Spring 5-2020

Reversible Inhibition of Mycobacterial DnaB Protein Splicing by Zinc

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HONORS THESIS

Certificate of Approval

Reversible Inhibition of Mycobacterial DnaB Protein Splicing by Zinc

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May 2020

Approved to fulfill the requirements of HON 437

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Examination Approval Page

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Project Title: Reversible Inhibition of Mycobacterial DnaB Protein Splicing by Zinc

Department: Biology

Date of Defense: April 23rd, 2020

Approval by Examining Committee:

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Submitted in partial fulfillment
of the requirements
of the Murray State University Honors Diploma

Gabrielle Hardison

May 2020

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ABSTRACT

Inteins are emerging as post-translational regulatory elements, undergoing conditional protein splicing in response to a variety of environmental cues. Inteins are seamlessly removed by self-splicing from exteins, or flanking portions of the host protein, which they interrupt. DnaB of *Mycobacterium smegmatis*, a helicase essential for replication, harbors two inteins known as DnaBi1 and DnaBi2, each with discrete structural characteristics and insertion positions. DnaBi1 was used here to design a reporter system which links splicing with resistance to the antibiotic kanamycin. We built a construct that strictly requires catalytically active DnaBi1 for survival in the presence of kanamycin and used this construct to probe for factors that influence splicing *in vivo* of *M. smegmatis,* the native context of the intein. We show that zinc, biologically relevant during mycobacterial pathogenesis, is a potent inhibitor of DnaBi1 *in vivo*, and using a separate reporter system, that zinc inhibits DnaBi1 *in vitro*. We present the crystal structure of DnaBi1 bound to zinc, and finally propose that DnaBi1 splicing inhibition by zinc may be important during mycobacterial infection.

INTRODUCTION

Inteins, also known as internal proteins, autocatalytically splice from their host proteins. They are capable of serving as on and off switches that can control protein activity upon splicing through conditional protein splicing (CPS) (1). Depending on the environment in which an intein resides, these elements can act as environmental sensors that ultimately modulate protein function before splicing is initiated (2). In other words, an intein can act as a pause button to allow the environment to return to normal conditions whenever stressors are present. Although inteins are analogous to introns, they are distinctive in their ability to become translated and removed at the protein level.

Inteins are divided into three separate classes depending on their catalytic mechanisms. Several inteins fall under the class one inteins, where there is one branched intermediate during splicing. In our research we focused on class three inteins, which are unique because they encompass two separate branched intermediates in the splicing mechanism. This protein splicing reaction involves the rearrangement of N- and C-terminal ends of inteins, which are joined onto exteins. The rearrangement of these fragments of the intein allows for the formation of an active splicing area within the protein, interceding the formation of a bond between exteins. This in turn provides the ability of the intein to break off of the protein via the previously mentioned N- and C- terminal ends (3).

While inteins are found in all three domains of life, a large portion of them are found within archaea and bacteria. This provides a great opportunity to study post-translational regulation in these biological units within prokaryotic organisms. For our research, we chose to study inteins within *Mycobacteria*, a bacterial genus enriched to inteins. In addition,

Mycobacteria are the carriers of human pathogens known to cause serious infectious diseases such as tuberculosis and leprosy (4). DnaB, the DNA helicase found within mycobacterial proteins, is essential and required for DNA replication. Importantly, the species *M. smegmatis* is studied in this project because it is a non-pathogenic species. Using the DnaB intein within the replicative helicase of this species, we created a reporter system that links splicing with resistance to kanamycin, which is an important bacterial aminoglycoside antibiotic (2).

In *M. smegmatis*, the DnaB helicase carries two separate inteins known as DnaBi1 and DnaBi2. We chose to look into the specific mycobacterial helicase intein DnaBi1 for many reasons. First, DnaBi1 of *M. smegmatis* shares approximately 68% sequence identity with an intein found in the same position as *M. leprae* DnaB. Also, DnaBi1 happens to splice slowly *in vitro*, providing an experimentally tractable system to allow one to study the precursor protein function prior to splicing (2). Lastly, this type of intein splicing allows the opportunity to discover what types of environmental factors and outside stressors can impact the splicing within the native host of *M. smegmatis* at the initiating catalytic nucleophile, an internal cysteine residue.

