Insights into the Thermotolerance of an *Escherichia coli* RNA Polymerase Mutant

by

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ABSTRACT

When exposed to abiotic stresses, *Escherichia coli* responds by activating various stress-mitigating pathways. Initiation of stress responses partially relies on the RNA polymerase (RNAP) to transcribe genes necessary to tolerate various stresses, including nutritional deprivation and heat exposure. Consequently, RNAP mutations impacting transcription can have pleiotropic effects on the cell physiology and the ability to tolerate stress.

Previously, while investigating antibiotic-resistant mutations arising in the absence of major antibiotic efflux pumps, four mutants containing alterations in the RNA polymerase beta subunit gene (*rpoB*) were isolated (Cho & Misra, 2021). Of the four mutants, one (RpoB58) was found to be thermotolerant, permitting homogenous, stable growth at temperatures up to 47°C, whereas the parental *rpoB* wildtype (RpoB-WT) was only able to do so up to 45°C. Additionally, RNA-Seq analysis indicated that the RpoB58 mutant had a 'stringent' profile that is normally seen under nutritionally deprived conditions. To better understand the regulatory pathways used to confer stress tolerance, this thesis sought to further characterize and investigate the intracellular mechanisms contributing to the thermotolerance conferred by the *rpoB58* mutation. The RpoB58 mutant was found to be significantly more tolerant to both continuous heat stress (up to 47°C) and short-term heat (55°C) and ethanol (25%) exposure. Additionally, the RpoB58 mutant tolerated the absence or depletion of major heat shock chaperones DnaJ and DnaK that normally play key roles during temperature stresses by reducing protein misfolding. RNA-Seq data and reporter gene assays showed reduced expression of genes involved in protein synthesis. A similar reduction in the expression of protein synthesis

genes was observed when cells were grown in growth-limiting minimal media. Interestingly, growth in minimal medium rescued the Δ*dnaJ* defect like the *rpoB58* mutation. Based on these data, it was proposed that a decrease in protein synthesis, whether caused by *rpoB58* or the growth medium, would result in less growth-inhibiting protein misfolding and aggregation, especially at higher growth temperatures where proteins are susceptible to denaturation and aggregation. As a result of these investigations, a possible mechanistic insight was provided as to how the *rpoB58* mutation confers thermotolerance.

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This thesis would not have been possible without the people that have supported me throughout the process. First, thank you Dr. Rajeev Misra for allowing me to join and work in your lab. As a result of working in your lab, I have become a better researcher and critical thinker that can also appreciate how a single mutation can lead to a complex investigative journey. I would also like to thank my fellow lab members for their assistance, specifically Keilen Kelly who has patiently answered my endless questions provided fantastic feedback on my experiments and written work. Thank you to my former lab members, Maria Predtechenskaya and Nan Qiu, for your support and encouragement during my experimental and writing struggles.

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CHAPTER 1

INTRODUCTION

1.1 Overview of Antibiotic Resistance

Undoubtedly, as described by the World Health Organization and the Centers for Disease Control and Prevention (CDC), antibiotic resistance remains a global health concern (Michael et al., 2014). Aside from the misuse of antibiotics and the unavailability of new drugs, there also remains a lack of thorough understanding as to how resistance develops (Aslam et al., 2018). Just as quickly as a new antibiotic (either natural or synthetic) is introduced, some form of antibiotic resistance is identified (Graham, 2017).

As described by Blair et al. (2015), there are three general categories for molecular mechanisms of antibiotic resistance: 1) minimizing the intracellular concentration of antibiotics, 2) genetically mutating the target of the antibiotic, and 3) inactivation of the antibiotic. While the last category of resistance, inactivation of the antibiotic, is a critical way in which bacteria can actively develop antibiotic resistance it will not be relevant for this study.

1.2 Antibiotic Resistance Through Efflux Pumps

In the first mechanism category, bacteria can maintain antibiotic resistance through efflux pumps which are able to actively transfer antibiotics out of the cell thereby decreasing the intracellular concentration of antibiotics. Although some efflux pumps are limited by the substrate they are able to expel, others are able to transport a wide range of different antibiotics (Blair et al., 2015). Efflux pumps that are able to expel a wide range of different antibiotics are known as multidrug resistance (MDR) efflux pumps. In a Gram-negative bacterium *E. coli*, the resistance-nodule-division (RND) family serves as

one of the major transporters for multiple types of drugs. Within the RND family is the constitutively active tripartite AcrAB-TolC system that serves to expel novobiocin, beta-lactams, erythromycin, among many other types of antibiotics (Swick et al., 2011). The AcrAB-TolC system has been well-studied and is known to confer antibiotic resistance (Ma et al.,1995). In the event that the AcrAB-TolC system becomes inactivated, efflux pumps that normally remain dormant or weakly expressed, like AcrEF, can become activated through mutations (Misra et al., 2015; Alon Cudkowicz & Schuldiner, 2019). The activation of AcrEF-TolC, a homologue of AcrAB, can confer similar levels of antibiotic resistance as AcrAB-TolC (Misra et al., 2015).

As efflux pumps can play a major role in the survival of a cell during antibiotic stress, discovering ways of targeting such resistance mechanisms are being investigated. However, since new antibiotics are infrequently discovered, some have turned to analyzing the possibility of extending the efficacy of existing ones. Thus, research has also focused on the utilization of efflux pump inhibitors (EPIs). EPIs block the efflux pump proteins from expelling antibiotics that have entered the bacteria, thus increasing the internal buildup of antibiotics and enhancing the antimicrobial activity (Sharma et al., 2019). However, while EPIs can block some efflux pumps, mutations can also arise to circumvent the inhibited efflux pumps (Delcour, 2009).

1.3 Origin of RpoB Mutants

In the absence of major efflux pump AcrAB, efflux pump AcrEF can often become mutationally activated to compensate. However, since AcrAB and AcrEF share the same substrate specificity and sequence homologies, both efflux pumps can be inhibited by the same EPI (Misra et al., 2015). Thus, the complete functional inhibition of

AcrAB and AcrEF by an EPI was considered equivalent to a genetic inactivation of the genes coding for the two pumps. Based on this premise, both AcrAB and AcrEF were genetically inactivated and the resulting strain was then exposed to antibiotics to isolate resistant mutations. Two antibiotics, novobiocin and erythromycin, were used in the selection because they employ different mechanisms of action. Novobiocin inhibits DNA gyrase and topoisomerase IV (Smith et al., 1967), while erythromycin inhibits protein synthesis by binding to the 50S ribosomal subunit (Weisblum et al., 1995). Exposure to the two mechanistically-different antibiotics ensured that antibiotic- and cell target-specific mutations would not be selected for.

The minimum inhibitory concentrations (MIC) of the Δ*acrAB* Δ*acrEF* strain for novobiocin and erythromycin were 0.5 μg/mL. In contrast, MICs of $AcrAB^{+}AcrEF^{+}$ parent strain against novobiocin and erythromycin were 64-128 μg/mL and 128 μg/mL, respectively. The concentration used for the isolation of antibiotic resistant mutants was 2.5 μg/mL for both antibiotics. The resistant mutants, which arose after 24 to 48 hours of incubation, were then purified on lysogeny broth agar (LBA) containing 1.25 μg/mL each of novobiocin and erythromycin. The resulting twenty stable antibiotic resistant mutants were further characterized by determining efflux pump dependence and whole genome sequencing. Four of the twenty mutants had missense mutations in *rpoB* (Cho & Misra, 2021).

