THE ROLE OF CLPC2 IN MYCOBACTERIAL STRESS RESPONSE

by

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ABSTRACT

Bacteria of the pathogenic *Mycobacterium* genus are significant for their ability to cause disease and, indeed, to thrive in the hostile and challenging host environments. This sturdiness is especially characteristic of *Mycobacterium tuberculosis* (Mtb), the causative agent of human tuberculosis (TB). The resilience and ability of Mtb to withstand nutrient deprivation as well as oxidative, thermal, and proteotoxic stress are attributed to its complex stress response mechanisms, which have also been implicated in its tolerance to antibiotic treatment. (Dawan & Ahn, 2022; Moukendza Koundi et al., 2024) Moreover, the substantial global burden of TB cases tolerant to available antibiotics poses a serious public health threat globally. Consequently, efforts to develop new drugs are urgently needed to combat this public health crisis.

Clp proteases, a class of ATP-dependent enzymes that plays a central role in bacterial protein homeostasis and quality control, have emerged as key regulators of bacterial pathways, including stress responses in a wide cross-section of bacterial pathogens. (Lupoli et al., 2018) Whereas Clp proteases are dispensable in many bacteria, these enzymes are absolutely essential for viability in mycobacteria, making them attractive novel drug targets against drug-resistant *M. tuberculosis.* (d'Andrea et al., 2022; Hoi et al., 2023; Lupoli et al., 2018; Schmitz & Sauer, 2014)

Previous work in the Schmitz lab has centered around characterizing and understanding the role of ClpC1, an unfoldase component of mycobacterial Clp proteases, in substrate recognition and proteolytic regulation. ClpC1 is a

particularly appealing target because it is absolutely essential for mycobacterial survival and it lacks a direct ortholog in humans. (Choules et al., 2019; Lunge et al., 2020; Schmitz & Sauer, 2014; Weinhäupl et al., 2022a) Several naturally occurring antibiotics, including rufomycin and cyclomarin A, are known to potently dysregulate ClpC1 activity and kill Mtb cells in culture, further validating ClpC1 as a viable drug target. (Choules et al., 2019; Taylor et al., 2023) However, our limited understanding of ClpC1's physiological roles and range of substrates in *M. tuberculosis* complicates efforts to develop clinically viable ClpC1-targeting drugs. Recent studies in the Schmitz lab and others have expanded our understanding of ClpC1 physiology by establishing that mycobacteria post-translationally phosphorylate arginine residues of some cytosolic proteins (Ogbonna et al., 2022; Suskiewicz et al., 2019), and that these phosphoarginine modifications (pArg) are directly recognized by the N-terminal domain of ClpC1 as markers for proteolysis. (Hoi et al., 2023; Taylor et al., 2023) However, the details and regulation of arginine phosphorylation in mycobacteria remains poorly understood and is an area of active research.

Interestingly, mycobacteria also possess a homolog of ClpC1, termed ClpC2, which is not known to be directly involved in proteolysis. ClpC2 bears strong sequence and structural homology to the N-terminal domain of ClpC1, sharing ~40% sequence identity. (Taylor et al., 2023). ClpC2 has been found to be a transcriptional regulator that represses transcription of the *clpC2* promoter (Taylor et al., 2023). Interestingly, some ClpC1-targeting antibiotics, including rufomycin, bind to ClpC2, block its interaction with DNA, and cause strong ClpC2

overexpression. The accumulated ClpC2 serves as a molecular sponge by sequestering antibiotics from ClpC1 (Hoi et al., 2023a; Taylor et al., 2023). Recent evidence from the Schmitz lab demonstrates that ClpC2, like ClpC1, binds phosphoarginine, and that pArg similarly blocks DNA operator binding and relieves repression by ClpC2. Based on these initial studies, our overarching hypothesis is that ClpC2 functions as a pArg-sensor that responds to cellular pArg levels and regulates downstream transcriptional programs during stress.

This project tests aspects of this hypothesis by addressing two major gaps in our understanding of the physiological interplay between pArg and ClpC2 in the model mycobacterium *Mycolicibacterium smegmatis*. In Chapter 3, we use RNA-seq to identify transcripts that are differentially expressed upon antibiotic-induced dysregulation of ClpC2 or deletion of the *clpC2* coding region. Results of this experiment show that ClpC2 only regulates its own operon. Unexpectedly, these data also suggest a ClpC2-independent connection between antibiotic-induced dysregulation ClpC1 and transcriptional regulation of other genes.

Overall, these studies provide new information on the behavior of ClpC2 and expand our understanding of the role of phosphoarginine in mycobacteria. Our findings may aid efforts to develop ClpC1-targeting therapeutics that avoid cross-targeting ClpC2. Moreover, our results provide new unexpected insight into the essential role of ClpC1 in mycobacteria.

CHAPTER 1. Introduction

1.1. Drug-Resistant Tuberculosis: A Severe and Growing Public Health Threat

Tuberculosis (TB) is a highly communicable infectious disease caused by the bacterial pathogen *Mycobacterium tuberculosis* (Mtb). TB primarily affects the lungs, causing necrotic granulomas within lung tissue, but Mtb infection can also spread to other vital organs of the body, such as the brain, spine, or heart, depending on the stage of the infection. (Wang et al., 2020; WHO, 2024) The most common source of TB infection occurs when an infected person expels Mtb in respiratory droplets and unsuspecting individuals inhale such aerosolized material. The severity and progression of the infection can be influenced by certain predisposing factors, most importantly HIV/AIDS. (Alsayed & Gunosewoyo, 2023; WHO, 2024) TB currently infects one-quarter of the world's population and causes over a million deaths annually (Schön et al., 2017; WHO, 2024), ranking as the leading cause of death worldwide from a single infectious agent, with drug-resistant tuberculosis alone accounting for 13% of tuberculosis-related deaths (Farhat et al., 2024; Liebenberg et al., 2022; WHO, 2024).

Common symptoms of TB include intense coughing, often with blood, chest pain, night sweats, and severe weight loss, among others. Symptoms may also vary according to the stage and site of infection. (CDC, 2025; WHO, 2022) Although most TB cases are treatable, treatment typically requires a 4 - 6-month antibiotic regimen, with a combination of first-line (including isoniazid, rifampin, pyrazinamide, and ethambutol) and second-line antibiotics (including kanamycin, capreomycin, and streptomycin) to achieve treatment goals. (Sotgiu et al., 2015) The mortality rate of active TB infections is as high as 50% if left untreated (WHO, 2024).

The emergence of drug-resistant tuberculosis has further worsened its global burden by impeding treatment and control efforts, resulting in increased mortality rates and economic waste-associated treatment costs. (Soodejani et al., 2024) Drug-resistant tuberculosis arises from infection with *M. tuberculosis* strains that have undergone mutations to develop tolerance to available clinical anti-TB drugs. (Farhat et al., 2024; WHO, 2010) Drug-resistant TB is grouped into subclasses based on the extent of drug resistance: multidrug-resistant TB (MDR-TB) is characterized by resistance to at least one of the most potent first-line drugs, isoniazid or rifampin; extensively drug-resistant TB (XDR-TB) strains are additionally resistant to any fluoroquinolone and at least one of the three injectable second-line drugs, including amikacin, kanamycin, or capreomycin; and the recently emerged strains classified as pre-extensively drug-resistant TB

(pre-XDR-TB) exhibit resistance to first-line and second-line drugs. (Domínguez et al., 2023; Seung et al., 2015; WHO, 2022, 2024)

Challenges associated with drug-resistant TB include poor treatment outcomes, severe side effects, increased mortality rate, and economic loss, among others. This global public health emergency, therefore, necessitates the urgent need to discover new drugs with improved treatment outcomes against these drug-resistant infections.

1.2. Clp Proteases as Central Regulators of Bacterial Proteostasis

1.2.1. Protease structure and function

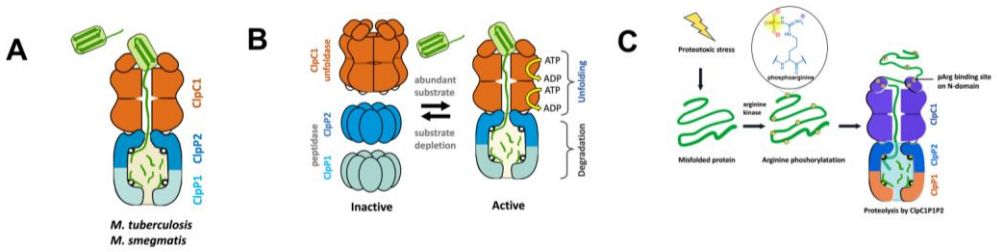


Figure 1 Cartoon of proteases showing its structural components and assembly A) Shows the structural components of the Clp protease with the unfoldase(blue) sitting on the surface of the peptidase chamber, ClpP1P2(pink & purple), delivering substrate(green) into the peptidase chamber. B) Shows the inactive Clp protease components, the unfoldase(pink), the peptidase, P2(blue) and P2(purple) assembling into an active complex. C) Show the effect of proteotoxic stress(yellow) resulting in misfolded protein(green) which is autophosphorylated or labeled by pArg (circled) and marked for degradation by the Clp protease complex

In the relentless global fight against tuberculosis, mycobacterial Clp proteases have emerged as promising novel drug targets. (d'Andrea et al., 2022) Maintenance of the bacterial proteome is important for the overall function and survival of all bacteria. Clp proteases (originally termed caseinolytic proteases) comprise one of the five classes of bacterial ATP-dependent proteases, which are functionally analogous to the 26S proteasome in eukaryotes. (Beardslee et al., 2021) (Clp proteases also occur in some

eukaryotic organelles.) These large enzymatic machines consist of a hexameric ATP-dependent unfoldase (ClpC1 or ClpX in mycobacteria) and a tetradecameric self-assembled peptidase chamber lined with serine active sites (ClpP1P2 in mycobacteria) that together select, unfold and degrade cytosolic protein targets. Clp proteases help enforce general protein homeostasis and quality control. (Beardslee et al., 2021; Kress et al., 2009; Schmitz & Sauer, 2014) Additionally, Clp proteases regulate diverse cellular pathways, including virulence factor expression, stress responses, cell cycle progression, and antibiotic resistance in different species of bacteria. (Frees et al., 2007; Gottesman, 2019).

The ability to selectively degrade damaged, misfolded, and unneeded proteins confirms their role as key regulators of protein homeostasis and proteome maintenance, both of which are significant in bacterial adaptation and survival during stress. (Illigmann et al., 2021). Nevertheless, Clp proteases are individually non-essential in most well-studied bacteria, perhaps due to functional overlap with other ATP-dependent proteases in the cell. By contrast, all enzymatic components of the Clp proteases have been shown to be absolutely essential for cell viability in mycobacterial species, (Famulla et al., 2016; Ollinger et al., 2012; Schmitz & Sauer, 2014) although the physiological reason for their essentiality remains unknown. The inability to knock out or knock down Clp protease components complicates efforts to probe their function in cells, and relatively few specific proteolytic substrates or pathways

have been identified to date (Ogbonna et al., 2022a, 2023; Schmitz & Sauer, 2014).