A splicing dependent reporter KISR, or kanamycin intein splicing reporter, was constructed before the study began by building a scheme that is capable of disrupting the bacterial aminoglycoside phosphotransferase gene, known as the KanR protein, with the DnaBi1 intein (2). This reporting system can be used for several purposes, but importantly acts as a tool that is able to search for the outside factors that can directly affect the splicing of inteins depending on kanamycin resistance or sensitivity. In our instance, we found that DnaBi1 of *M. smegmatis* must splice out of the kanamycin sensitive precursor protein in order to

survive in the presence of kanamycin. We nicknamed the splicing reporter “Splice or Die”, making it a simplistic way of stating how the intein needs to splice for *M. smegmatis* to demonstrate resistance to the antibiotic.

Zinc, a divalent cation (2+), acts as an outside stressor that is capable of inhibiting the splicing of inteins. Further, the concentration of zinc influences mycobacterial pathogenesis and development of infectious diseases (3). This concentration typically increases in the presence of macrophages so that zinc can be engulfed and used for combating the invasion of bacterial pathogens in an organism. Using an independent reporter system, called a MIG reporter, to match our *in vivo* work, we show how small increases in zinc concentration affect splicing of DnaBi1 (2).

Unlike KISR, the MIG reporter does not require use of the KanR protein. We were able to test the role of zinc inhibition based on micromolar concentrations of the metal cation. A splicing assay was used to model how these increasing concentrations of zinc began to inhibit splicing of the intein in the precursor protein compared to the ligated extein (GFP-containing) product. The non-native maltose binding protein component ensured the prevention of aggregated proteins; meanwhile, the green fluorescent protein helped in visualizing spliced products on the assay.

The MIG reporter also allowed us to test for reversibility of zinc inhibition in intein splicing without interruption from kanamycin resistant proteins. To complete this work, the chelator ethylenediaminetetraacetic acid (EDTA) was used in another splicing assay. EDTA is capable of binding and holding on to specific metals via chelation. When EDTA is bound to metals, especially cations like zinc, this chemical prevents the effects of the metal in the system

in which it is present (2). Even with increasing concentrations of zinc present in a system, this compound possesses the ability to halt the effects of the stressor and allow splicing to proceed as expected. Remarkably, the MIG reporter plays a critical role in linking *in vitro* results with what occurs in the cell.

To propose exactly how the metal cation enters and binds to DnaBi1 of *M. smegmatis*, a crystal structure was solved. This crystalline structure is useful in determining exactly where and how zinc potently, yet reversibly, binds the intein. In our structure, we demonstrate how the highly conserved nucleophilic nature of cysteine can be impacted by the tight binding of zinc.

My specific contribution to this project involved splicing assays using the MIG reporter. We present this data in the context of the larger project as a whole in order to provide greater understanding of the significance of the work.

MATERIALS AND METHODS

Bacterial Strains and growth conditions

To prepare *Escherichia coli* strains DH5-alpha, MG1655(DE3), and BL21(DE3) competent cells, following growth to mid-log, cultures were previously separated into centrifuge tubes and placed them on ice for 20 minutes. After centrifuging again at 4000RPM at 4°C for 10 minutes, we resuspended the pellets with 100 mL of ice cold $CaCl_2$. The samples were then incubated on ice for 30 minutes before repeating the previous centrifuge and combining the finished pellets via resuspension in 5 mL of ice cold CaCl2 with 15% glycerol. These strains were grown in Luria Broth (LB) with aeration at 250 rpm. Next, these transformants were plated onto the LB agar

plates using the correlating antibiotic, then incubated overnight at 37°C. For plasmid transformation, we used heat shock to increase the uptake of DNA in each of our samples.

In vitro MIG reporter

In order measure results for the zinc-mediated inhibition of *M. smegmatis* DnaBi1 and *M. leprae* DnaB, our main project was to utilize a previously developed reporter system using maltose binding protein (MBP)-intein-green fluorescent protein (GFP), or MIG as non-native exteins. We transformed each of the plasmids of MIG into BL21(DE3) cells. To overexpress the protein, cells were subcultured into fresh LB at a 1:100 dilution and grown overnight. We grew the cultures at 37°C to an OD600 of approximately 0.5, moved them to 30°C and then induced with 0.5 mM Isopropyl β-d-1-thiogalactopyranoside (IPTG) for 1 hour to allow for appropriate protein expression.