1.4 Rifampin Often Results in RNAP Mutations

To become antibiotic resistant, bacteria may also mutate the antibiotic's intended target so that the antibiotic can no longer stably bind. Rifampin (also known as rifampicin) is one such antibiotic that inhibits prokaryotic RNA synthesis resulting in

RNAP-specific mutations. Through RNAP mutations, bacteria can develop resistance against rifampin (Campbell et al., 2001). In *E. coli*, rifampin-resistant mutants almost always accumulate mutations in the gene encoding for the RNA polymerase beta subunit (*rpoB*). Structural studies have suggested that the antibiotic binds to the active area of the beta subunit, blocking RNA elongation and consequently preventing synthesis of bacterial proteins (Campbell et al., 2001). To develop rifampin resistance, bacteria mutate the rifampin binding region on RNAP, destabilizing rifampin binding.

In *E. coli*, rifampin resistance is often located in four clusters (also known as rifampicin resistance determining regions [RRDRs]) in the RpoB protein: the N-terminal cluster, cluster 1 (amino acids 509-503), cluster 2 (563-572), and cluster 3 (687) (Landick et al., 1990; Campbell et al., 2001). Aside from rifampin, other *rpoB* mutations have arisen due to antibiotics such as ciprofloxacin, which inhibits bacterial DNA topoisomerase and gyrase (Pietsch et al., 2016). In addition to demonstrating resistance against rifampin, RpoB mutants can be cross-resistant to other antibiotics which may share similar binding sites. For example, RpoB mutants were also found to be resistant to the antibiotic sorangicin A, a broad-spectrum antibiotic chemically unrelated to rifampin, which can bind to the same areas of RNAP (Campbell et al. 2005).

The mutation of this study, *rpoB58* (G449V), is not located in the aforementioned rifampin clusters. Previous work in our lab has further demonstrated that RpoB58 mutant does not increase resistance against rifampin (Cho & Misra, 2021).

1.5 Pleiotropy of RpoB Mutants

Unlike eukaryotic organisms, there is only one type of RNA polymerase (RNAP) in *E. coli* that transcribes all genes. It is composed of multiple subunits with a catalytic

core referred to as the complex β′βα2ω. Alone, the *E. coli* RNAP does not recognize specific promoters. With the addition of sigma factors, the formed RNAP holoenzyme can initiate transcription (i.e. RNA synthesis) from specific subsets of promoter sequences. Sigma factors are utilized under different conditions with σ^{70} (RpoD) being responsible for transcribing most of the genes necessary for growing cells and maintaining necessary pathways. For this reason, it is referred to as the housekeeping sigma factor.

Although frequently associated with rifampin resistance, *rpoB* mutations can also have widespread implications on the physiology of the cell due to their impact on cell-wide transcription. For example, these mutations have been also associated with features such as thermoinducible filamentation, alteration of associated transcription factors, optimized growth on minimal media, and transcription itself (Vinella & D'Ari, 1994; Conrad et al., 2010; Satory et al., 2013; Heisler et al., 1996; Zhou et al., 2013). For the purposes of this study, we have primarily examined the thermotolerance of the RpoB58 mutant alongside its 'stringent' profile.

1.6 Stringent Response

Among the previously isolated *rpoB* mutations, there were mutations with notable conformational changes. These RpoB mutants have been noted to behave as if they were in "stringent" conditions similar to that described by others investigating *rpoB* and *rpoC* (which encodes for the beta prime subunit of RNAP) (Zhou & Jin, 1998). During amino acid starvation conditions when there is a lack of available nutrients, the stringent response would be triggered resulting in the negative regulation of a particular set of genes, most of them being involved in protein and nucleic acid synthesis. Previous RNA-

Seq data on RpoB58 indicated that genes involved with nucleic acid synthesis had been downregulated while those involved in amino acid synthesis were upregulated (Misra & Cho, 2021). This particular expression pattern was similar to cells experiencing stringent conditions. During such starvation conditions when there is low availability of amino acids, the alarmone guanosine pentaphosphate (pppGpp) accumulates subsequently binding to RNAP and causing inhibition of ribosomal and transfer RNAs and other genes involved with growth. Separately, genes involved with stress and survival, such as *rpoS*, encoding the sigma factor σ^{38}, are upregulated (Gentry et al., 1993).

Sigma factors such as σ^{38} (*rpoS*) are part of the accessory factors that RNAP relies on for the transcription of particular promoters in response to environmental changes. Another critical component that binds to RNAP to modulate the stringent response is DksA (DnaK suppressor A). DksA is a transcription factor which binds to the secondary channel of RNAP thereby affecting transcription initiation (Parshin et al., 2015). It can also assist with the positive regulation of some amino acid biosynthesis promoters while negatively regulating ribosomal RNA expression (Lyzen et al., 2016). Additionally, DksA facilitates the binding of (p)ppGpp to one of its RNAP sites (Ross et al., 2016). Cells lacking DksA are unable to grow on minimal medium missing certain amino acids (Paul et al., 2005). During stringent conditions, when there is nutritional deprivation, (p)ppGpp inhibits transcription of ribosomal promoters. Suppressors of Δ*dksA* auxotrophy have been mapped to *rpoB* (Brown et al., 2002). Previous work on the aforementioned four isolated *rpoB* alleles has found that they are able to grow on glycerol minimal medium (without amino acid supplement) in the absence of DksA, further

supporting the adaptation of the mutant RNAPs to a more 'stringent' state (Cho & Misra, 2021).

1.7 Heat Shock Response

In response to elevated temperatures (>42°C), the heat shock response (HSR) is triggered, leading to an increase in the synthesis of approximately 20 proteins that exhibit a large (10-20 fold) transient increase (Lemaux et al., 1978; Yamamori et al., 1978). Most of the 20 or so heat shock proteins (HSPs) are under the control of sigma factor RpoH (σ^{32}). These HSPs include chaperones DnaK and DnaJ which assist with proper protein folding by binding to naive proteins and assisting with the refolding of proteins by limiting non-productive interactions (Ellis & Van der Vies, 1991; Hendrick & Hartl, 1995). HSPs also include proteases which degrade misfolded or abnormal proteins that can occur as a result of protein denaturation at higher temperatures. If left alone, the misfolded proteins can clump together forming aggregates. Within aggregates, some proteins can remain harmlessly in a misfolded state until conditions become more favorable (Leuenberger et al., 2017). However, if the amount of aggregates becomes too great for the cell to process it can be quite toxic and fatal to the cell. Consequently, HSPs play a key role in proteostasis even at normal temperatures where they are required for proper functioning of proteins involved with cell growth (Herendeen et al., 1979; Yamamori & Yura, 1978). Interestingly, increases in HSPs have been noted after exposure to ethanol, carbon starvation, and other environmental stresses further pointing to the role of heat stress genes in the general maintenance of the cell (Jenkins et al., 1991).

While molecular chaperones such as DnaK and DnaJ can assist with preventing protein aggregation and misfolding, they are not the only cellular factors that contribute to the thermotolerance of a cell. Other factors such as decreased protein synthesis would result in a less likelihood of aggregation and misfolding within the cell which would ultimately lead to extended survival during heat stress.

1.8 Specific Aims

As a result of the noted thermotolerance of the RpoB58 mutant, this thesis investigated the effects of continuous heat stress and short-term exposure to heat and ethanol using quantitative growth assays. Additionally, due to the potential dependence on the HSR pathway, we also aimed to determine the involvement of molecular chaperones, DnaK, DnaJ, and CbpA, in RpoB58 and RpoB-WT backgrounds. Lastly, the utilization of *in vivo* gene expression assays and different growth conditions have resulted in further mechanistic insights into *rpoB58*-mediated thermotolerance.

CHAPTER 2

RESULTS

2.1 Aim 1. Characterizing Tolerance to Heat and Ethanol

Of all four *rpoB* mutations isolated as described above, *rpoB58* conferred the greatest thermotolerance, as seen by its ability to allow formation of homogeneous single colonies on a lysogeny broth agar (LBA) plate at 47°C. In contrast, the parental strain expressing the wildtype (WT) *rpoB* allele failed to form homogenous single colonies beyond 45°C. When plated at a low growth temperature (30°C), cells expressing *rpoB58* displayed a small colony phenotype unlike its WT counterpart (Fig. 1).