1.3. Clp Proteases as Novel Drug Targets

The essentiality of mycobacterial Clp proteases makes them particularly promising antibacterial targets. Architecturally, the mycobacterial Clp unfoldase (ClpX or ClpC1) associates with the Clp peptidase, ClpP1P2, by specifically docking on the surface of ClpP2 through a weak and flexible interaction made by each unfoldase I/LGF loop. (Beardslee et al., 2021; Poulton et al., 2022b; Raju et al., 2014; Schmitz et al., 2014). The interaction between the AAA+ chaperone or unfoldase and the peptidase barrel, ClpP, forms the Clp protease complex such as ClpXP, ClpCP, etc. The Clp protease ClpC1P1P2 has been identified to be strictly essential in *M. tuberculosis*, presumably by regulating the homeostasis of several growth-essential proteins. (Lunge et al., 2020). Importantly, ClpC1 is equally strictly required for normal cell growth in the non-pathogenic model mycobacterium *Mycolicibacterium smegmatis*. (Lunge et al., 2020)

Several classes of antibiotic-like compounds have been identified that disrupt the normal function of Clp proteases in multiple bacterial species (Famulla et al., 2016; Hong et al., 2023; Malik & Brötz-Oesterhelt, 2017). Importantly, such dysregulation of Clp proteases has been observed to kill *Mycobacterium tuberculosis* in culture and in animal models of infection,

although no compound has yet been developed into a clinically viable drug (Baker & Sauer, 2006; Lupoli et al., 2018). The different classes of Clp protease-targeting compounds bind to different sites on the protease and exert different mechanistic consequences on activity. (Famulla et al., 2016; Schmitz & Sauer, 2014).

1.4. The Mycobacterial ClpP1P2 Peptidase as a Drug Target

The mycobacterial Clp peptidase is made up of two heptameric rings that self-assemble, stacking face-face to form a tetradecameric barrel-shaped degradation chamber. In most model bacteria, including *Escherichia coli* and *Bacillus subtilis*, two identical ClpP heptamers assemble to form a homomeric 14-mer barrel. By contrast, *M. tuberculosis* and other actinobacteria have two separate heptamers, ClpP1 and ClpP2, that form a heteromeric ClpP1P2 complex. (Schmitz et al., 2014) Each peptidase subunit possesses a chymotrypsin-like Ser-peptidase active site, with 14 peptidase active sites lining the interior of the barrel. Access to the degradation chamber is controlled by narrow entry portals at the top and bottom of the barrel. The Clp peptidase alone can non-specifically cleave some small peptides that freely diffuse through the entry portals. However, degradation of larger folded protein substrates requires the association of an ATP-dependent unfoldase, which can unfold native proteins and translocate the resulting denatured polypeptides through the entry portals and into the degradation chamber. ClpP1P2 is an essential component

of mycobacterial Clp proteases, and compounds have been described that kill *M. tuberculosis* by dysregulating ClpP1P2 by different mechanisms.

Compounds that target Clp peptidases work by one of two distinct mechanisms of action. Some inhibit peptidase activity by covalently modifying active site serine residues (eg, boronate peptides, beta lactones, and phenyl esters) (Gersch et al., 2013; Queraltó et al., 2023). However, the most widely studied class of compounds targeting the Clp peptidase are the acyldepsipeptides (ADEPs), which are synthetic mimics of naturally occurring cyclic peptides produced by streptomyces bacteria. ADEPs dysregulate Clp peptidase activity by binding to the peptidase surface and preventing docking of the unfoldase, thus preventing assembly of the full proteolytic complex. (Famulla et al., 2016)

Some Clp peptidase targeting compounds are capable of killing mycobacteria, but none are potent. Additionally, all classes of compounds that target the peptidase exhibit cross-reactivity with human mitochondrial ClpP, creating the possibility of interfering with normal mitochondrial protein homeostasis during treatment.

1.5. The ClpC1 Unfoldase as a Drug Target

Clp unfoldases form ring hexamers and possess either a single AAA+ ATPase module per subunit, resulting in a type-1 unfoldase as found in ClpX, or two AAA+ ATPase modules per subunit, making a type-2 unfoldase as in

ClpA, ClpC, etc. (Beardslee et al., 20S21; Kress et al., 2009). Clp unfoldases recognize substrates either i) directly via short “degron” sequences, as with the co-translationally appended ssrA tag, ii) indirectly through an adaptor, as with ClpS-mediated delivery of N-end rule substrates to ClpAP, or iii) through posttranslational modifications, such as phosphoarginine. (Beardslee et al., 2021; Presloid et al., 2024; Schmitz & Sauer, 2014) Once engaged, substrates are mechanically unfolded through power strokes driven by rounds of ATP hydrolysis, and denatured substrate polypeptides are ultimately translocated into the degradation chamber of the associated Clp peptidase for hydrolysis. (Beardslee et al., 20S21; Kress et al., 2009).

While all mycobacterial Clp enzymes are essential for viability, the ClpC1 unfoldase is notable because it is the only component that lacks a close ortholog in humans, making it a compelling selective target for development of antibacterial drugs that do not interfere with protein homeostasis in humans. Like most type-II Clp unfoldases, ClpC1 possesses a small family-specific globular N-terminal domain that is thought to play a significant role in substrate recognition and overall functional regulation. The ClpC1 N-terminal domain also serves as the binding site for several classes of antibiotics that dysregulate ClpC1 and effectively kill mycobacteria, including the natural product antibiotics (NPAs) rufomycin, cyclomarin A, lassomycin, and ecumicin. (Weinhäupl et al., 2022b, 2025) The specific mechanisms by which these cyclic peptides dysregulate ClpC1 and cause cell death are poorly understood, but all are

known to modulate the ATPase and proteolytic activities of ClpC1 to varying extents. (Bhanot et al., 2023; Choules et al., 2019; Weinhäupl et al., 2022b)

Although the roles of Clp proteases in the selective degradation of misfolded proteins, protein homeostasis and quality control, regulation of stress adaptation, pathogenesis, and virulence are generally well-studied across bacteria, (Hoi et al., 2023a; Poulton et al., 2022; Schmitz & Sauer, 2014) their functions in mycobacteria are less well understood and just a few of their specific substrates and physiological functions have been characterized to date. This leaves a gap in our knowledge and thus limits our ability to fully exploit them for therapeutic purposes.

1.6. Mycobacterial Stress Response Mechanisms: An Adaptation and Survival Strategy

1.6.1. Major Stressors Encountered by Mycobacteria tuberculosis

Unlike most fast-growing bacteria, such as *Escherichia coli*, whose major means of survival depends on rapid replication, *Mtb* has evolved to utilize robust stress response mechanisms that enable it to sense environmental pressure and deploy “a fitting response” to curtail and counter them (Tripathi et al., 2020). This ability of *Mtb* to tolerate multiple stressors or hostile environments is critical to its survival and pathogenicity, and allows it to persist in lung cells despite continual assault by the host immune system (Tripathi et al., 2020). *Mtb* has been previously reported to tolerate certain extreme environmental conditions,

such as oxidative stress in the form of reactive oxygen species (ROS), which occurs mainly in macrophages, and can cause damage to DNA and proteins; hypoxia, caused by the low available oxygen inside granulomas, leading to dormancy; low pH, such as acidic environment that is common in phagosomes; as well as nitrosative stress caused by nitrite oxide (NO) and reactive nitrogen species (RNS) generated by macrophages. This high level of stress tolerance, together with the low metabolic activity of *Mtb* cells within granulomas, is thought to contribute to the general ability of *Mtb* to tolerate antibacterial drugs and partially explain the need for months-long treatment regimes. (Dechow & Abramovitch, 2024; Timm et al., 2003; Tripathi et al., 2020).

1.7. Key Stress Response Mechanisms In Mycobacteria

1.7.1. Stringent Response Promotes Survival, Virulence, And Antibiotic Tolerance in *Mtb* During Stress

The stringent response is a conserved global adaptation mechanism in bacteria that enables them to adapt and survive numerous stressors induced by the host environment. It is characterized by the accumulation of regulators and alarmones, particularly guanosine tetra- / pentaphosphate, (p)ppGpp (Gavrish et al., 2014), synthesized by the Rel enzyme (Danchik et al., 2021; Gupta et al., 2021). In *Mtb*, elevated levels of (p)ppGpp and poly(P) occur during stress occasioned primarily by lack of amino acids. It can also result from other forms of stress. This leads to the downregulation of rRNA and ribosomal protein

expression, shifting metabolic activities to promote long-term survival, virulence, and antibiotic tolerance. However, in *E. coli*, stringent response targets the downregulation of growth-related pathways by altering metabolism and transcription. (Danchik et al., 2021; Gupta et al., 2021; Traxler et al., 2008). Deletion of the Rel enzyme and depletion of CarD, a conserved transcription regulator in *Mtb* and *Mycolicibacterium smegmatis*, has been reported to impair the stringent response and cell viability, and significantly increase tolerance to antibiotics. (Danchik et al., 2021; Dutta et al., 2019).

In conclusion, the stringent response mechanism plays a vital physiological role in mycobacteria's survival during stress by influencing resource allocation towards virulence, antibiotic resistance, etc, and triggering the repression of growth-associated pathways. The disruption of the key mediator of stringent response, Rel, and the transcriptional regulator, CarD, would negatively affect the survival of Mtb as well as decrease its susceptibility to antibiotics (Danchik et al., 2021; Dutta et al., 2019; Gupta et al., 2021).

1.7.2. SOS Response in Mycobacteria Enhances Survival, Persistence, and Antibiotic Resistance Under Genotoxic Stress.

Save Our Soul (SOS) is the most studied form of the DNA damage response (DDR) mechanism found in bacteria. Classical SOS is a conserved regulatory mechanism in bacteria that is activated in response to DNA damage caused by genotoxic stress. Generally, SOS response halts the cell cycle while

inducing DNA repair. (Gao et al., 2023) The goal of the SOS response is to preserve overall genome integrity. (Gao et al., 2023)

The SOS response is controlled by the repressor LexA and the DNA-binding protein RecA. Under normal conditions, LexA represses transcription of a suite of SOS genes. In the event of DNA damage, RecA accumulates on single-stranded DNA (ssDNA), where it becomes activated, inducing the autocleavage of LexA. Cleavage of LexA relieves repression and activates transcription of SOS response genes, including genes responsible for DNA repair, DNA damage tolerance, and cell-cycle arrest. (Gao et al., 2023; Kamat & Badrinarayanan, 2023; Little & Mount, 1982; Simmons et al., 2008)

A significant consequence of SOS response in *M. tuberculosis* is its link to mutagenesis-driven antibiotic resistance and persistence resulting from the LexA-controlled activities of DnaE2 polymerase. (Wipperman et al., 2018) Recent studies have established that SOS plays a major role in antibiotic resistance caused by chromosomal mutations; for example, rifampin resistance (Boshoff et al., 2003; Wipperman et al., 2018). Additionally, it has been observed that low-fidelity polymerase that arise from the activation of SOS response could enhance the resistance of *Mtb* to fluoroquinolones by creating resistance-causing mutation (Dos Vultos et al., 2009)

1.8. Phosphoarginine (pArg) As A Regulatory Signal for Proteolysis And Stress Response In Mycobacteria.

Bacterial survival in a hostile and changing environment depends on a highly regulated system of protein homeostasis and stress response. Recent studies have shown that, in some bacteria, posttranslational modifications (PTMs) play a key regulatory role in adjusting stress response vis-a-vis protein homeostasis.