We resuspended cell pellets in our MIG lysis buffer (50 mM Tris pH 8.0, 20 mM NaCl, 10% glycerol) and lysed them on ice using a tip sonicator. Each culture sample was then pelleted for 10 minutes at 4000 rpm at 4°C. The soluble portion of lysate was taken to be assayed. We took a sample at t=0, taking this sample lysate to be incubated and stored at -20°C for the remainder of experimental procedures. Our sample lysate of *M. smegmatis* DnaBi1 was stored and incubated at 16°C for approximately 16 hours, while the lysate of *M. leprae* DnaB was incubated for approximately 24 hours. The indicated compound was added to lysate at the desired concentrations immediately prior to incubation. DnaBi1-MIG samples were incubated for 5 hours.

Splicing assays and visualization

Samples were run using 8-16% Tris-Glycine semi-native gels (Bio-Rad). To do this, we took each mixed sample lysates 1:1 with Laemmli buffer with 1% β-mercaptoethanol. Each lysate gel ran for approximately 30 minutes at 200 volts. In order to visualize the ligation of GFP-containing extein portions of the splicing assay, we took our completed gel and placed it into an Amersham Imager 680 (GE Healthcare) and ran scanned images to produce a visualization of spliced products. To quantitate results, ImageJ programming was used, then followed by analysis with GraphPad Prism (v7.02)

Addition of EDTA and reversibility in DnaBi1

Using zinc and EDTA treatments to support the idea that zinc-mediated binding is in fact reversible, samples were treated with 2x excess of EDTA following an hour incubation with zinc. Samples were run on an additional protein splicing assay on separate 8-16% Tris-Glycine semi-native gels (Bio-Rad) using Laemmli sample buffer (Bio-Rad) with 1% β-mercaptoethanol. Upon completion of the splicing on gel, the Amersham Imager 680 (GE Healthcare) was used to visualize GFP-containing products. Quantitative analysis was done using ImageJ and GraphPad Prism (v7.02).

RESULTS

Construction of DnaB intein splicing reporter in mycobacteria

A splicing-dependent selection scheme was constructed by interrupting the bacterial aminoglycoside phosphotransferase gene (KanR protein) with the DnaBi1 intein whereby catalytically active DnaBi1 is required for splicing. The DnaBi1 intein (both WT and C118A variants) was inserted individually ahead of each of the sixteen serine residues found in the KanR

protein. A range of phenotypes were observed related to kanamycin resistance, including no resistance, splicing-independent resistance, and the desired splicing-dependent resistance (2). Ultimately, we chose KD154 (Ser154 of KanR used as +1 residue) for the splicing reporter because it strictly requires splicing for survival in the presence of kanamycin.

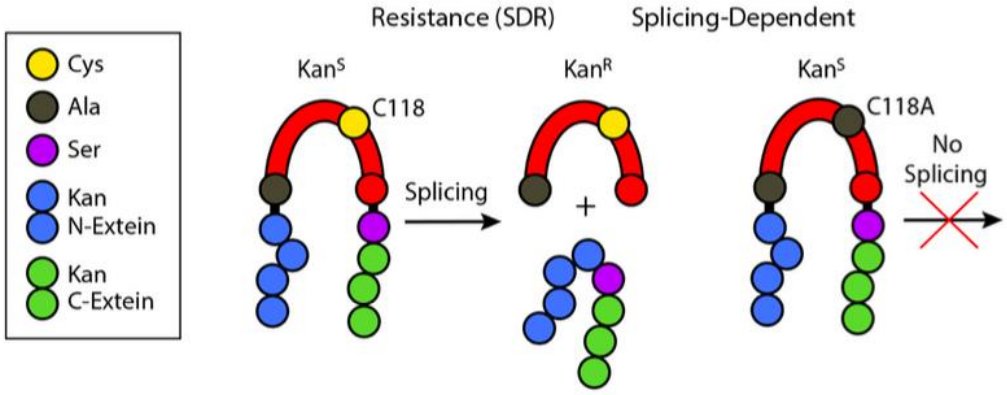

Figure 1. Spicing-dependent genetic reporter. Kanamycin resistance occurs when the catalytically active intein interrupts the KanR protein. When the intein cannot splice due to mutation, the KanR protein cannot function and the bacteria dies off in the presence of the antibiotic.