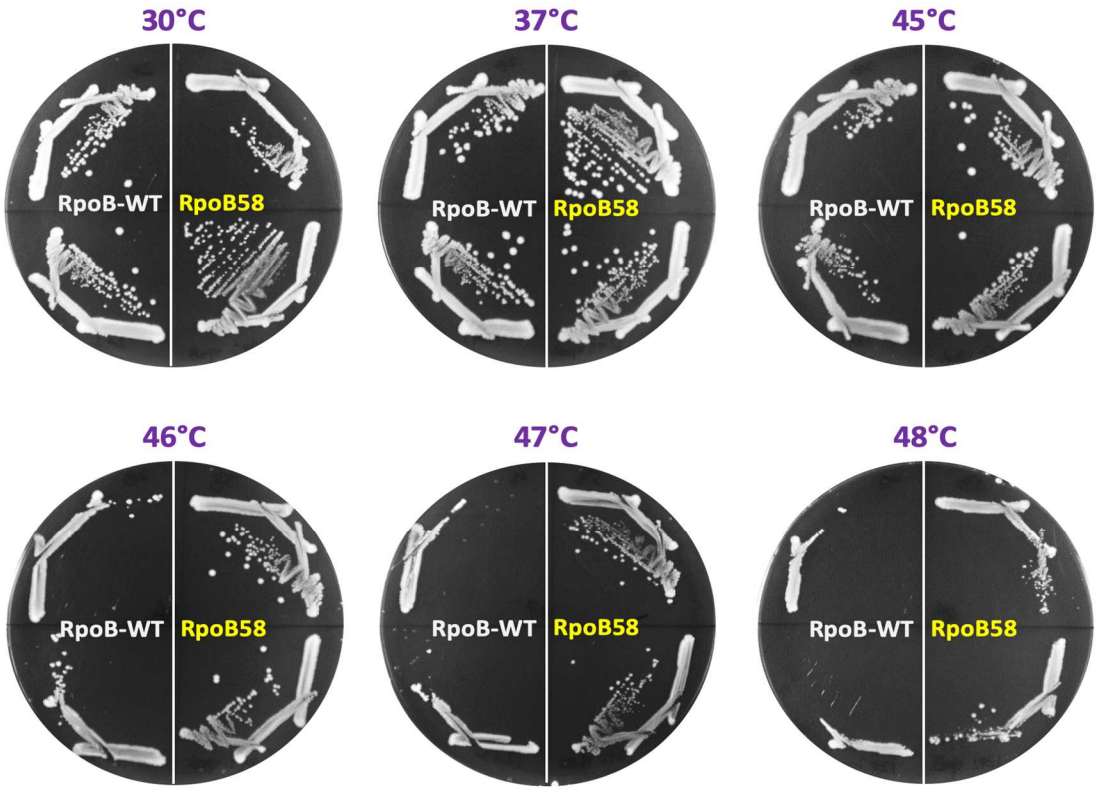

Figure 1. RpoB-WT and RpoB58 at incrementally increasing temperatures on LBA plates. Temperatures were tested at 30, 37, 45-48°C on LBA with plates incubated for approximately 24 hours.

To quantify the effect of temperature on bacterial growth, cultures were grown in a liquid medium (lysogeny broth; LB) and growth rates were determined by periodically withdrawing samples to measure optical density (OD at 600 nm; OD_{600}). Based on the growth phenotype on solid media, it was expected that *rpoB58* would also allow for better growth rate than *rpoB*-WT in liquid cultures at elevated temperatures. However, in liquid cultures, the results were opposite; i.e., the growth rates of the RpoB58 mutant were consistently lower than the RpoB-WT parental strain across all temperatures (Fig. 2; Table 1). Despite the lower growth rates, the mutant cultures, however, contained a slightly higher number of viable cells (determined from the last sample time point) than the parental cultures (Table 1).

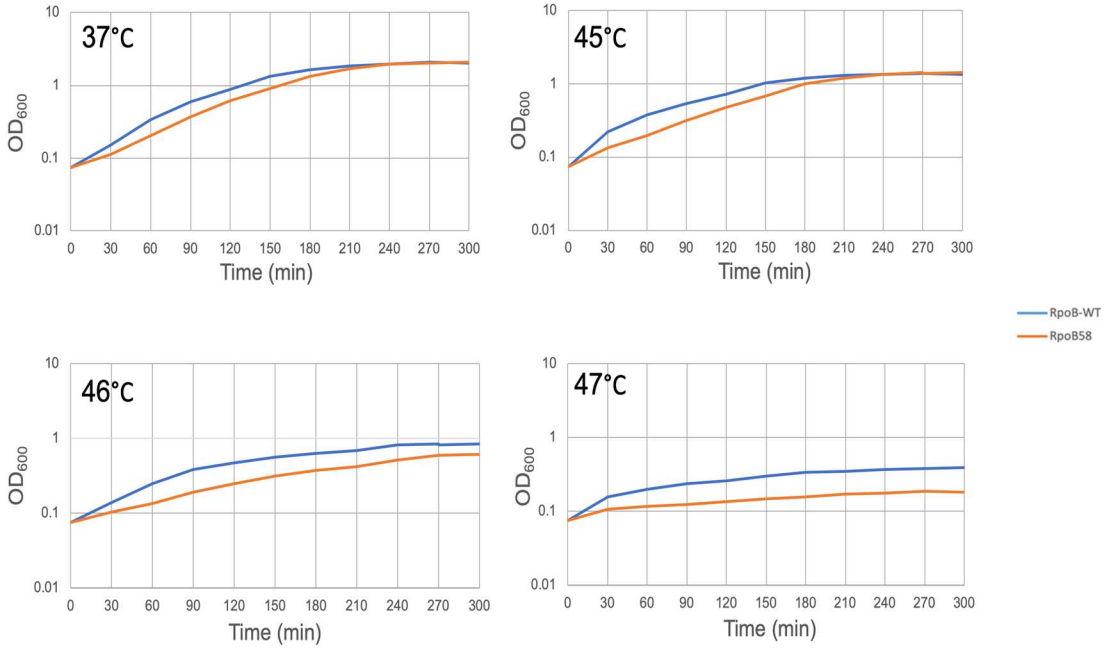

Figure 2. Liquid (LB) semi-log growth curves of RpoB-WT vs. RpoB58 at 37°C, 45-47°C. Growth curves were plotted using the average of three biological replicates per strain at each temperature until cultures reached stationary phase (after approximately five hours). RpoB-WT had a higher OD_{600} reading than the RpoB58 strain at every time point.

Table 1. Growth trends in the terminal OD_{600} reading, growth rate, and cell viability at various growth temperatures

37°C	Ending OD	Growth Rate	CFU/mL
RpoB-WT	2.018	$0.2263e^{0.442x}$	4.16×10^9
RpoB58	2.080	$0.1301e^{0.4991x}$	5.64×10^9
45°C	**Ending OD**	**Growth Rate**	**CFU/mL**
RpoB-WT	1.385	$0.1639e^{0.3754x}$	4.80×10^8
RpoB58	1.442	$0.0881e^{0.4165x}$	7.85×10^8
46°C	**Ending OD**	**Growth Rate**	**CFU/mL**
RpoB-WT	0.890	$0.3498e^{0.1448x}$	1.12×10^8
RpoB58	0.689	$0.1652e^{0.1941x}$	1.99×10^8
47°C	**Ending OD**	**Growth Rate**	**CFU/mL**
RpoB-WT	0.393	$0.1493e^{0.1325x}$	4.14×10^7
RpoB58	0.233	$0.0989e^{0.0768x}$	5.20×10^7

The ending (terminal) OD_{600} reading was when the cultures reached stationary phase. Growth rate was determined using the exponential trendline from the linear portion of the semi-log growth curve. Cell viability (CFU/mL) was obtained by serially diluting the ending OD_{600} (stationary phase) cultures and plating at 37°C.