Several lines of evidence have shown that phosphoarginine (pArg), a form of PTM, marks protein substrates for proteolysis during proteotoxic stress in *Bacillus subtilis* (Helmann et al., 2001; Kirstein et al., 2005; Trentini et al., 2016). Early studies demonstrated that proteotoxic stress (such as heat shock) induce the protein kinase McsB to phosphorylate the master stress-response regulator CtsR, thereby lifting repression of heat shock genes, including Clp protease genes (Derré et al., 1999; Helmann et al., 2001; Kirstein et al., 2005). Later studies found that CtsR is an arginine kinase, and that phosphoarginine modifications on CtsR target it for destruction by the ClpCP protease (Schmidt et al., 2014; Trentini et al., 2016). It is now understood that autophosphorylation of McsB induces formation of a cage-like complex that phosphorylates a wide array of misfolded proteins during stress (Cai et al., 2024; Elsholz et al., 2010; Soshnikova & Duboule, 2009; Suskiewicz et al., 2019; Trentini et al., 2016). Phosphoarginine sites on protein substrates are recognized by two highly conserved pArg binding sites on the non-catalytic ClpC N-terminal domain (Schmidt et al., 2014; Trentini et al., 2016). This pArg-linked quality control

pathway appears to be conserved across the phylum Firmicutes (synonym Bacillota).

The Schmitz lab recently demonstrated posttranslational phosphoarginine modifications also exist in *Mycolicibacterium smegmatis*, a representative of the phylum Actinobacteria (synonym Actinomycetota). Using an integrated workflow that combined phosphopeptide enrichment and shotgun phosphoproteomics, pArg sites were identified on a small number of cellular proteins. Additionally, sequence analysis reveals that pArg binding sites are strongly conserved across actinobacterial ClpC1 orthologs, and that the mycobacterial ClpC1 NTD indeed binds pArg and pArg-bearing model substrates. Together, these data suggest that phosphoarginine modifications are conserved across the entire phylum, and may play a similar role in protein quality control and stress response (Ogbonna et al., 2022). However, specific conditions that elevate cellular pArg levels have not yet been identified.

1.9. ClpC2 As A Repressor And Stress-Induced Molecular Sponge For ClpC1.

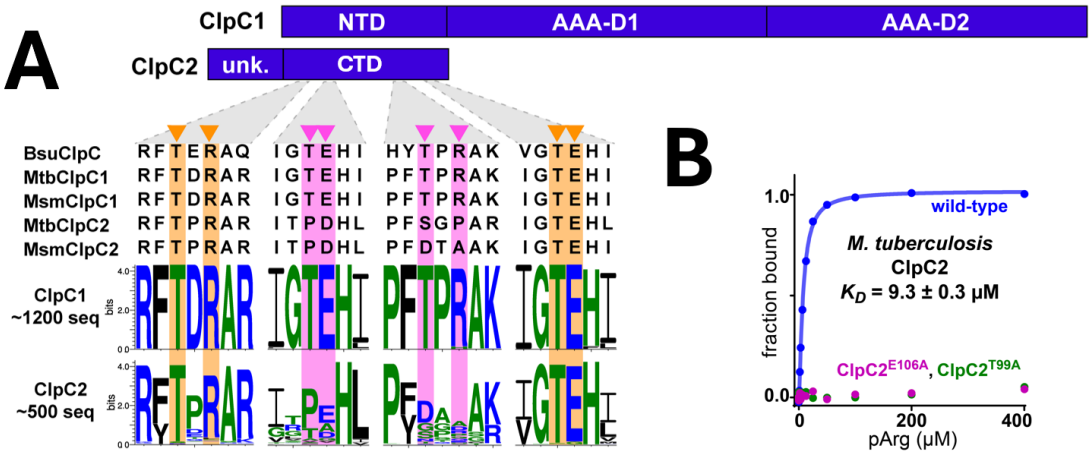


Figure 2 **ClpC2 bears homology to the NTD of ClpC1. A)** Domain organization of ClpC1 and ClpC2, illustrating homology between the ClpC1 NTD and ClpC2 CTD. Sequences and sequence logos illustrate conserved residues in two phosphoarginine binding sites (orange and pink shading). **B**) Microscale thermophoresis binding curve showing that ClpC2 binds free phosphoarginine, but mutations to the conserved binding site residues abolishes binding. Figure by Karl Schmitz. Binding data collected by Henry Anderson.

ClpC2 is a small protein with partial homology to the N-terminal domain (NTD) of ClpC1 (**Fig. 2A**), encoded by mycobacteria and some other members of the phylum Actinobacterium. It is the first gene in a putative multi-citronic operon. The ClpC2 C-terminal domain (CTD) consists of approximately 160 amino acid residues and has about 42% sequence identity to the NTD of ClpC1. (Hoi et al., 2023b) Crystal structures show that the NTD of ClpC2 adopts a novel fold and stabilizes dimer formation. (Taylor et al., 2023) ClpC2 lacks ATPase

modules, does not interact with ClpP1P2, and is not known to play a direct role in proteolysis.

Under normal physiological growth conditions, it has been shown that ClpC2 exists as a dimer and functions as a transcriptional repressor, binding to an operator sequence in the promoter region of the *clpC2* gene and repressing transcription. Interestingly, the extensive sequence similarity between the ClpC2-CTD and the ClpC1-NTD allows ClpC2 to bind some ClpC1-targeting antibiotics (eg, rufomycin). Binding of these antibiotics dissociates ClpC2 from DNA, relieves repression, and allows high-level expression of ClpC2. This accumulated ClpC2 appears to serve as a molecular sponge, partially protecting ClpC1 from the effects of ClpC1-targeting antibiotics, and reducing antibiotic potency by about 10-fold. (Hoi et al., 2023; Taylor et al., 2023).

Importantly, recent studies have also shown that ClpC2 possesses one out of the two conserved pArg binding sites found in mycobacterial ClpC1 and retains the ability to bind pArg (**Fig. 2B**). Interestingly, a deeper understanding of the mechanism of evolving evidence of the interaction between ClpC2 and pArg and its significance in mycobacterial physiology during stress remains unknown to us. Addressing this knowledge gap is important in developing new antitubercular therapeutics targeting the Clp protease.

Research Objectives

Based on prior studies and the preliminary data above, our global hypothesis is that i) some stress conditions cause elevated cellular pArg levels, ii) ClpC2 is a pArg-sensitive transcriptional regulator, and iii) binding of pArg to ClpC2 relieves repression and leads to elevated transcription of downstream targets. The following aim and sub-aims specifically examine the transcriptional repertoire of ClpC2 and the contribution of pArg binding sites to ClpC2's regulatory functions.

Aim 1 Identify Genes Regulated by ClpC2 Under Stress Conditions in Mycobacteria

In Aim 1, I tested the hypothesis that ClpC2 transcriptionally regulates multiple genes during stress. Utilizing *Mycolicibacterium smegmatis*, a non-pathogenic surrogate for *M. tuberculosis*, and building on initial investigations by Dr. Patrick Beardslee and Pratistha Kandel, I set out to:

1.1 Identify Phenotype Associated With *clpC2* Deletion

To test the hypothesis that *clpC2* deletion impairs tolerance of proteotoxic stress, I compared the tolerance of wild-type and Δ*clpC2* strains to multiple stress conditions, including proteotoxic, oxidative, redox, nitrosative, and heat shock. The most consistent phenotype was observed in a combination of heat shock and redox stress.

1.2 Identify Stress Conditions That Activate ClpC2-dependent Transcription

To test the hypothesis that stress conditions relieve transcriptional repression by ClpC2, I used a reporter developed by Pratistha Kandel in which the *clpC2* promoter regulates expression of the red fluorescent protein mCherry. Reporter activity was assayed by fluorescence and by α-mCherry immunoblot in wild-type and Δ*clpC2* cells, under normal growth and stress conditions, and in the context of *clpC2* complementation. Rufomycin, cyclomarin A drives strong *clpC2*- dependent transcription, while diamide, L-can and SNP show moderate levels of strong *clpC2*-dependent transcription.

1.3 Perform RNA-seq Under Stress Conditions To Identify Genes Regulated By ClpC2

To test the hypothesis that ClpC2 regulates multiple transcripts, used RNA sequencing to compare the transcript levels in wild type and Δ*clpC2* that were untreated or treated with rufomycin. The data were analyzed within R and Python environments, revealing the absence of a wider ClpC2-dependent regulon beyond its own operon.

CHAPTER 2. Materials and Methods

2.1. Bacterial Strains and Growth Conditions.

Cell-based experiments were conducted in *Mycolicibacterium smegmatis* mc^{2}155 (ATCC 700084) and derived strains. Cells were grown in Middlebrook 7H9 broth (HiMedia) supplemented with 0.05% (w:v) Tween 80 (Fisher). The *clpC2* deletion strain was created by Dr. Patrick Beardslee and Pratistha Kandel by CRISPR methods (M. Y. Yan et al., 2020; M.-Y. Yan et al., 2017) and verified by whole-genome sequencing (Plasmidsaurus).

2.2. Cloning & Plasmids

Gibson Assembly was used by Pratistha Kandel to construct an integrating P*clpC2*-mCherry reporter construct consisting of the *Escherichia coli* ColE1 origin of replication, mycobacterial L5 integrase cassette, and KanR resistance cassette from PLJR962 (Rock et al., 2017), along with the *M. smegmatis clpC2* promoter region (P*clpC2*) followed by a sequence encoding mCherry.

A non-integrating *clpC2* complementation plasmid was constructed by Gibson Assembly from a ColE1 *E. coli* origin of replication, the pAL5000 mycobacterial origin of replication and hygromycin resistance cassette from pUV15tetORm (Guo et al., 2007), and the *M. smegmatis clpC2* gene. PCR-based mutagenesis was used to create versions of the plasmid incorporating R56A or T100A point mutations that reduce oligomerization or pArg binding,

respectively, as previously characterized by Henry Anderson. Plasmid sequences were confirmed by whole-plasmid sequencing (Plasmidsaurus or Genewiz).

2.3. Minimum Inhibitory Time (MIT) Assay

M. smegmatis cells were grown at 37°C with 250 rpm shaking for 3 days in Middlebrook 7H9 + Tween 80 in 5 mL cultures. Cells were sub-cultured at a 1:60 dilution and grown at the same temperature and shaking conditions overnight for ~16 hours until mid-log phase. The sub-cultured was used to inoculate a 2 mL culture at OD_{600} of 0.2. Cultures were treated with 5 mM diamide (TCI) and incubated at 53°C with 200 rpm shaking on a plate shaker (Fisher). Samples were collected at the time of addition and at one-hour increments for 5 hours. A 10-fold serial dilution was prepared, and dilutions spanning 10^{-1} - 10^{-6} of the original culture were spot-plated on square petri dishes Middlebrook agar. Plates were incubated at 37°C for 3 days, after which plates were imaged.