Inhibition of DnaBi1 splicing by zinc in vivo

In past studies, metal divalent (2+) cations have been shown to be major influencers in direct binding and regulation during protein splicing. Specifically, DnaBi1 splicing can be regulated via redox states of catalytic cysteine residues (5). Based on previous constructs of splicing dependent resistance, it was hypothesized that alternate conditions could also influence DnaB splicing. Saying this, we chose to test splicing within the native host against the presence of the divalent cation zinc. Survival of *M. smegmatis* cells with plasmids of KanR (without intein) or KD154 (intein interrupting KanR using S154 as the +1 residue) was recorded in the absence of

kanamycin and zinc, presence of kanamycin only, presence of zinc only and finally in the presence of kanamycin and zinc (Fig. 2).

We used sublethal concentrations of kanamycin (300 μg/mL) and $Zn(OAc)_2$ (100 μM) to avoid toxicity that would be unrelated to splicing inhibition. Low concentrations of zinc were used, while the concentration of kanamycin was chosen to be high enough to where a reduction in the active enzyme in splicing inhibition was able to be detected. We found that when both kanamycin and zinc are present in our plates, *M. smegmatis* cells expressing interrupted KanR was reduced by approximately a 200-fold. This splicing mechanism and results can be compared to indirect links of DnaB helicase function with zinc homeostasis in the native host.

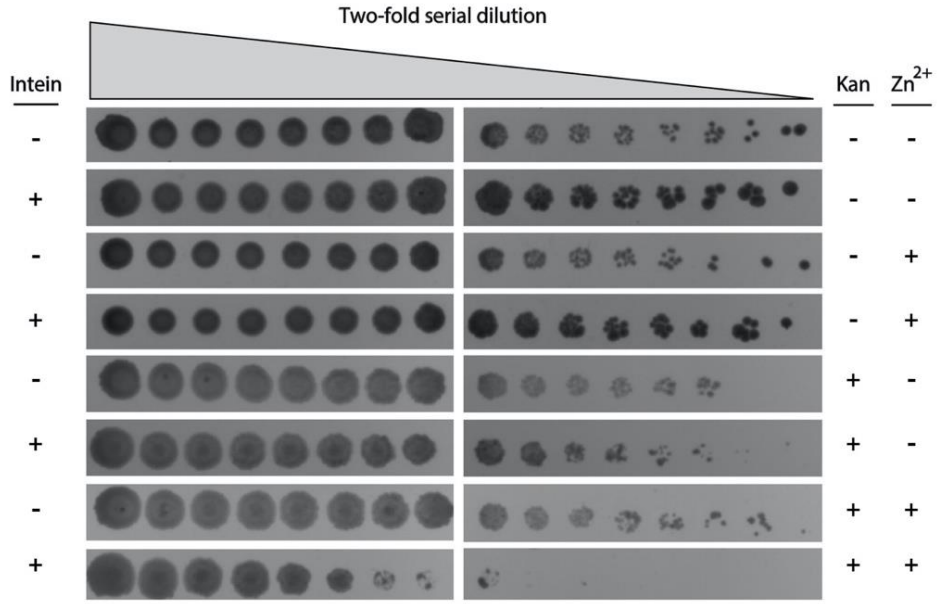

Figure 2. The survival of *M. smegmatis* is demonstrated in spot titers, measured by the plasmid expression of either KanR or KD154 in the absence of kanamycin and zinc, presence of zinc only, presence of kanamycin only, or the presence of both kanamycin and zinc. The concentration of kanamycin was 300 µg/mL and $Zn(OAc)_2$ was 100 µM where present.