To test whether the higher growth rates displayed by the parental strain was due to accumulation of temperature-tolerant revertants, samples from liquid cultures were re-tested for growth on the solid medium at all four temperatures. The results matched that seen when directly streaking onto the plates at higher temperatures (Fig. 1); i.e., unlike RpoB58, RpoB-WT cultures from 47°C liquid cultures were unable to grow on the solid medium at 47°C and grew poorly at 46°C (Fig. 3). Therefore, the higher OD readingss of RpoB-WT cultures were not due to the accumulation of thermotolerant mutants. These results demonstrated that the higher OD readings and growth rates of WT cultures do not reflect a higher number of viable cells than the RpoB58 mutant.

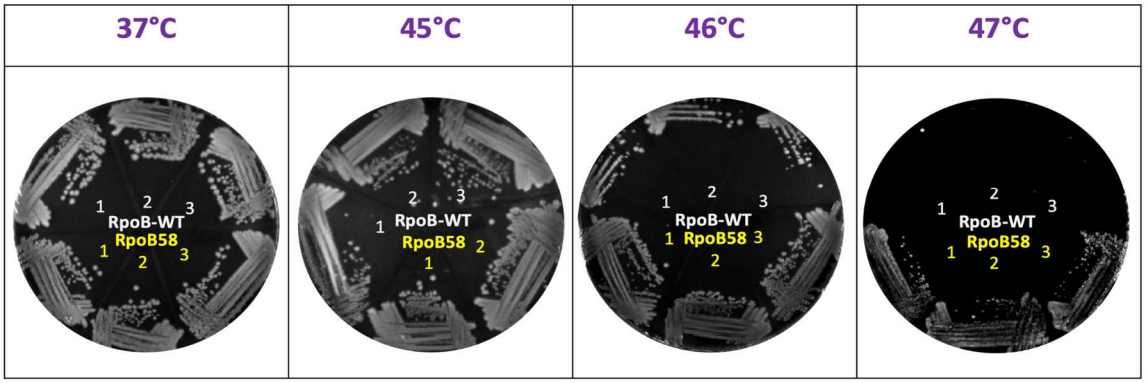

Figure 3. Transferred endpoint liquid cultures were incubated on LBA at the respective temperatures. Plates inoculated with stationary phase endpoint cultures (from Figure 2) were incubated 24 hours at the indicated temperature.

The incongruent results between the growth curves and the plating on solid media at higher temperatures led us to investigate cell morphology using electron microscopy. This was, in part, based on previous studies that have reported filamentous (elongated) cell phenotypes seen at higher growth temperatures and when cells are physically stressed (Van Derlinden et al., 2008; Rizzo et al., 2020). Scanning electron microscopy (SEM) demonstrated that at higher growth temperatures, WT cells were significantly more filamentous than RpoB58 mutant cells (Fig. 4). Quantification of the length and width of the bacterial cells using ImageJ software showed that the length of the WT cells was on average 3.26-fold greater and the width 1.58-fold greater at 46°C than at 37°C. In contrast, the length of the RpoB58 mutant increased by 2.40-fold at 46°C than 37°C and the width increased by 1.40-fold (Fig 4; Table S2). A direct comparison of length and width between WT and mutant cells showed that while dimensions were almost identical at 37°C, WT cells were 1.30-fold longer and 1.22-fold wider than the mutant cells at 46°C (Fig 4; Table S2). These results suggest that higher cell dimensions of WT cells at elevated temperatures contributed to higher OD readings of WT cells over mutant cells.

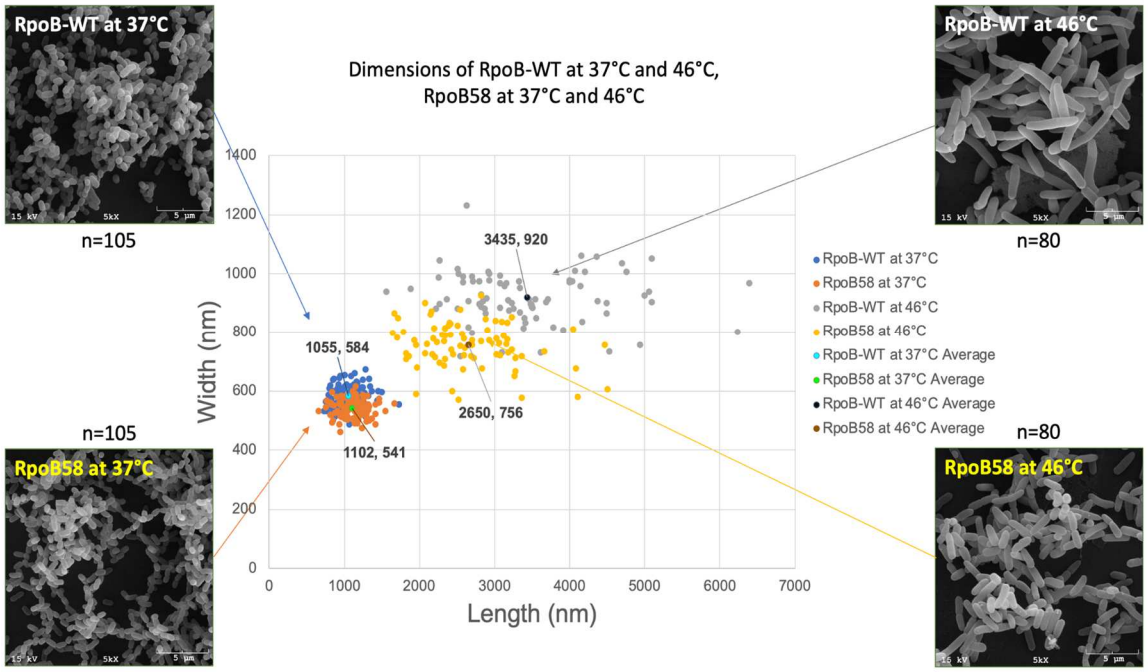

Figure 4. SEM images and scatterplot of cell dimensions of RpoB-WT and RpoB58 at 37 and 46°C. For samples at 37°C, the dimensions of 105 cells. Due to limited images, the dimensions of only 80 cells at 46°C were examined. Additional details on cell dimensions can be found in Table S2.

We sought to determine if the RpoB58 mutant would demonstrate higher thermotolerance than WT to short-term extreme high temperature heat shock. A heat shock temperature of 55°C was chosen as this is well above the growth temperature of *E. coli* and would immediately halt essential enzyme-mediated cellular processes, such as protein and nucleic acid synthesis. In other words, a greater tolerance to this lethal temperature would require a preexistent cellular state rather than an adaptive state. Cells grown at 37°C were exposed to 55°C and samples were periodically withdrawn to determine cell viability. As seen in the survival curve, the RpoB58 mutant displayed 100-fold higher viability than RpoB-WT after 20 minutes of 55°C exposure (Fig. 5A).

Past literature indicated a similar cellular response to that of heat when *E. coli* cells are exposed to ethanol (Neidhardt et al., 1984; VanBogelen et al., 1987; Brissette et

al., 1990). As the RpoB58 better tolerated heat shock, it was tested whether they could tolerate ethanol shock. When exposed to 25% (v/v) ethanol, the RpoB58 mutant again displayed 100-fold greater viability than the RpoB-WT parental strain (Fig. 5B).