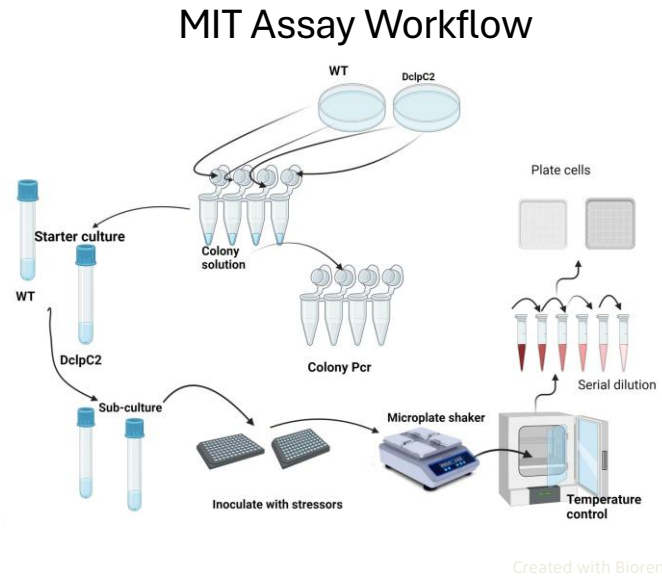


Figure 3 Schematic Diagram Showing The Minimum Inhibitory Time Assay Workflow.

2.4. Reporter Stress Assay & Fluorescence Measurement.

M. smegmatis cells were transformed with the integrating P*clpC2*-mCherry reporter plasmid. Transformants were used to inoculate 5 mL cultures in Middlebrook 7H9 + Tween 80 media with 20 µg/mL kanamycin. Cultures were incubated at 37°C with 250 rpm shaking for 3 days. Cells were sub-cultured at a dilution factor of 1:60 and grown at the same temperature and shaking conditions overnight for ~16 hours until mid-log phase. Sub-cultured cells were

pelleted at 3,000 x g for 5 minutes and resuspended in fresh media to OD_{600} of 0.1. This culture was used to inoculate 150 µL samples in a black 96-well plate with a transparent bottom (Corning) at a starting OD_{600} of 0.05, with a range of rufomycin concentrations ranging from 0 - 2 µM. (Rufomycin was a kind gift from Dr. Scott Franzblau and the Institute for Tuberculosis Research at University of Illinois at Chicago.) Plates were incubated with periodic shaking in a plate reader (Tecan Spark) at 37°C. mCherry fluorescence (540 nm excitation, 610 nm emission) and OD_{600} were measured over 16 hours. Data was analyzed by calculating RFU/OD values, and data was represented on a line graph in Prism (Graphpad).

2.5. RNA Extraction

To check the ability of ClpC2 to regulate genes under stress conditions, *M. smegmatis* mc^2155 was grown at 37°C for 4 days with shaking at 250 rpm in 25 mL 7H9 Middlebrook broth with 0.05% (w:v) Tween 80. Cells were sub-cultured at 1:100 and grown at 37 °C for ~16 hours until mid-log phase. The sub-culture was diluted into fresh media to OD_{600} of 0.05. A final volume of 25 mL was added to a flask with 0.5 µM rufomycin. Treated cells and their corresponding controls were incubated with shaking at 37 °C for 8 hours. After 8 hours, cells were harvested, OD_{600} was taken, and the cell concentration for each sample was calculated and cells normalized to the highest OD_{600}. The cells were pelleted at 4000 x g for 30 minutes. Pelleted cells were resuspended

in the same volume of lysis buffer. Resuspended cells were lysed partly as described by (Taylor, 2022) in 1.7 mL tubes containing 250-500 mg 0.15 mm zirconium oxide beads using a bead beater (Disruptor Genie) as previously described (Tükenmez et al., 2021). The lysate was cleared by centrifugation at 8,000 x g for 10 min, the supernatant was collected, and the total RNA was purified using a GeneJET RNA Cleanup and Concentration Micro Kit (Thermo). RNA concentration and quality were determined by using a Qubit kit and a Qubit Fluorometer and by running samples on a Sybr Gold gel. For RNA-seq analysis, RNA samples were fragmented into 200 - 300 bp fragments by sonication (Diagenode Megaruptor).

2.6. RT-qPCR

To determine relative regulation by ClpC2, primers were designed for qPCR that amplified a ~150 bp region in *clpC2*, MSMEG_2521, or MEMEG_0708, and the reference housekeeping gene *sigA*. 20 ng per sample of RNA was reverse transcribed into complementary DNA (cDNA) using SuperScript IV First-Strand Synthesis System (Invitrogen). RT-qPCR was performed using the PowerTrack SyberGreen Master Mix kit (Invitrogen) in a CFX96 Real-Time System (BioRad) thermocycler with an initial 2 minute 95 °C denaturing step, followed by 95 °C for 30 seconds, 60 °C for 1 minute, 65 °C for 5 seconds, running a total of 45 cycles in all. Data analysis to determine relative

gene expression was determined using the Ct method and with *sigA* as the reference gene (Livak & Schmittgen, 2001).

2.7. RNA-seq Sequencing and Analysis.

Rufomycin-treated and untreated wild-type and Δ*clpC2* RNA samples were sent to the University of Delaware DNA Sequencing & Genotyping Center for RNA-seq analysis. Fragment analysis, bacterial ribosomal RNA depletion, and library preparation were performed by the Core. Samples were analyzed by 2x75 paired end sequencing on an Element Aviti instrument. The sequenced data was evaluated by FastQC (Simon Andrews, 2010) for and MultiQC (Ewels et al., 2016) to assess read quality. Final reads were 83 bp in length, with an average of 43.9 million sequences collected per sample, with ~82% of reads having a quality score of Q30 or greater. Reads were trimmed with FastP (Chen et al., 2018), enforcing an average quality score threshold of 25; 72% of sequences survived FastP trimming. Reads were mapped to the *Mycobacterium smegmatis* reference genome (NC_008596.1) using STAR, with 83% of reads successfully mapped. Reads were normalized and the total read count per sample was presented in the form of Reads Per Kilobase Per Million using HTseq (Grigorov et al., 2023). Genes with fewer than 10 total reads across samples were filtered out. Differentially expressed genes were identified using DESeq2 (Love et al., 2014) in R or PyDESeq2 in python (Muzellec et al., 2023; L. Wang et al., 2009). Genes were considered differentially expressed if

the adjusted p-value was ≤ 0.01 and the log2 fold change (log2FC) value was equal to 5.

2.8. Immunoblots

To interrogate stress conditions that upregulate pArg, *M. smegmatis* cells carrying the P*clpC2*-mCherry reporter were grown at 37 °C for 4 days with shaking at 250 rpm in 25 mL 7H9 Middlebrook + Tween 80. Cells were sub-cultured at 1:100 and grown at 37 °C overnight for ~16 hours until mid-log phase. The sub-cultured cells were diluted to OD_{600} 0.3 in fresh media. A final volume of 45 mL was added to a flask with treatments, including 0.5 µM rufomycin, 0.5 µM cyclomarin A, 7.5 mM L-canavanine, 5 mM diamide, 2.5 mM of sodium nitroprusside, 5 mM sodium nitrite, 55 mM L-azetidine, and 53 °C heat-shock. Treated cells and their corresponding controls were incubated for shaking at 37 °C (except heat-shock and heat-shock + 5 mM diamide samples, which were incubated at 53 °C) for 8 hours with time points taken at 0,3, and 8 hrs, where OD_{600} was recorded, and cells were harvested at 0.3 OD_{600} across all samples throughout the 8 hours period. Harvested cells were pelleted at 4000 x g for 30 minutes. Resuspended cells were lysed as partly described by (Gabrielle Taylor, 2022) in 1.7 mL tubes containing 300 mg 0.15 mm zirconium oxide beads using a disruptor gene bead according to (Tükenmez et al., 2021) for 7 cycles with one 1 minute mechanical disruption of samples and 1 minute incubation on ice. The lysate was centrifuged at 8,000 x g for 10 minutes to

obtain the supernatant. Samples were loaded and separated on 15% precast SDS-PAGE gel (Genscript) and transferred to PVDF membranes by an iBlot3 transfer divide (Thermo) at 25 volts for 3 minutes, following the manufacturer's instructions. The membrane was blocked with 0.05% w/v non-fat milk in TBST for 1 hour at room temperature. The membrane was washed three times in TBST for five minutes each and incubated in α-mCherry monoclonal primary antibody 5A6 (Thermo) in TBST with 0.05% w/v BSA for 1 hour at room temperature, and washed three times in TBST for 5 minutes each. The membrane was then incubated in HRP-conjugated goat α-mouse IgG secondary antibody (Thermo) for 1 hour at room temperature. The membrane was washed three times in TBST for five minutes each. The secondary antibody was detected using Supersignal West Pico Plus chemiluminescent substrate (Thermo Scientific), and imaged on a chemidoc (Biorad).

CHAPTER 3.Results

3.1. Identify phenotype Associated With *clpC2* Deletion Under Stress

While the essential mycobacterial ClpC1 unfoldase has received extensive attention as a target for novel antibacterial drugs, the small non-essential non-proteolytic protein ClpC2 has been investigated only recently. The C-terminal two-thirds of ClpC2 shares homology with the ClpC1 N-terminal domain (NTD). Studies have shown that the ClpC1-targeting compound cyclomarin A (CymA), which potently kills mycobacteria in culture and in animal models of infection, also binds with high affinity to ClpC2.

Although non-essential, ClpC2 is conserved across mycobacteria and a subset of actinobacterial species. While ClpC2 clearly confers partial resistance to some ClpC1-targeting antibiotics, it is not clear how widespread these antibiotics are in nature or if antibiotic defense is the primary physiological role of ClpC2. Interestingly, ClpC2 also possesses one of the two conserved phosphoarginine (pArg) binding sites present on the ClpC1 NTD, which are spatially separate from the site of CymA binding. In *Bacillus subtilis* and other firmicute bacteria, pArg modifications play a role in proteotoxic stress response by marking misfolded proteins for destruction by the ClpCP protease. Prior studies from the Schmitz lab have shown that pArg modifications exist in mycobacteria (Ogbonna et al., 2022a). More recent unpublished work by Henry Anderson in the Schmitz lab demonstrates i) that ClpC2 binds pArg, and ii) that pArg binding relieves transcriptional repression by ClpC2 *in vitro*. While the

physiological regulation of pArg in mycobacteria remains poorly understood, these preliminary data lead us to hypothesize that pArg-dependent regulation of ClpC2 contributes to the proteotoxic stress response.

3.1.1. *clpC2* Deletion impairs survival under severe stress

Maintaining protein homeostasis is vital for bacterial survival, particularly during proteotoxic stress. In some Mycobacterium species, such as *Mtb* and *Msm*, ClpC2, a partial homolog of the essential Clp protease, ClpC1, has been reported to play a crucial stress-responsive modulating or regulatory role. Recent studies have highlighted the physiological and functional significance of ClpC2 in stress adaptation related to proteotoxicity, which is antibiotic-induced. (Gabrielle Taylor, 2022; Taylor et al., 2023) This raises the question of the significance of clpC2 deletion in mycobacteria during severe stress.

