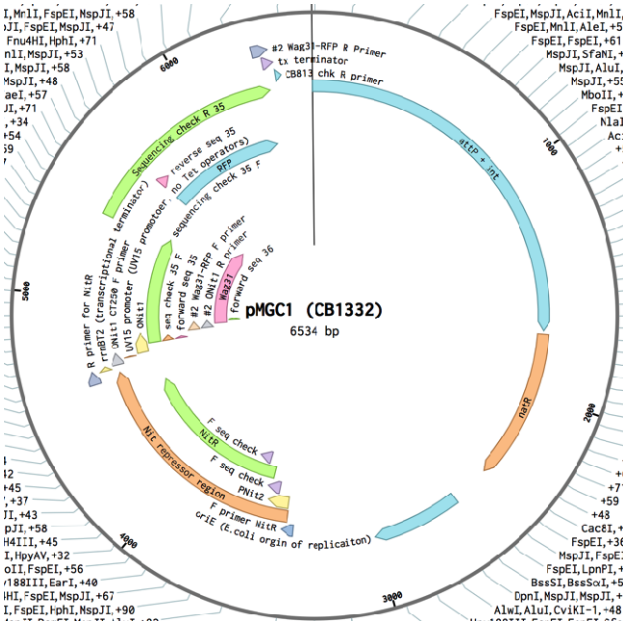


Figure 2.3: Plasmid Map



2.3.1 Electrophoresis Transformation

The strain, CB1166, with the strong ATC SepIVA depletion promoter were grown up in 5mL of 7H9 media + ADC and 5µL of strep and 2.5µL of hyg. They were placed in an incubator at 37°C until they were turbid with cell growth. Do to unforeseen circumstances some culture spilled out of the tubes during incubation, and 7H9 +ADC (no additional antibiotics) was added to re-attain the experimental conditions of the cultures. The cells were centrifuged to separate the cell mass from the media. They were then washed twice in a 10% glycerol solution. The cells were placed in a 2 mm-gap electroporation cuvette and 1µL of the created plasmid was added. The cells were then shocked in an electroporator. These were spread on a LB nuo 20 µg/mL petri dish and set in the incubator at 37°C until the colonies were visible. This culture was then grown in 5mL of 7H9 +ADC media with 5µL of strep, 2.5µL of hyg, and 2.5µL of nat. It was placed in the incubator at 37°C. The culture was tested via fluorescent microscopy as detailed in the following chapter, and then frozen down for storage in -80°C and labelled CB1332.

CHAPTER 3

FLUORESCENT MICROSCOPY

3.1 Background

Fluorescent microscopy was chosen for this experiment as a way to observe both the localization of DivIVA and the location of newly synthesized peptidoglycan in the cell wall. The NIS Elements software used allows for one frame of the microscopy image to have an overlay of three different types of pictures; a phase-contrast image, a green fluorescent image called GFP, and a red fluorescent image called mCherry. This allows for one cell to be analyzed with the information that each type of image provides. During imaging, a slide with the cells is placed on the microscope stage, then the amount, focus, and spacing of light is adjusted for even lighting on the frame. As images are taken, different wavelength ranges are chosen depending on the type of imaging (full spectrum, GFP, and mCherry). The fluorescent molecules will divulge the location of DivIVA the peptidoglycan structure. After the images are taken, the frame will be shifted at least two full frame lengths away from previously imaged frames, to prevent low fluorescent yield due to photo bleaching.

3.2 Induction

Three replicates of the CB1332 M*smeg* strain were individually grown up from the frozen stock in 5mL of 7H9 + ADC media with 5µL of strep, 2.5 µL of hyg, and 2.5 µL of nuorseothricin (nuo). They were incubated in 37°C until the culture was turbid andvisibly in stationary phase. They were then diluted as to reached log phase (having an optical

density [O.D.] between 0.1 and 0.5 O.D.) in a total of 5mL of 7H9 + ADC broth, and incubated at 37°C. Each of the cultures in log phase were then diluted again as to reach log phase, but this time into two test tubes of 5mL 7H9 + ADC per replicate. One test tube per replicate was induced with 2.5 ul/5mL of ATC and set in the incubator at 37°C. The other test tube was not induced, and was put in the incubator at the same time. After approximately 16 hours, a 1mL portion from all six tubes were put into a labeled Eppendorf tube. Each tube was induced with 1µL of 10^{-6} IVN. All six Eppendorfs were placed in the incubator at 37°C for the final three hours.