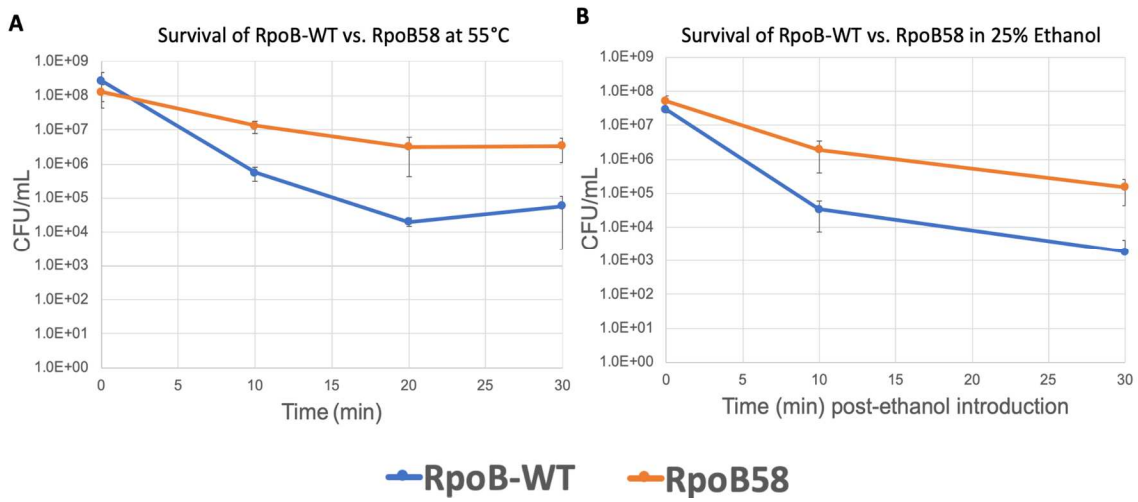

Figure 5. Heat (55°C) and ethanol (25%) shock survival curves of RpoB-WT and RpoB58. CFU/mL was calculated from colonies that grew after plating the cultures on LBA at 37°C.
A) Heat shock was performed exposing 37°C fresh exponential phase cultures to 55°C. Samples were withdrawn every ten minutes, diluted, and plated on LBA.
B) Ethanol shock survival curve after exposure to 25% (v/v) ethanol. Samples were withdrawn after ten and 30 minutes and plated after dilution on LBA.

2.2 Aim 2. Characterization of Chaperone Involvement

Molecular chaperones, also known as heat shock proteins (HSPs) play a vital role in maintaining cell viability at high temperatures by lowering protein misfolding. Expression of the HSP genes is under the control of a specialized sigma factor σ^{32}, also known as RpoH. A major HSP complex, comprised of chaperones DnaJ-DnaK-GrpE, plays a crucial role not only in protein folding but also in regulating HSP gene expression by controlling the proteolysis of σ^{32}. Since RpoB58 could better tolerate exposure to high temperatures, we tested whether the mutant can also better tolerate the absence of DnaJ or the depletion of DnaK and DnaJ. For this, the *dnaJ* gene was deleted from RpoB-WT and RpoB58 backgrounds. At 30°C, both RpoB-WTΔ*dnaJ* and RpoB58Δ*dnaJ* showed no growth (i.e., colony size) difference compared to their *dnaJ*$^{+}$ counterparts (Fig. 6). However, at 37°C and 42°C, RpoB-WT Δ*dnaJ* was unable to form single colonies (Fig. 6). On the other hand, RpoB58Δ*dnaJ* was able to grow stably - forming homogeneous colonies at 42°C and even at higher temperatures (i.e., up to 45°C). Beyond 45°C, RpoB58Δ*dnaJ* began to display unstable, heterogeneous growth (data not shown) demonstrating a lack of dependence on chaperones unless at more extreme temperatures.

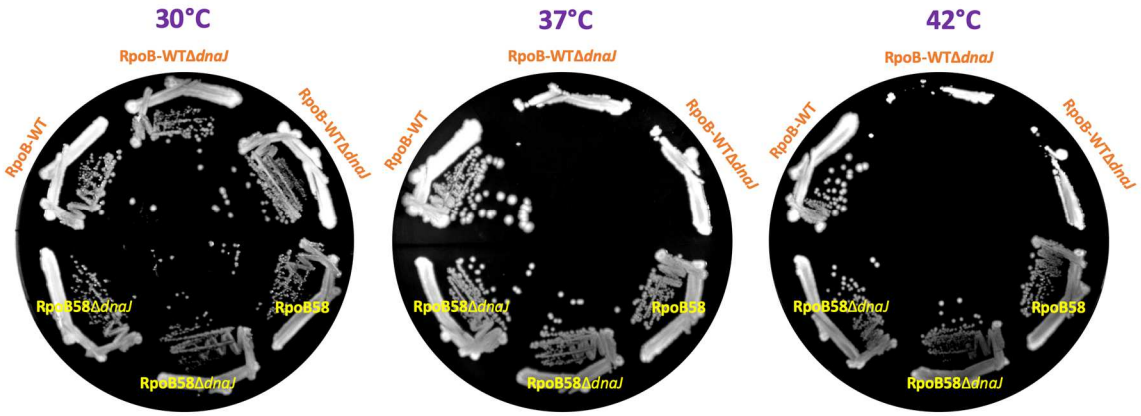

Figure 6. Effect of Δ*dnaJ* on plate growth at 30, 37, and 42°C on RpoB-WT and RpoB58.

DnaJ was originally discovered for its role in the replication of bacteriophage lambda (Sunshine et al., 1977). Consequently, cells lacking DnaJ are phage lambda resistant. We asked whether the *rpoB58* mutation, which overcomes the temperature sensitive phenotype of *dnaJ* deletion (see above), could also overcome the absence of DnaJ and allow lambda growth. When tested by the phage cross-streak assay, both the RpoB-WT and RpoB58 mutant expressing DnaJ were sensitive to lambda, as their growth abruptly stopped where the phage had been streaked. However, neither the RpoB-WTΔ*dnaJ* strain nor the RpoB58Δ*dnaJ* mutant was killed by lambda, as both strains grew past where the lambda phage had been streaked (Fig. 7). These results indicated that *rpoB*58 did not change the cell physiology to substitute for all DnaJ functions.

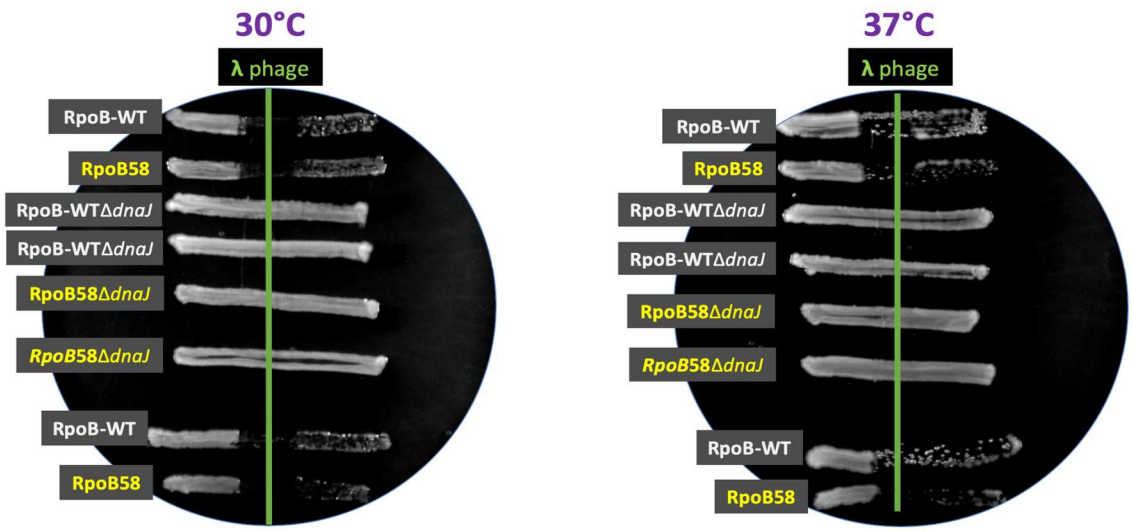

Figure 7. Lambda phage cross-streak assay for Δ*dnaJ* strains on LBA at 30 and 37°C. Lambda (λ) phage was streaked directly down the middle of the plate (as indicated by the green line). Strains RpoB-WT, RpoB58, RpoB-WTΔ*dnaJ*, and RpoB58Δ*dnaJ* were streaked horizontally across the vertical phage streak prior to 24-hour incubation at the indicated temperature.