In one of the studies, it was demonstrated that *clpC2* deletion in *Mycolicibacterium smegmatis* severely impacted bacterial viability and survival on exposure to a naturally occurring antibiotic, Cyclomarin A, through a mechanism that suggests that ClpC2 acts as a molecular protector and provides some buffer against Cyclomarin A-induced toxicity. (Hoi et al., 2023b) Similarly, in a separate but closely related study, ClpC2 was found to autoregulate the transcription of its gene under basal conditions by binding to its operator sequence at the promoter region. However, upon exposure to antibiotics such

as Cyclomarin A, ClpC2 will preferentially bind antibiotics by unbinding from the operator sequence of its DNA, hence relieving repression and leading to overexpression of its gene. This positions ClpC2 into a form of “molecular sponge” with the ability to sequester ClpC1 from the toxicity of ClpC1-targeting antibiotics. (Taylor et al., 2023) Together, these studies point to a ClpC2-dependent stress-responsive mechanism for targeting ClpC2 alongside ClpC1 as a potential strategy for developing new antitubercular therapies.

To test the impact of *clpC2* deletion on cell survival during proteotoxic stress, I performed a minimum inhibitory time (MIT) assay by monitoring the viability of cells across a 4 h time course of heat shock (53°C) on wild-type (WT) cells and a strain harboring knockout of the *clpC2* coding region (Δ*clpC2*). Surprisingly, both wild-type and Δ*clpC2* strains withstood heat shock with no major reduction in viability up to 4 h (**Fig. 4A**). These findings conflict with prior studies, which found that Δ*clpC2* cells exhibited reduced tolerance to similar heat shock conditions (Hoi et al., 2023b). The difference may be due to the method for construction of *clpC2* knockout. Our studies utilized a clean deletion of the *clpC2* coding region generated by CRISPR methods. Hoi and colleagues replaced *clpC2* with a gentamicin resistance cassette, which may disrupt expression of the downstream genes in the *clpC2* operon.

We reasoned that stronger proteotoxic stress may manifest a *clpC2*-dependent phenotype. Thus, we repeated heat shock experiments with the inclusion of 5 mM diamide, which disrupts the intracellular redox environment

(**Fig. 4B**). Under these conditions, we found that the Δ*clpC2* cells exhibited reduced viability, compared to wild-type, after 3 h. When diamide was tested at the normal growth temperature of 37°C, there was no significant difference in sensitivity to the treatment among the two strains, even at higher concentrations (**Fig. 4C**). Additionally, other conditions tested either completely killed the cells (in both strains) simultaneously or did not show a significant difference in sensitivity. It was unclear what other effects the treatments might have on cells beyond differential growth in terms of phenotypic expression.

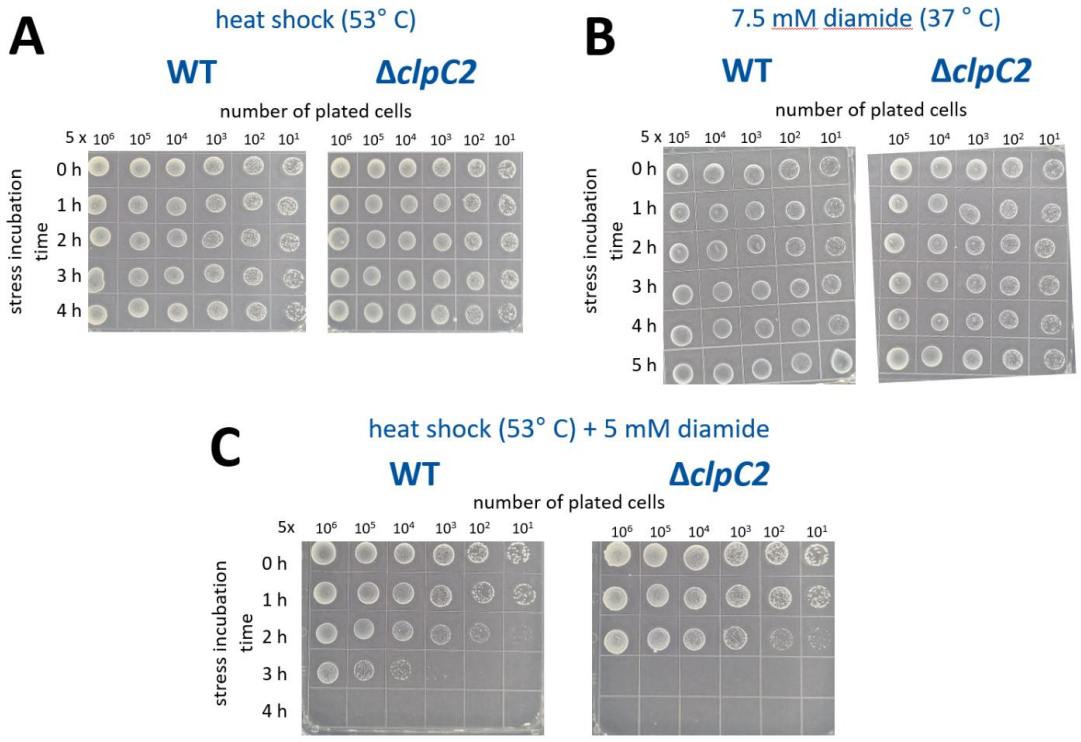


Figure 4 **Comparison of growth and survival of *M. smegmatis* wild-type and Δ*clpC2* in stress conditions.** Mid-log phase wild-type or Δ*clpC2 M. smegmatis* cultures were treated for the indicated time with A) 53° C heat shock, B) 7.5 mM diamide, or C) a combination of 53° C heat shock and 5 mM diamide for the indicated amount of time. After treatment, ten-fold serial dilutions of culture with the indicated number of cells was spot plated on agar plates.

3.2. *clpC2*-Dependent Transcription And Phosphoarginine Upregulation During Stress.

Stress adaptation and response mechanisms in mycobacteria are controlled by a strongly regulated interaction between protein homeostasis and a network of transcriptional programs, with ClpC1 of the essential Clp protease complex (ClpC1P1P2) playing a central role. While ClpC1 has been established to play a dominant role in mycobacterial proteostasis, emerging biophysical and biochemical evidence shows that ClpC2, its partial homolog, plays a transcriptional regulatory role rather than an enzymatic role under stress conditions. (Hoi et al., 2023b; Taylor et al., 2023)

One line of evidence suggests that ClpC2 modulates transcription via a stress-response mechanism that accumulates damaged phosphoarginine-tagged protein during proteotoxic, antibiotic-induced, or oxidative stress. In this study, it was demonstrated that ClpC2 binds pArg in a specific and conserved domain. (Hoi et al., 2023b) We think that this interaction between ClpC2 and pArg disrupts its oligomeric state, thereby causing it to depress its transcription, resulting in unknown downstream transcriptional events. Additionally, another line of evidence shows that ClpC1-targeting antibiotics, such as rufomycin, bind ClpC2, resulting in upregulation of ClpC2-mediated transcription. (Taylor et al., 2023) In concert, these findings support our hypothesis that ClpC2 functions as a pArg-responsive transcriptional regulator during stress. This section interrogates the specific role of ClpC2 transcriptional regulation

during antibiotic-induced stress using the mCherry reporter in vitro assay apart from cell viability.

3.2.1. Rufomycin Treatment Results in ClpC2-Dependent Transcription as Determined by Reporter Assay.

To investigate the physiological impact of stress on M. smegmatis cells beyond cell viability, as tested above, we sought to understand if stress conditions relieve clpC2-dependent transcriptional repression using a fluorescent reporter. To achieve this, I used an integrative reporter construct (PclpC2-mCherry) designed by Pratistha Kandel that consisted of the M. smegmatis clpC2 promoter followed by a cassette encoding the red fluorescent protein mCherry. Wild-type *M. smegmatis* cells transformed with PclpC2-mCherry exhibit low fluorescence (**Fig. 5**, left panel, gray trace), but fluorescence increased after treatment with 0.5 μM rufomycin (**Fig. 5**, left panel, black trace), consistent with the expected mechanistic ability of rufomycin to block ClpC2 from binding its DNA operator.

Cells lacking clpC2 (Δ*clpC2*) exhibited constitutive expression of mCherry (**Fig. 5**, right panel, red trace). To confirm that ClpC2 is responsible for repression of the reporter, I constructed a non-integrating complementation plasmid incorporating the endogenous promoter and clpC2 coding region. Introduction of the complementation plasmid into Δ*clpC2* carrying the PclpC2-mCherry reporter reduced fluorescence to levels observed in untreated wild-

type (**Fig. 5**, right panel, orange trace). Treatment of complemented ΔclpC2 cells with 0.5 μM rufomycin elevated fluorescence, similar to wild-type (**Fig. 5**, right panel, purple trace). These data confirm the P*clpC2*-mCherry reporter is responsive to ClpC2-dependent repression.

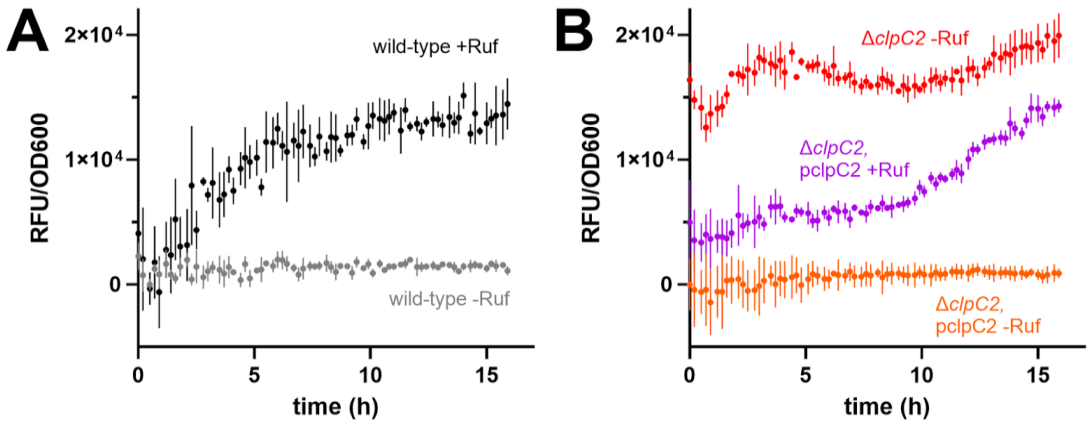


Figure 5 **Reporter assay shows *clpC2* upregulation during rufomycin treatment**. A) Wild-type *M. smegmatis* cells carrying an integrating P*clpC2*-mCherry reporter construct were treated with 0.5 µM rufomycin (black) or left untreated as a control (gray) at a starting OD_{600} of 0.05 and incubated at 37°C with shaking in a plate reader for 16 h. mCherry fluorescence and OD_{600} were measured over the course of the assay. mCherry expression was monitored by calculating the RFU per OD_{600} and plotted against time. Rufomycin induces mCherry fluorescence. B) *M. smegmatis* cells harboring a clean deletion of *clpC2* (Δ*clpC2*) were transformed with an integrating P*clpC2*-mCherry reporter alone and RFU/OD_{600} was monitored in the absence of rufomycin (red). *M. smegmatis* Δ*clpC2* harboring P*clpC2*-mCherry was additionally transformed with a non-integrating complementation plasmid incorporating the promoter and coding region of *clpC2* (pclpC2), and was monitored in the presence (purple) or absence (orange) 0.5 µM rufomycin as in A. Deletion of *clpC2* causes constitutive mCherry fluorescence. Complementation restores near wild-type behavior.