3.3 NADA Staining

In order to show where peptidoglycan synthesis took place, it is helpful to be able to visualize the incorporation of peptidoglycan. This can be done by using a fluorescent technique called NADA staining. In NADA staining, D-amino acids that are covalently bound to the fluorescent molecule, 7-nitrobenzofurazan, are used by cell's to crosslink their peptidoglycan (Kuru, 2012). This results in the ability to visually see the sites of recent peptidoglycan synthesis in a wide range of bacteria, using fluorescent microscopy (Kuru, 2012). This technique was used on each of the six tubes of induced cells. To each tube, 1µL of the NADA stain (10mM) was added. Each tube was vortexed and they sat for ~2min at room temperature. They were then spun for 1 min in the minicentrifuge at maximum speed. The supernatant was discarded and the remaining cells were resuspended in 1mL of phosphate buffer saline + Tween80 (1X PBS, 250L of 20% tween 80 / 100mL). Then 100mL of 16% para-formaldehyde was added to the tubes, in order to fix the cells. The tubes were incubated at room temperature for 10 min, then spun for 1 min at maximum speed. The supernatant was discarded and the ATC induced cells were resuspended in

100μL of phosphate buffer saline + Tween80, while the non-ATC induced cells were resuspended in 350μL. The cells were then placed on microscope slides for imaging.

CHAPTER 4

CONCLUSION

4.1 Summary of Findings

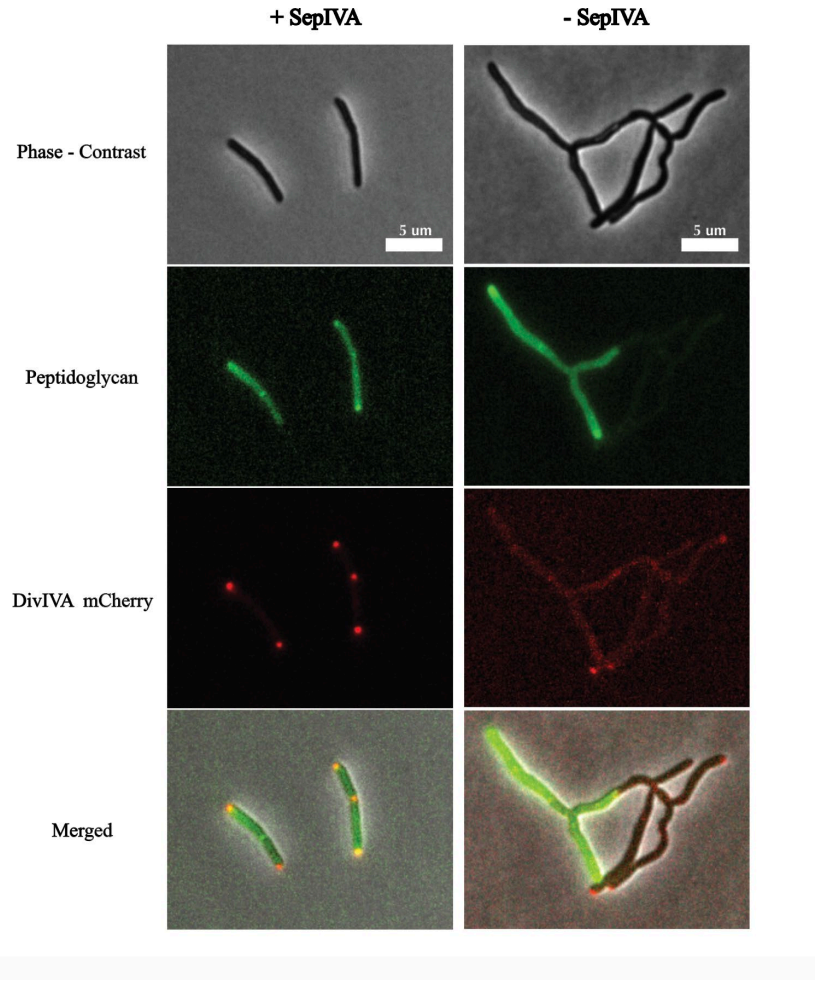
Cells that had normal amounts of the SepIVA protein had a normal rod-shaped morphology, with DivIVA localizing to the poles and septation site of the cell. Cells that had depleted amounts of the SepIVA protein had a high number of dead cells, and a mixture of cells with branched and rod-shaped morphology. In these cells, DivIVA was very faintly localized at the poles and in some cases at the septation site of the cell. The polar signal from the RFP tagged DivIVA was remarkably brighter in cells with SepIVA compared to cells with depleted SepIVA. These results demonstrate that DivIVA localizes to the cell poles, as expected for cell wall precursor incorporation, in the presence and relative absence of SepIVA.