In *E. coli*, curved DNA protein A (CbpA) has been shown to share a significant functional overlap with DnaJ (Ueguchi et al., 1995). Cells deleted for the *cbpA* gene do not display any noticeable growth defect; however, a Δ*cbpA*Δ*dnaJ* mutant is considerably more growth defective than the *dnaJ* mutant alone (Ueguchi et al., 1995). To test whether the *rpoB58* mutation can overcome the severe growth defect phenotype of Δ*cbpA*Δ*dnaJ*, *cbpA* was also deleted from the RpoB-WTΔ*dnaJ* and RpoB58Δ*dnaJ* backgrounds. Strikingly, the *rpoB58* mutation was still able to reverse the severe temperature growth defect observed in the WT background (Fig. 8).

Figure 8. Strains with *cbpA* and *dnaJ* deletions on 30 and 37°C LBA. For the 30°C plate, images were taken after 48 hours of growth whereas the 37°C was incubated for 24 hours.

To further assess the RpoB58 mutant's lack of reliance on HSPs, a strain was obtained in which the expression of both *dnaK* and *dnaJ* could be simultaneously depleted. A genetic construct in which the expression of *dnaKJ* has been placed under the control of an IPTG-inducible promoter was introduced in RpoB-WT and RpoB58 backgrounds (denoted by P_{IPTG}*dnaKJ*). For maximum expression of *dnaKJ*, 100 µM of IPTG was required. When IPTG was present both strains grew well at all three incubation temperatures (Fig. 9). In the absence of IPTG, when only basal levels of *dnaK* and *dnaJ* were being expressed, the RpoB58 mutant was able to form homogenous colonies at all three temperatures. In contrast, RpoB-WT P_{IPTG}*dnaKJ* failed to form any single colonies, even at the lower 30°C temperature. This further confirms the proteostatic differences found between the RpoB58 mutant and the RpoB-WT strains. Furthermore, it supports the notion that there are other mechanisms, independent of chaperones DnaK and DnaJ, which are contributing to the thermotolerance phenotype of the RpoB58 mutant.

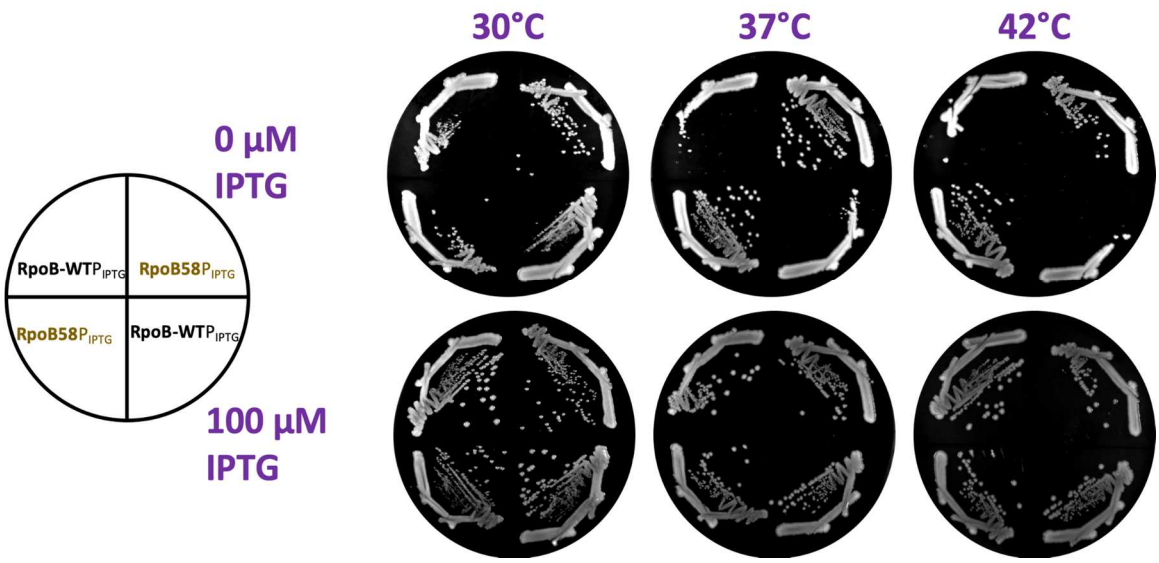

Figure 9. Growth of P$_{IPTG}$(*dnaKJ*) strains on 0 µM, 100 µM IPTG LBA. RpoB-WTP$_{IPTG}$ is abbreviated for RpoB-WT P$_{IPTG}$*dnaKJ*. Similarly, RpoB58P$_{IPTG}$ is abbreviated for RpoB58P$_{IPTG}$*dnaKJ*. All plates were imaged after 24 hours with the exception of 30°C plates which required 36 hours of incubation to form single colonies.
Top row: Without IPTG at 30, 37, and 42°C.
Bottom row: With 100 uPM IPTG which would be considered full induction of *dnaK* and *dnaJ*.

Cells lacking DnaJ are also known to form long filaments (Sell et al., 1990; Caplan et al., 1993). We asked whether the *rpoB58* mutation could reverse the phenotype of a *dnaJ* null mutation (Δ*dnaJ*). Cell morphology of Δ*dnaJ* cells was analyzed by SEM from cultures grown at 30°C and 42°C. At 30°C, both RpoB-WTΔ*dnaJ* and RpoB58Δ*dnaJ* cells appeared similar, except the presence of occasional extremely filamentous cells in the WT background (Fig. 10). At 42°C, RpoB-WTΔ*dnaJ* cells became extremely filamentous whereas the RpoB58Δ*dnaJ* cells were significantly less so.