Table 1 Summary of Stress Conditions and Reporter Activity

	Concentration Tested	Mechanism of Action	Remark
Hydrogen Peroxide	0.2–10 mM	Oxidative stress via ROS generation	No significant induction
Tert-Butyl Hydroperoxide (T-BHP)	0.25–1 mM	Lipid peroxidation and oxidative damage	No significant induction
Diamide	1–10 mM	Thiols oxidation, disulfide stress	No significant induction in reporter activity, but showed a phenotype with 53 degrees Celsius heat shock.
Heat Shock (53°C)	60 min - 8 hours	Protein misfolding and aggregation due to elevated temperature	Moderate induction on blot
Heat Shock + Diamide (5 mM)	30–60 min + 5 mM diamide	Combined thiol oxidative stress and protein denaturation	Slightly elevated induction
SDS	0.25–2%	Disruption of membrane integrity	No significant induction
Ethanol	2–5%	Protein and membrane denaturation	No significant induction
NaCl	0.3–1 M	Osmotic stress	No significant induction
Azetidine-2-carboxylic acid (AZC)	2.5–55 mM	Proline analog induces protein misfolding	No significant induction
L-Canavanine	0.25–7.5 mM	Arginine analog; protein misfolding	No significant induction

CuSO4	1–6 mM	Metal-induced oxidative and proteotoxic stress	No significant induction
NiSO4	1–6 mM	Metal-induced oxidative and proteotoxic stress	No significant induction
Sodium Nitroprusside (SNP)	0.1–2.5 mM	Nitrosative stress via NO release and protein nitrosylation	No significant induction
D-Cycloserine	300 µg/mL	Inhibits cell wall synthesis (D-Ala racemase and ligase inhibitor)	No significant induction
Sodium Nitrite	5 mM	Acidified nitrite generates reactive nitrogen species (RNS)	No significant induction
Rufomycin	0.5–2 µM	Targets ClpC1, induces proteotoxic stress and transcriptional reprogramming	Strong induction in WT in blots and reporter assay

I next used wild-type cells carrying the P*clpC2*-mCherry reporter to test for stress conditions that physiologically relieve ClpC2-dependent repression. Tested conditions included the oxidizing agents hydrogen peroxide and tert-butyl hydroperoxide (T-BHP), diamide, sodium dodecyl sulfate (SDS), ethanol, NaCl, azetidine-2-carboxylic acid (AZC), L-canavanine (L-can), $CuSO_4$, and $NiSO_4$. (**Table 1**) None of these conditions showed significant reporter fluorescence above baseline. Thus these physiological proteotoxic stress do not

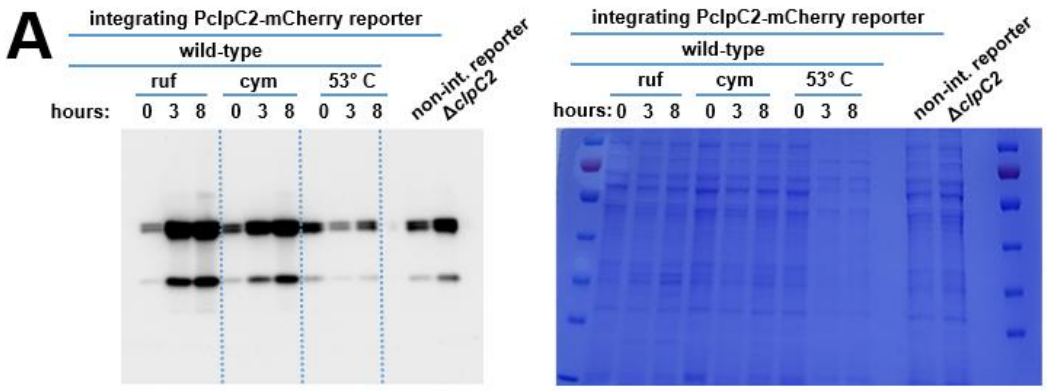
elevate ClpC2-dependent transcription to levels as high as induced by Ruf treatment. However, it is possible that physiological stress elevates ClpC2 expression to modest levels that are not well detected in this assay, as might be expected since upregulated ClpC2 should be able to further repress transcription, providing a negative feedback mechanism that avoids high level overexpression.

3.3. Western blots reveal that multiple stressors drive mild upregulation of ClpC2.

We sought to use Western Blots as an alternative and more sensitive method to investigate transcription at the protein level. To achieve this, I utilized a non-integrating version of the mCherry reporter construct engineered by Pratistha Kandel, which produces brighter mCherry signal than the integrating version. Cells with this reporter were treated with 0.5 μM rufomycin, 0.5 μM cym A, 53°C heat shock alone, 53°C heat shock + 5 mM diamide, 5 mM diamide only, 7.5 mM L-can, 55 mM AZC, 2.5 mM $NaNO_2$, 5 mM sodium nitroprusside (SNP), and controls for 0, 3, or 8 hours. Lysates from these samples were probed with anti-mCherry primary antibodies and imaged with Goat anti-mouse secondary antibodies using an ECL substrate (**Fig. 6**).

As expected, rufomycin and cyclomarin A drove strong overexpression of the reporter. Qualitatively, heat-shock, diamide, L-can, SNP, and $NaNO_2$ drove incrased mCherry expression at the 8 h time point, suggesting that

multiple stresses can relieve transcriptional repression by ClpC2, possibly through elevation of pArg.



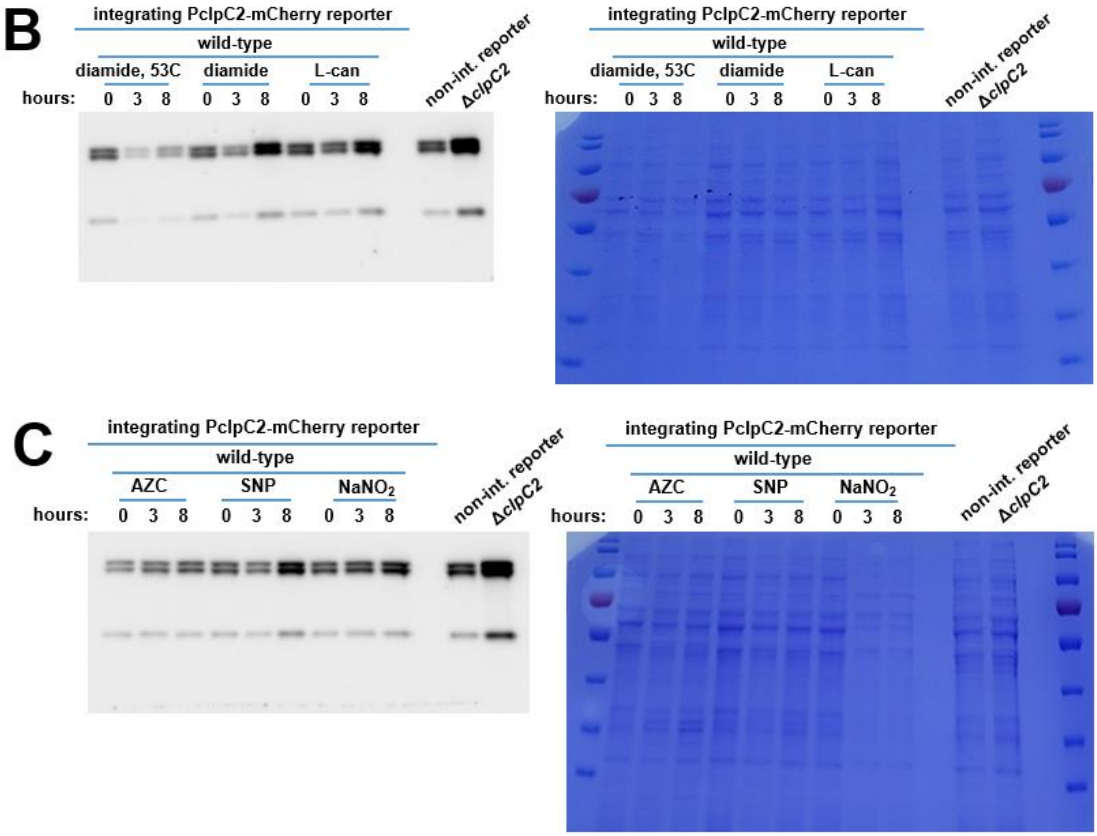

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Figure 6 **Western blot shows *clpC2* upregulation during rufomycin, cyclomarin A, diamide, SNP treatment**. **A-B)** Wild-type *M. smegmatis* cells carrying a non-integrating P*clpC2*-mCherry reporter construct were treated with 0.5 µM rufomycin, 0.5 µM cyclomarin A and 53°C heat shock with two untreated controls at a starting OD_{600} of 0.3 and incubated at 37°C (except for heat shock) with shaking for 8 h, taking time points at 0 h, 3 h and 8 h . mCherry expression was determined by incubating blots with α-mcherry primary antibody. Rufomycin and cycomarin A induces strong mCherry fluorescence from the third and eight hour**. C-D**) Wild-type *M. smegmatis* cells carrying a non-integrating P*clpC2*-mCherry reporter construct were treated 5mM diamide plus 53°C heat shock, 5 mM diamide only and 7.5 mM with two untreated controls at a starting OD_{600} of 0.3 and incubated at 37°C (except for heat shock) with shaking for 8 h, taking time points at 0 h, 3 h and 8 h . mCherry expression was determined by incubating blots with anti-mcherry primary antibody. 5mM diamide only and 7.5 mM induces moderate mCherry fluorescence at the eight hour**. E-F**) Wild-type *M. smegmatis* cells carrying a non-integrating P*clpC2*-mCherry reporter construct were treated 55mM AZC, 2.5 mM SNP and 5 mM NaNO2 with two untreated controls at a starting OD_{600} of 0.3 and incubated at 37°C with shaking for 8 h, taking time points at 0 h, 3 h and 8 h. mCherry expression was determined by incubating blots with anti-mcherry primary antibody. 2.5mM SNP induces fair mCherry fluorescence at the eight hour.

Prior data demonstrates that ClpC2 is a repressor that regulates transcription of the *clpC2* operon. (Taylor et al., 2023) We reasoned that ClpC2 may also regulate additional genes in the *M. smegmatis* genome. We therefore scanned the *M. smegmatis* genome for sequences similar to the reported consensus ClpC2 operator sequence (**Fig. 7A**, upper black sequence), and identified several possible ClpC2 operator sequences within the promoter

regions of six additional genes: MSMEG_0708 (LysR-family transcriptional regulator), MSMEG_1619 (uncharacterized protein), MSMEG_2521 (homolog of amiC amidase), MSMEG_3766 (uncharacterized protein), MSMEG_5988 (translation initiation factor IF-2), and MSMEG_6622 (cytochrome P450 monooxygenase).