Inhibition of DnaBi1 splicing by zinc in vitro

Now that we have gathered our results for the *in vivo* splicing, we wanted to test the zinc mediated DnaBi1 splicing inhibition *in vitro*. In order to do so, we utilized separate reporter system known as a MIG (MBP-Intein-GFP) splicing reporter. Here, an intein is to be flanked out of the system by the non-native extein components of maltose binding protein (MBP) and green fluorescent protein (GFP). GFP, a useful tool in protein assays and reporter systems, aids in visualization of products like the precursor protein and ligated exteins. The intact GFP structure provided a signal for detection via gel fluorescence after semi-native polyacrylamide

gel electrophoresis, or PAGE, was run (Fig. 3). Protein assays were used to visualize the way a protein splices and at which concentrations the precursor protein loses the flanked inteins.

We overexpressed the cells of DnaBi1-MIG and following lysis by sonication, we incubated in the presence of a range of zinc concentrations. Splicing was monitored over time at 16°C. Without zinc present in the system, DnaBi1-MIG splicing proceeds to near completion (Fig. 3). On the contrary, when zinc is present, even at low micromolar concentrations, we found that inhibition of DnaBi1 is potent, with an IC_{50} of ~10-25 μM (Fig. 3).

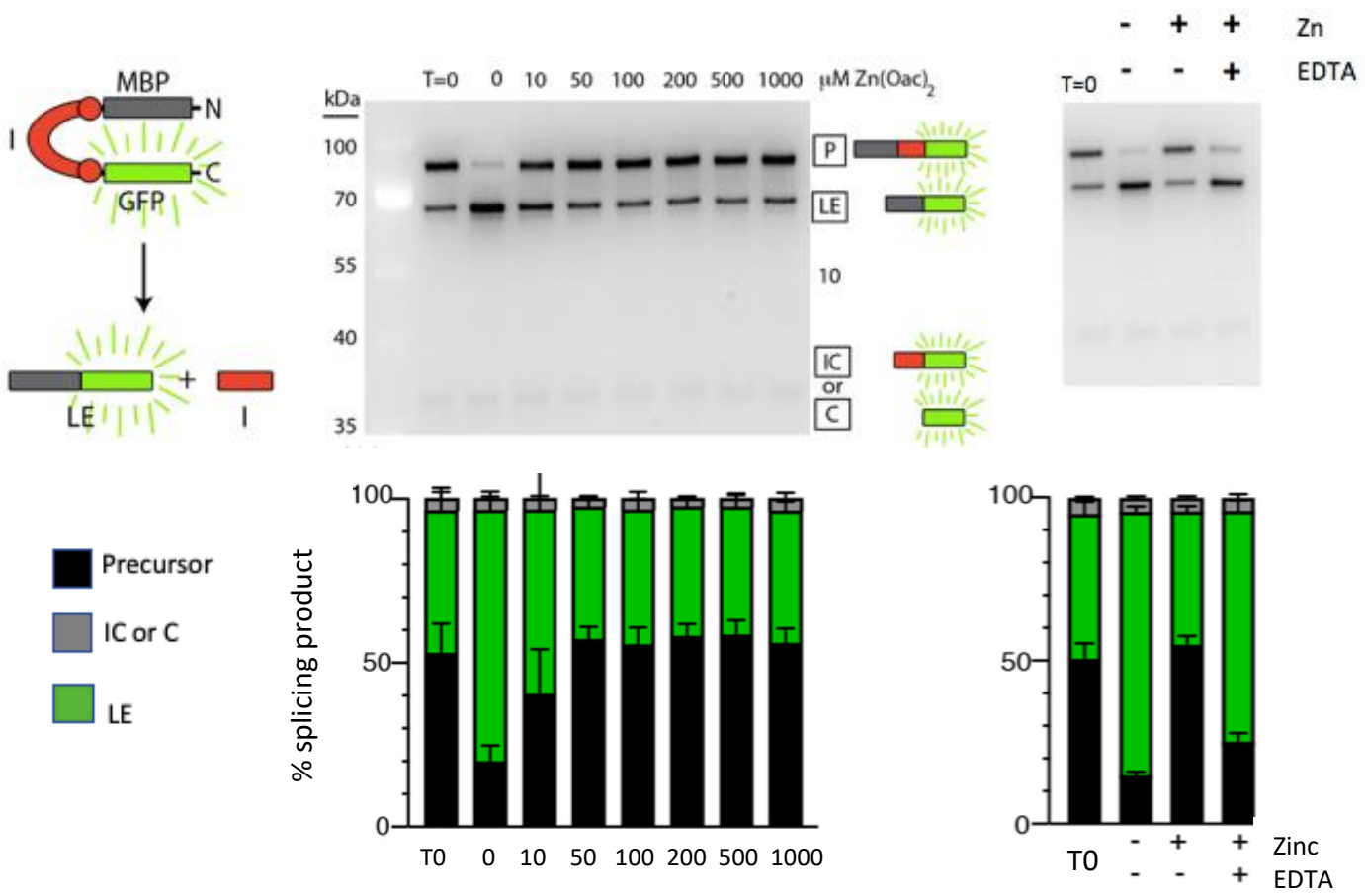

Figure 3. (Top) Using the MIG reporter, a protein splicing assay was used to show the effect of low micromolar zinc concentrations on the splicing activity and amount of ligated extein from DnaBi1 in *M. smegmatis*. To test the reversibility, another splicing assay was used with either the absence of zinc and EDTA, presence of zinc only, or the presence of both zinc and EDTA.

(Bottom) Results were quantitated based on IC_{50} values of the percent spliced product at each specific concentration of zinc and/or EDTA for both the MIG and EDTA assays.

Reversible inhibition of zinc