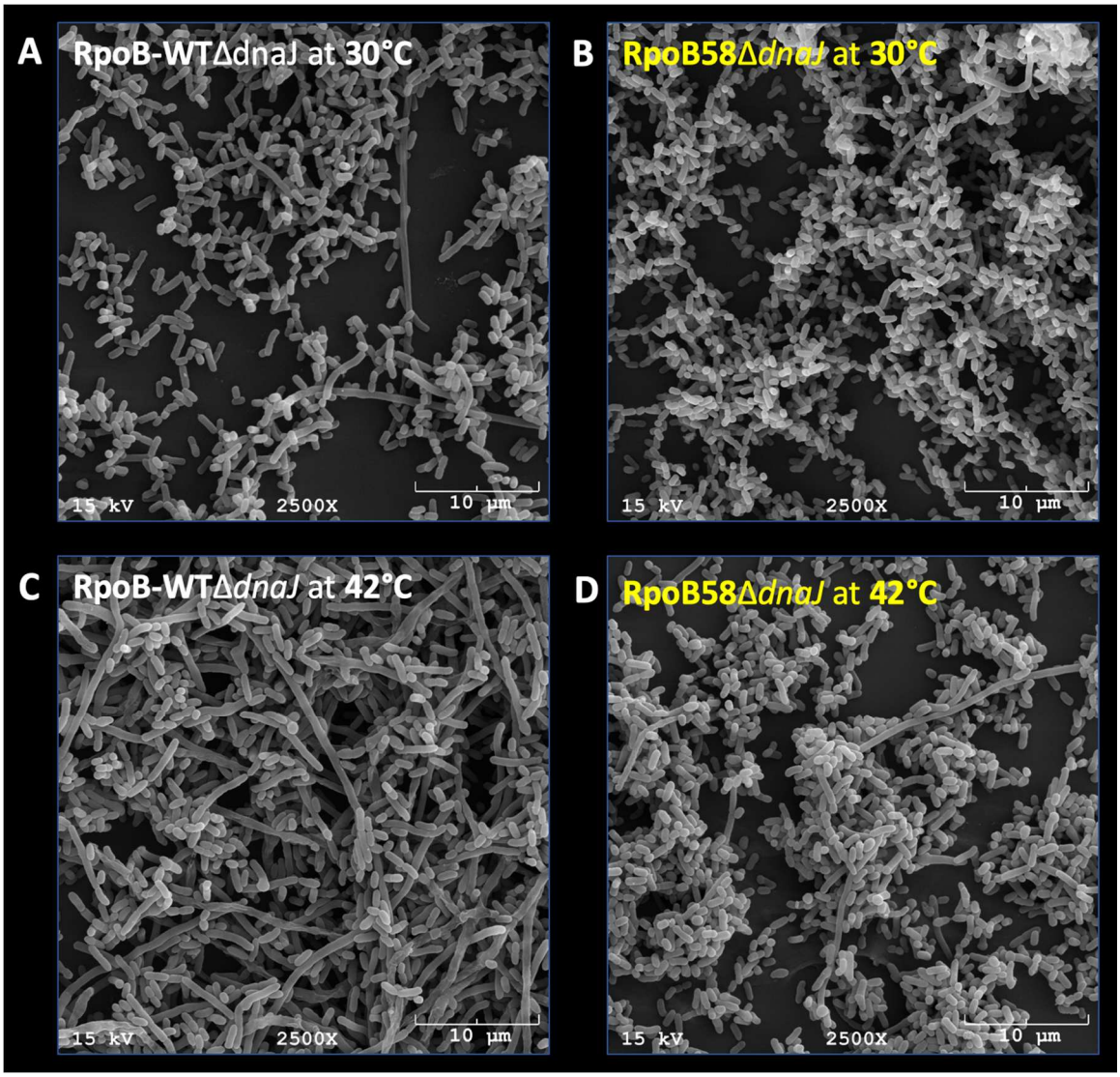

Figure 10. SEM images of Δ*dnaJ* cultures grown at 30 and 42°C. Cells had been preserved in 2% glutaraldehyde before imaging at 2500x magnification. Left to right, top to bottom: Image of strain A) RpoB-WTΔ*dnaJ* at 30°C B) RpoB58Δ*dnaJ* at 30°C C) RpoB-WTΔ*dnaJ* at 42°C and D) RpoB58Δ*dnaJ* at 42°C.

2.3 Aim 3. Investigating Possible Mechanisms Associated with the Thermotolerance Phenotype of the RpoB58 Mutant

RNA-Seq analysis was carried out in our lab to assess the gene expression profile that could provide a clue concerning the mechanism of *rpoB58*-mediated antibiotic resistance (Cho & Misra, 2021). The gene expression profile of the RpoB58 mutant resembled that of bacteria undergoing stringent response, which included the characteristic reduced expression of genes involved in protein and nucleic acid synthesis along with increased expression of genes involved in various stresses (Durfee et al., 2008). RNA-Seq data had not indicated the increased expression of HSP genes which could explain the thermotolerance phenotype of the RpoB58 mutant. However, since expression of genes involved in protein synthesis was down-regulated (Fig. 11), it was hypothesized that reduced protein synthesis could result in lower protein misfolding/aggregation, thus lowering the need for molecular chaperones like DnaK and DnaJ.

To independently verify that genes involved in protein synthesis are indeed down-regulated by *rpoB58*, a *rrnB* P1::*lacZ* gene fusion construct was employed where activity of the P1 promoter of the ribosomal RNA operon (*rrnB*) could be measured by assaying β-galactosidase (LacZ) activity. The ribosomal RNA operon (*rrnB*) P1 promoter was chosen as it has been extensively utilized as an environmentally responsive promoter that indicates protein synthesis activity (Tedin et al., 1995). Further, a previous lab publication had utilized *rrnB* P1::*lacZ* fusions to demonstrate the decreased expression of ribosomal RNA operon transcription as an effect of the *rpoB58* mutation (Cho & Misra, 2021). The *rpoB*-WT and *rpoB58* alleles were transferred by P1 transduction into

a VH1000 strain containing the *rrnB* P1::*lacZ* construct. The presence of the *rpoB58* allele consistently lowered *rrnB* P1::*lacZ* activity by about 40% (Fig. 11) supporting the RNA-Seq data of reduced expression of genes involved in protein synthesis.

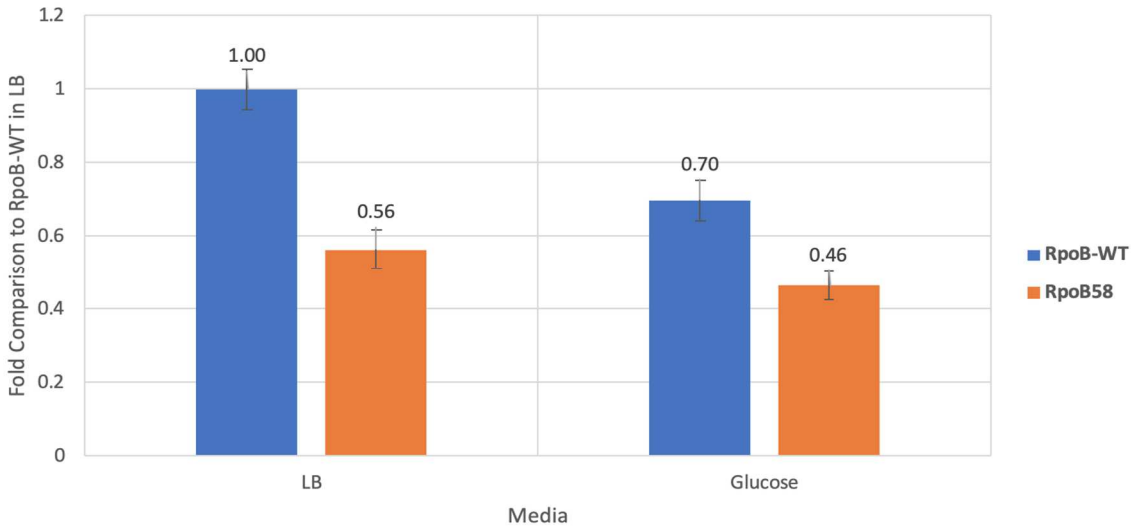

Figure 11. Activity of *rrnB* P1 promoter in RpoB-WT and RpoB58 at 37°C. Strains had been tested in LB (left side) and glucose minimal medium (right side). Results were normalized to the Miller units obtained for RpoB-WT in LB.

Protein synthesis varies with different growth conditions (Koch, 1980; Tao et al., 1999; Jin et al., 2012; Li et al., 2014). For example, it is well known that faster bacterial growth in a rich medium (e.g. LB) is tied to higher protein synthesizing capacity. Conversely, in a minimal medium (e.g. M63-glucose), both bacterial growth rate and protein synthesizing capacities are low. If protein synthesizing capacity is low in a minimal medium, then the growth defect associated with the absence of DnaJ (Fig. 12) might be reversed when the RpoB-WTΔ*dnaJ* strain is grown on a minimal medium. The effect of media on protein synthesis was determined by comparing *rrnB* P1::*lacZ* activities from RpoB-WT and RpoB58 cells grown on rich LBA and glucose minimal (M63) media. In the RpoB-WT background, *rrnB* P1::*lacZ* activity was 25% lower in the

minimal medium compared to the rich medium. In the RpoB58 background, where *rrnB* P1::*lacZ* activity was already 40% lower compared to the WT strain, growth in the minimal medium further reduced the *rrnB* P1::*lacZ* activity by 10% (Fig. 11). These results supported previous reports that growth in minimal medium reduces protein synthesis. Moreover, the effects of growth medium and *rpoB58* on protein synthesis were additive. When RpoB-WTΔ*dnaJ* cells were tested for medium-dependent growth defects, they formed homogeneous colonies on minimal medium but not on rich medium (Fig. 12). This supported the hypothesis that lowering protein synthesis either by *rpoB58* or by culturing cells in a minimal medium can overcome the growth defect associated with the absence of molecular chaperone DnaJ.