To assess whether these genes are similarly regulated under conditions that elevate *clpC2* transcription, we designed qPCR primers for two genes carrying possible ClpC2 operator sites, MSMEG_2521 and MSMEG_0708, as well as *clpC2* and the housekeeping protein *sigA* as a reference. Cells were heat-shocked for 8 hours at 53°C, lysed, cDNA was prepared from cellular RNA, and transcript levels were assessed by qPCR. Analysis of qPCR data obtained showed that heat-shock induced *clpC2* expression >30-fold, consistent with its upregulation during strong stress (**Fig. 7B**). Additionally, both MSMEG_0708 and MSMEG_2521 were upregulated >5-fold in heat-shocked wild-type cells compared to unstressed cells (**Fig. 7C**). By contrast, levels of MSMEG_0708 and MSMEG_2521 increased less than 2-fold in *clpC2* cells upon heat-shock (**Fig. 7D**). These data support the hypothesis that ClpC2 regulates a wider set of transcriptional targets during stress conditions, although we cannot rule out indirect effects.

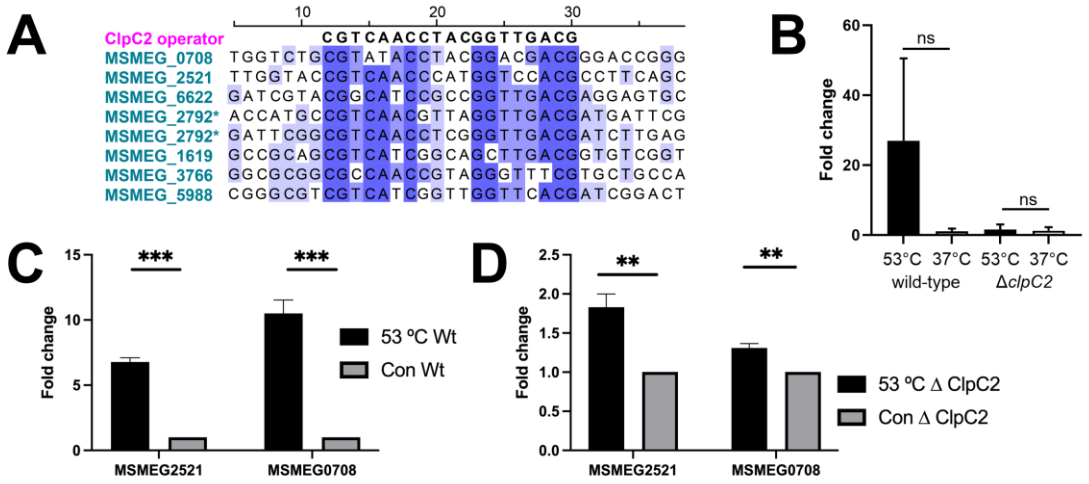

Figure 7 **Relative Expression of MSMEG_0708 and MSMEG_2521 Upon Heat Shock in *M. smegmatis***. **A**) The *M. smegmatis* genome was scanned for sequences similar to the published ClpC2 operator consensus sequence (top). The listed sequences occur within putative promoter regions. The promoter region of *clpC2* (MSMEG_2792) contains two adjacent operator sequences. **B**) WT and Δ*clpC2* cells were incubated at 53°C or 37°C for 8 h. Expression levels of *clpC2* were measured by qPCR and compared with *sigA*, the mycobacterial housekeeping sigma factor. **C**) Wild type cells were incubated at 53°C or 37°C for 8 h. Transcription of MSMEG_0708 and MSMEG_2521 was assessed by qPCR and compared to *sigA*. Both exhibited significant upregulation during heat shock. **D)** Δ*clpC2* cells were incubated at 53°C for 8 hours, or at 37°C as a control. Expression levels of MSMEG_0708 and MSMEG_2521 were measured by qPCR and compared to *sigA*. While statistically significant, MSMEG_0708 and MSMEG_2521 exhibit a lower level of heat shock induced upregulation in Δ*clpC2* than in wild-type. P-values were determined by two-tailed Student's unpaired T-test. ** ≤ 0.01; *** ≤ 0.001.

3.4. Transcriptomic Changes in Response to Stress Show That ClpC2 Upregulates The Transcription of Its Gene, While Other Essential Genes Are Downregulated in a ClpC2-independent Manner.

Given the presence of multiple candidate *clpC2* operator sequences in the *M. smegmatis* genome (**Fig. 7A**) and the ClpC2-dependent upregulation of two of these during heat-shock (**Fig. 7C**), we sought to use RNA-seq to identify the full set of transcripts regulated by ClpC2. Although *clpC2*, MSMEG_0708 and MSMEG_2521 are upregulated by heat shock, we chose not to use this stress condition for RNA sequencing (RNA-seq) experiments, as it is likely to modulate numerous ClpC2-independent pathways in the cell. Instead, we treated cells with rufomycin, which is known to mechanistically block ClpC2 binding to DNA. While rufomycin treatment ultimately kills *M. smegmatis* in culture, the time course of killing is relatively slow. With 0.5 μM rufomycin treatment, substantial loss in culture viability is not observed until 5 hours. Thus, for RNA-seq we chose to analyze transcript levels after 6 hours of treatment.

Wild-type or Δ*clpC2* were grown until mid-log phase (OD_{600} ~0.75) and were either untreated or treated with 0.5 μM rufomycin for 6 hours, with four biological replicates of each sample (**Fig. 8**). Cells were harvested, RNA was extracted from lysates, RNA was fragmented to 200 - 300 bp fragments by sonication, and samples were sent to the UD Sequencing and Genotyping Core

for analysis. After kit-based ribosomal RNA depletion and adaptor ligation, samples were analyzed by 2x75 paired end sequencing, yielding an average of 44 million reads per sample. After trimming the dataset to an average sequence read quality of Q25, an average of 32 million reads per sample was retained (**Fig. 9**), corresponding to an average ~370-fold coverage of the *M. smegmatis* genome. Sequence reads were mapped to the *Mycolicibacterium smegmatis* reference genome. Differential gene expression was examined for Δ*clpC2* vs wild-type (**Fig. 10B**), treated vs untreated wild-type (**Fig. 11A**), and treated vs untreated Δ*clpC2* (**Fig. 11B**).

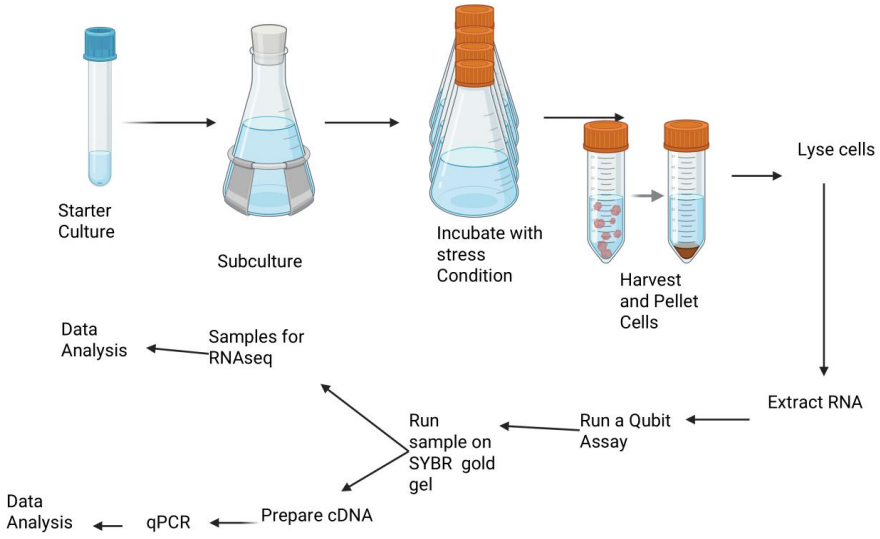

Figure 8 RNA-seq Sample Prep Workflow

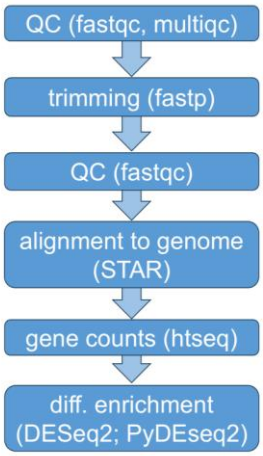

Figure 9 RNA-seq Data Analysis Workflow

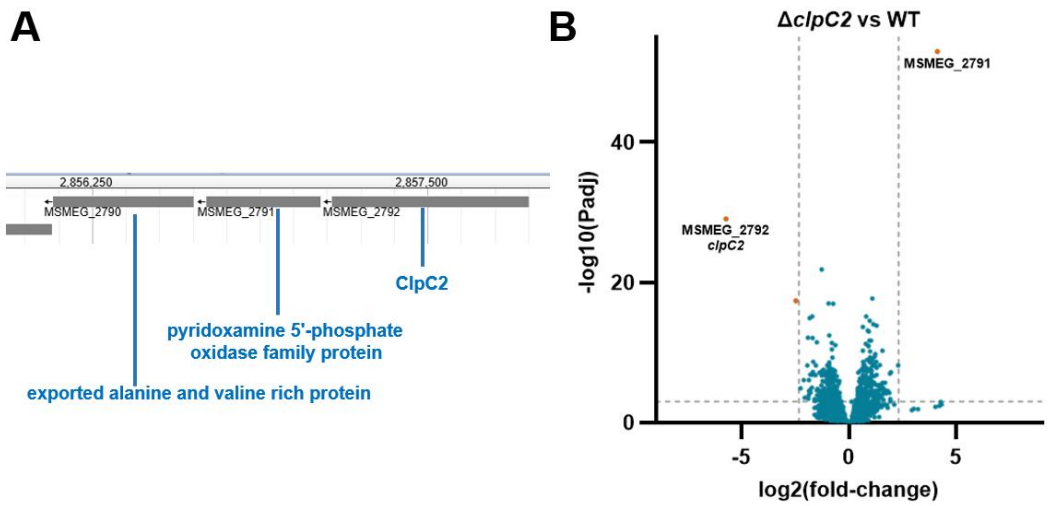

Figure 10 **Figure 3.5. Deletion of *clpC2* has minimal effects on gene expression under normal conditions.** A) Organization of the multicistronic *clpC2* operon, generated by the NCBI genome viewer. The operon runs right-to-left, with *clpC2* the first coding region after the promoter. In Δ*clpC2,* the ClpC2 coding region is removed, but the promoter and downstream coding regions are intact. B) RNA-seq comparison of mRNA transcripts in untreated Δ*clpC2*, compared to wild-type *M. smegmatis*, shown as a volcano plot. Dashed lines indicate adjusted P-value threshold of 0.001, and fold-change threshold of ± 5-fold. MSMEG_2792, encoding *clpC2*, appears as downregulated because of its deletion. MSMEG_2791, which follows *clpC2* in the operon, is upregulated in Δ*clpC2*.