Lastly, we tested whether or not this inhibition by zinc was reversible. To do this, we added the chelator ethylenediaminetetraacetic acid (EDTA) to the system. To start testing, DnaBi1-MIG was incubated with zinc to allow for binding. Then, we added the chelator EDTA to the system to measure its effect on splicing. EDTA was found to fully reverse the zinc inhibition, which provided the opportunity for the splicing reaction to proceed at the rate it would if no zinc was present in the system at all (Fig. 3). Our results indicated that the binding of zinc to the intein DnaBi1 was the reason for inhibition, and that this binding is reversible. Lastly, we linked our potent and reversible binding results to the way in which zinc binds to the crystalline structure of DnaBi1. In this structure, zinc is bound to a catalytic cysteine residue of the intein, which is responsible for the initial nucleophilic during the splicing mechanism. This result nicely explains inhibition of intein catalysis when zinc is present in the system (Fig. 4).

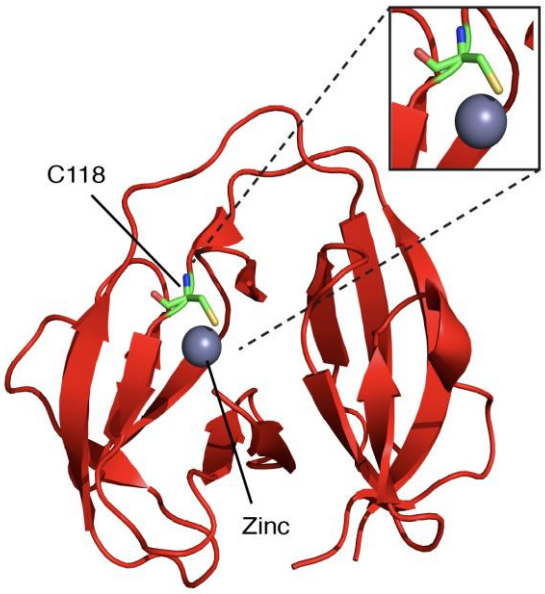

Figure 4. Crystal structure of DnaBi1-Zinc complex. In the crystal structure of DnaBi1, we demonstrate how zinc inhibits protein splicing through binding directly to the initiating nucleophile (C118) required for intein catalysis. Upper right box shows a magnification of zinc (gray) and C118 (sticks).

DISCUSSION

The concept of inteins as post-translational regulatory elements, rather than simply as molecular parasites, has emerged in recent years. In our research, we examined the potential role of DnaBi1 from *M. smegmatis* as an environmental sensor responsive to zinc stress. Based on previous studies, as well as what was gathered in our results, it has been shown that they can act as environmental sensors that modulate the function of a protein (5). It was our goal to run studies on the specific details of the outside stressor function and determine what characteristics were responsible for modulating the splicing within the DnaB replicative helicase of *M. smegmatis*.