The newly synthesized peptidoglycan of cells in both the control group and SepIVA depleted conditions had similar results with an even spread of faint signal throughout the cell wall, and slightly more concentrated signal at the cell poles. However, in cells with depleted SepIVA, there were several cells that had concentrated signal in the mid cell region, but not at septation sites. This demonstrates that the cell had newly synthesized peptidoglycan being incorporated throughout the cell for normal cell wall upkeep, as well as at the growing poles.

According to these findings, SepIVA does not seem to influence the localization of DivIVA in M*smeg*, since DivIVA localizes to the cell poles both in the absence and

presence of SepIVA. All of the above results were visually inspected and was not analyzed with computer software.

Figure 4.1: Microscopy Images of Cells With and Without SepIVA



4.2 Experimental Errors

The results of the microscopy imaging were not fit for computer analysis because of poor differentiation between the light emissions from fluorescence in the DivIVA protein, versus the background of the slide. While there was enough differentiation for visual analysis, the low quantity of the fluorescent signal prevented the use of computer software analysis, which gives the most unbiased and complete presentation of the results.

This may be due to low fluorescent emission by the resonating molecules, due to low protein yield or inadequate staining techniques. However, it may also be due to a technical problem, where the camera was unable to receive all of the emitted light from the fluorescent tags. Photobleaching is unlikely to have affected the results since precautions were made to prevent photobleaching by spacing out imaged frames, and only exposing each frame to the target wavelengths once.

Also, the high amount of dead cells in the SepIVA depleted samples provided a more limited understanding of the effect of SepIVA, since many of the cells were no longer viable. This is likely due to the process of SepIVA depletion by ATC induction lasting for too long.

4.3 Future Directions

In the future, this experiment should be repeated to increase the contrast between the cells and the background in the fluorescent images. This might be done by adjusting microscope and camera communication settings, or possibly by altering the fluorescent tag used on DivIVA. Additionally, in the future, the SepIVA depleted cells should have a shorter induction time, so as to decrease the number of dead cells in the sample.

Finally, more experiments can be done to further study SepIVA's role in M*smeg* by replacing the gene for DivIVA in the plasmid used in this experiment, with other division-related protein coding genes. Then, the same experiment can be repeated to understand SepIVA's influence on multiple proteins in the *Msmeg* division process. These experiments would continue to provide further insight into the function of SepIVA.

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BIOGRAPHICAL INFORMATION

Madeline Carlson graduated from the University of Texas at Arlington with an Honors Bachelor of Science in Microbiology. She will continue her education by earning her medical doctorate degree at McGovern Medical School. She plans to specialize in pathology and work full-time at a hospital. In addition, she hopes to continue her hobbies of dance and musical theatre. She is also trying to grow her hair out.