The *clpC2* gene (MSMEG_2792) is in the first position of a 3-gene operon (**Fig. 10A**). RNA-seq comparison of transcript levels in Δ*clpC2* to wild-type cells, showed that *clpC2 (*MESMEG_2792) was strongly downregulated – which makes sense given its deletion – while MSMEG_2791 was highly upregulated in Δ*clpC2*, as expected. This

data supports previous evidence of the role of clpC2 in transcriptional regulation of neighboring genes as part of an operon. However, no other genes were upregulated in Δ*clpC2*, including those with putative operators (**Fig. 7A**).

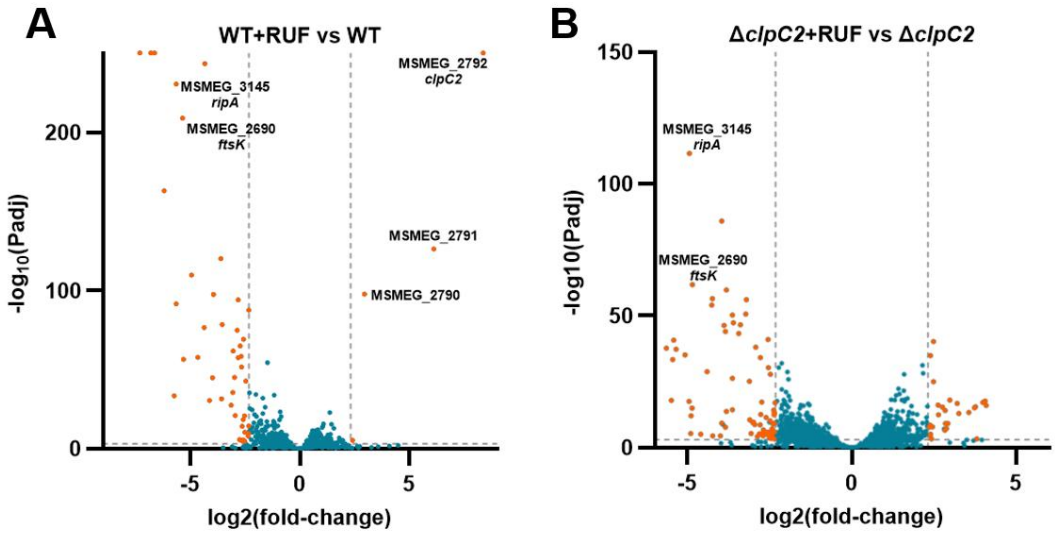

Figure 11 Up- and downregulation of genes upon rufomycin treatment. A) A volcano plot of RNA-seq data shows differences in transcript levels of wild-type *M. smegmatis* treated with 0.5 µM rufomycin, compared to untreated cells. Only the *clpC2* operon is strongly and significantly upregulated. However, 48 transcripts are downregulated, including the essential cell-division associated genes *ripA* and *ftsK*. B) A volcano plot of RNA-seq data shows differences in transcript levels in Δ*clpC2 M. smegmatis* treated with 0.5 µM rufomycin, compared to untreated Δ*clpC2*. Notably, a large number of genes are strongly downregulated, including *ripA* and *ftsK*. Dashed lines indicate adjusted P-value threshold of 0.001, and fold-change threshold of ± 5-fold.

We next examined changes in mRNA levels in wild-type cells treated with 0.5 μM rufomycin for 6 h, compared to untreated cells (**Fig. 11A**). (Prior studies by P. Kandel show that rufomycin does not cause cell death until after >12 h of treatment.) Only the *clpC2* operon (MSMEG_2792, MSMEG_2791, MSMEG_2790) was strongly upregulated under these conditions. Rufomycin treatment of *ΔclpC2* cells did not result in strong upregulation of any genes to the levels observed in wild-type. Since neither deletion of *clpC2* nor treatment with rufomycin lead to upregulation of genes outside of the *clpC2* operon, our data suggest that ClpC2 does not regulate a wide set of genes, contrary to our hypothesis.

Interestingly, and contrary to our expectations, a large set of genes were downregulated by rufomycin treatment of wild-type cells (**Fig. 11A**). Many of these, including the essential genes *ripA* (MSMEG_3145) and *ftsK* (MSMEG_2690), are involved in cell division and cell wall remodeling. Notably, a similar pattern of downregulation was observed in rufomycin-treated *ΔclpC2* cells (**Fig. 11B**).

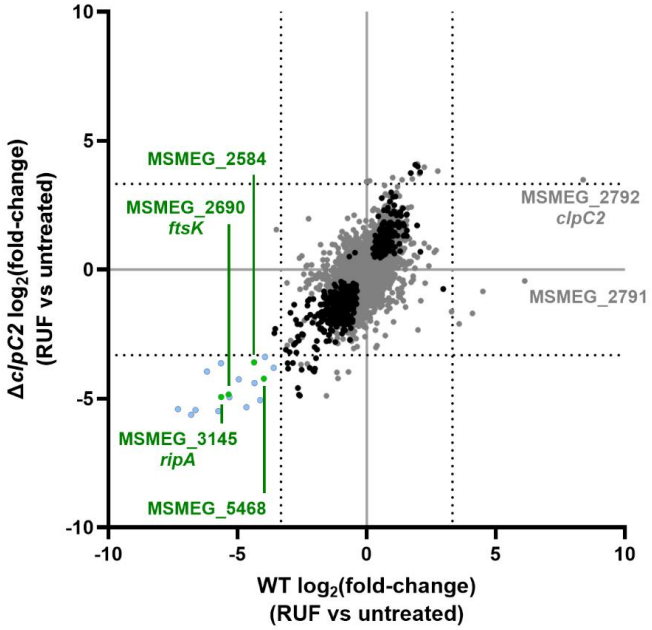

Figure 12 **Figure 3.7. Rufomycin treatment causes ClpC2-independent downregulation of genes involved in cell division.** The scatterplot shows transcript fold-change levels observed in rufomycin-treated wild-type and Δ*clpC2* cells. Dashed lines indicate a fold-change threshold of ± 5-fold. Gray points correspond to genes with P-adj > 0.001 in one or both samples (see Fig. 3.6). Black points correspond to genes with P-adj ≤ 0.001 in both datasets. Blue points have P-adj ≤ 0.001 and ≥ 5 fold-change. Labeled green points are essential genes.

We compared rufomycin-induced transcriptional changes in wild-type and *ΔclpC2* as a scatter plot (**Fig. 12**), and observed that the same set of genes are strongly and significantly downregulated in both strains. This indicates that rufomycin-induced downregulation of genes is independent of ClpC2. Since

ClpC1 is the only known target of rufomycin in *ΔclpC2* cells, our data suggest that ClpC1 at least regulates the transcription of a set of genes related to cell wall maintenance and division. The mechanistic connection between ClpC1, a component of an essential protease, and transcriptional regulation, remains unknown, but may involve proteolytic regulation of an unidientified transcriptional regulation.

CHAPTER 4.Discussion

The regulatory role of ClpC1 homolog, ClpC2, in bacterial proteostasis has been the subject of recent investigations. Of great interest also is the intricate stress response mechanism present in the pathogenic mycobacterial genus, which serves as an adaptive feature during stress. This study examines the regulatory function of ClpC2 in modulating the stress response mechanism in mycobacteria in response to various stressors. We demonstrate that the deletion of *clpC2* impairs mycobacterial growth under severe stress. This suggests that *clpC2* may play a secondary and supportive role in maintaining cell viability during stress. This finding supports the works of (Taylor et al., 2023) and (Hoi et al., 2023b) Both of whom had previously reported the role of *clpC2* in cell viability and survival during antibiotic and heat stress.

Although some of the conditions tested in the first part of our investigation did not yield a phenotype with respect to cell viability, they still demonstrate a limited or clear effect on cell death and survival. Therefore, we suspect that these conditions could still have a significant impact on transcriptional and protein levels. Therefore, we conclude that several of the stress conditions we tested have a unique impact, based on their mechanism of action, and such impacts may not necessarily be reflected in phenotypic expression. This observation could set the pace for dissecting some transcriptional programs controlled by ClpC2.

Another way we tested the regulatory role of ClpC2 was through a fluorescent reporter assay, in which we demonstrated that the well-known naturally occurring antibiotic, rufomycin, activates or upregulates ClpC2-dependent transcription. Again, this finding aligns with previous studies that have reported similar results. (Gabrielle Taylor, 2022; Hoi et al., 2023b; Taylor et al., 2023).

Additionally, we hypothesized that ClpC2 is a pArg-responsive transcriptional repressor involved in stress response in mycobacteria. We reasoned that, apart from ClpC1-targeting antibiotics such as rufomycin, which can bind ClpC2 during antibiotic stress and cause it to relieve repression, some of the stressors we tested earlier with respect to cell viability could upregulate cellular pArg levels, which would bind ClpC2 in a similar manner to antibiotics and cause by ClpC2. Notably, we observed that various stressors activate or upregulate ClpC2-dependent transcription, as confirmed by Western blot. Putting together, these findings highlight that ClpC2 is a pArg stress-responsive chaperone capable of mediating stress-induced transcriptional programs. It also suggests that ClpC2 may be part of a general stress survival network.

Furthermore, because ClpC2 is a transcriptional repressor, we anticipated a strong transcriptional response at the operator sites in response to stress. We therefore investigated the operator regions regulated by ClpC2. Our result revealed a differential transcriptional activity, with MSMEG0708 exhibiting significantly higher transcriptional activity than MSMEG2521 during

heat shock in wild-type cells. This suggests that ClpC2 may exert stress-specific control at specific operator sites or promoter regions.

Moreover, based on the data from previous evidence on the ClpC2 operator site. (Taylor et al., 2023) We computationally determined that there were multiple ClpC2 operator sites in the mycobacterial genome. With this additional piece of data, we reasoned that ClpC2 may be responsible for regulating multiple essential genes located in the same operon during stress. We investigated this by introducing antibiotic stress. Interestingly, our transcriptomic analysis revealed that ClpC2 is not a global transcriptional regulator, as previously thought, but rather influences a moderate subset of non-essential genes, including itself.

This finding narrows the function of ClpC2 to a more specific and specialized regulatory role, such as a sensor or modulator, and shifts our attention to ClpC1 as the possible global transcriptional regulator. Together, these positions ClpcC2 as a possible conditionally important factor in stress adaptation and survival without the ability to globally remodel the transcriptome.

Future Directions

To broaden our understanding of ClpC2-mediated stress adaptation in mycobacteria, future studies will examine mutants that disrupt ClpC2 oligomerization and phosphoarginine (pArg) binding. These variants will clarify

the structural requirements for their physiological and regulatory function. Proteomic approaches, particularly mass spectrometry, will be employed to assess global protein changes and directly quantify pArg-enriched peptides under defined stress conditions. Additionally, fluorescence microscopy will be used to monitor the subcellular localization and potential relocalization of ClpC2 in response to stress. Combined with ongoing transcriptomic and phenotypic studies, these approaches will provide a comprehensive framework for characterizing ClpC2's regulatory network and its role in stress tolerance pathways.

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