We show how the non-pathogenic *M. smegmatis* provides a model system for testing intein function and conditional splicing (4). For starters, the DnaBi1 intein of *M. smegmatis* was useful for testing the intein splicing due to its highly conserved properties, slow splicing, and experimentally tractable results (5). We were then able to compare our results of *M. smegmatis* to the similar composition of inteins found in *M. leprae* and *M. tuberculosis.* For our experiments, we found a pioneering result using splicing reporters to determine the impact inteins have on the protein function in the presence of antibiotic reporting systems in the native host.

The splicing reporters were a critical part to exploring the functions of inteins. Using KISR, we were able to perform *in vivo* tests to link survival of *M. smegmatis* to splicing of the DnaBi1 intein. Based on our newly discovered results, we show how the splicing mechanism of DnaBi1 was directly inhibited within the native host by the outside factor of zinc. Aside from kanamycin resistance and sensitivity, we demonstrate the significance of zinc being a highly

potent inhibitor within splicing of the inteins found in *M. smegmatis* and *M. leprae* via the MIG reporter. This reporter provided results that tested zinc's binding ability at notably low concentrations, showing the inhibition of spliced products from the precursor protein in our splicing assay. Lastly, after allowing zinc to incubate for an hour and have enough time to bind to the intein, we show how this binding process is reversible with the addition of EDTA as a chelator. It was reassuring to find linkage amongst the potent, direct, and reversible zinc binding process using these reporting systems (2). These results are fairly unfamiliar to intein research and projects performed thus far, paving the way for future research in conditional protein splicing.

To summarize, many experimental studies performed with inteins hold the potential to change future biotechnologies in multiple ways. Although once termed harmful and parasitic to genetic studies, we now know the benefits that come about relating intein function to conditional splicing, especially when in the native host (1). Inteins can act to modulate protein function in the presence of several outside stressors that are introduced in bacterial or archaeal systems. Importantly, their clustering within active sites of proteins involved in DNA replication, recombination, and repair plays raises intriguing questions as to the roles they may play in the cell (6). This can either hurt or help a biological system, with the inteins functioning as a critical on and off switch when it comes to stressors introduced to the environment.

We found this to be true in our experiment, showing how DnaBi1 splicing is easily manipulated when in the presence of the metal cation zinc. The compromised function of splicing can severely impact the survival of the organisms affected. According to our results, without splicing of the inteins, the proceeding mechanisms of post-translational regulation in

DnaB of the host can be paused, prohibiting replication and other important mechanisms of the protein within its host. Once permissive conditions return in the protein, then splicing can revert to normal and continue as if the stressor was not present (5). Regardless of the characteristics of the environment in which a prokaryotic intein resides, these results correlate the function of the helicase inteins to their importance in pathogenesis and development of infectious diseases.

ACKNOWLEDGEMENTS

This project was created in collaboration with the following individuals and their institutions:

Daniel Woods[a], Sweta Vangaveti[b], Ikechukwu Egbanum[a], Allison M. Sweeney[c], Zhong Li[d], Valjean Bacot-Davis[e], Danielle S. LeSassier[f], Matthew Stanger[a], Hongmin Li[a,d], Marlene Belfort[a,b], Christopher W. Lennon[c]

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First, I would like to give a special thanks to my research advisor, Dr. Christopher Lennon, and his fellow post-doctoral researchers and their institutions for their intensive lab research and background information on inteins. Without their hard work and dedication to these revolutionary biological elements, my thesis project would not be possible.

Secondly, I would like to acknowledge the importance of the MIG reporter used in the study. Assigned as my main contribution to the research project, this system helped explain the relative significance of micromolar concentrations of zinc and addition of EDTA in the potent and reversible binding of DnaBi1. Splicing inhibition of inteins is still reasonably unexplored, so the MIG reporter played a crucial role in analyzing intein behavior in the presence of specific environmental conditions and stressors.

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