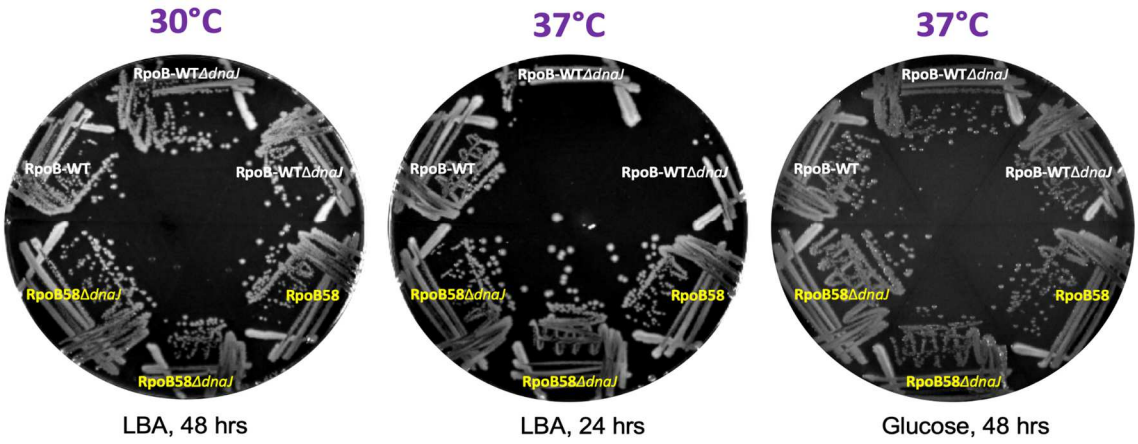

Figure 12. Effect of glucose minimal medium on Δ*dnaJ* at 37°C. The glucose plate had to be incubated for an additional 24 hours to see visible colonies.

CHAPTER 3

DISCUSSION

Due to the ever-increasing concern for antibiotic resistance, it is imperative that we gain a better understanding of how antibiotic resistant mutations can arise even without major efflux pumps, which would normally expel antibiotics. Previous investigations into mutations that could arise in the absence of major efflux pump AcrAB led to the isolation of four *rpoB* missense mutations that conferred elevated resistance against novobiocin and erythromycin in a MdtABC efflux pump-dependent manner (Cho & Misra, 2021).

Mutations in the RNAP can have a pleiotropic effect on the genes transcribed. Due to this potential pleiotropy, RNAP mutations can provide further insight into the regulation of various pathways within the cell. Additionally, RNAP mutations can also prove advantageous under different stress conditions, as seen with the significant thermotolerance of RpoB58 mutant. The RpoB58 mutant formed single colonies at temperatures as high as 47°C. In comparison, the parental *rpoB* wildtype, RpoB-WT, was unable to form homogenous colonies beyond 45°C (Fig. 1).

To quantify the level of thermotolerance of the RpoB58 mutant, we examined its growth in liquid media (LB). Surprisingly, the growth rate (as measured by culture turbidity at OD_{600}) of the RpoB58 mutant across various temperatures was always lower than RpoB-WT. However, when culture samples from the terminal time point were plated at 37°C to examine cell viability (measured in CFU/mL), the viability count of the RpoB58 mutant was slightly higher than the RpoB-WT parental strain (Table 1). Although the RpoB58 mutant had increased viability, it still did not account for the

differences between OD_{600} (culture turbidity) readings and cell viability. One possibility for this discrepancy was the difference in cell morphology. This was tested by scanning electron microscopy (SEM) of bacterial cells grown at 37°C and 46°C. When grown at 37°C, cells from both strains had almost identical dimensions. However, at 46°C, RpoB-WT cells became extensively filamentous compared to the 46°C-grown RpoB58 cells. Consequently, OD_{600} cannot be used as a proxy for cell growth at higher temperatures. Bacterial cells are known to become filamentous (elongated without septa formation) in stressed environments because of the impairment of essential cell division proteins such as FtsZ (filamenting temperature-sensitive mutant Z) (Ricard & Hirota, 1973; Walker et al., 1975). There is a possibility that FtsZ remains functional at higher growth temperatures in the RpoB58 background, as morphology does not appear to be as impacted as in the RpoB-WT cells. As further discussed below, the protein folding environment in the RpoB58 mutant appears to have changed, since the mutant overcomes the temperature-sensitive filamentous phenotype normally associated with the absence of DnaK and DnaJ molecular chaperones (McCarty & Walker 1994; Paciorek et al., 1997). Further work is required to test the effect of high temperature on FtsZ's functionality.

At high growth temperatures (e.g. at 42°C), bacterial cells often rely upon the heat shock response for survival. Such response leads to the production of the heat shock proteins (HSPs) that are either molecular chaperones or proteases. DnaK, DnaJ, and GrpE constitute one such heteromeric molecular chaperone complex. Without DnaK or DnaJ, the level of misfolded, non-functional proteins is expected to rise. Continued accumulation of misfolded proteins may eventually cause toxicity, due to the loss of protein function and build-up of protein aggregates, particularly at elevated growth

temperatures (>37°C) (Schramm et al., 2019). Consequently, WT cells lacking DnaK or DnaJ display a temperature sensitive phenotype (i.e., unable to form single colonies on a rich medium) (Paek & Walker, 1987; Sell et al 1990). Remarkably, unlike WT, the RpoB58 mutant tolerated the absence of DnaJ and the depletion of DnaKJ (Fig. 6, 9). However, while *rpoB58* could suppress the temperature-sensitive phenotype of cells lacking DnaJ, it could not overcome the lambda-resistant phenotype of the Δ*dnaJ* mutant. This indicated that changes in the gene expression profile alone by *rpoB58* is not sufficient to substitute for all DnaJ functions, including assisting in the functional assembly of the lambda DNA replication machinery (Sunshine et al., 1977; Liberek et al., 1988).

Another possibility for the temperature-dependent growth defect of cells lacking DnaJ or DnaKJ must be considered. The DnaKJ-GrpE complex is known to regulate σ^{32} levels by controlling its FtsH-mediated proteolysis (Liberek et al., 1992). Consequently, when the DnaKJ-GrpE complex is disrupted, σ^{32} level rises dramatically (Gamer et al., 1996; Tatsuta et al., 1998). This leads to a higher number of RNAP-σ^{32} holoenzyme complex formation and consequently, higher expression of heat shock genes, perhaps at the expense of housekeeping genes whose expression is dependent on the RNAP-σ^{70} holoenzyme complex. Consistent with this view, overproduction of σ^{70} overcomes the temperature-sensitive phenotype of a Δ*dnaK* or Δ*dnaJ*Δ*cbpA* mutant (Shiozawa et al., 1996). In further support of this, suppressor mutations in *rpoH*, which encodes for σ^{32}, have been isolated that fail to fully induce heat shock response (Zhou & Gross, 1992). Taken together, it appears that without DnaKJ, a cumulative effect of protein misfolding and an aberrant regulation of the heat shock and housekeeping genes may contribute to a

severe temperature growth defect in RpoB-WT. This then leads to the question of how *rpoB58* overcomes the growth defect associated with DnaKJ depletion.

Several possibilities are worth considering: first, *rpoB58* may alter the intracellular protein folding environment to compensate for the loss of DnaKJ chaperones. However, the RNA-Seq data did not provide any immediate clues as to how *rpoB58* can achieve this. For example, although expression of genes of several stress pathways was up-regulated, none appears to be directly involved in protein folding. Second, it is possible that the RpoB58 RNAP is attenuated to σ^{32}, consequently preventing a severe imbalance between heat shock and housekeeping gene expression when DnaKJ levels are depleted. Future RNA-Seq analyses from chaperone-deficient, RpoB-WT and RpoB58 backgrounds may test this possibility. Third, *rpoB58* lowers protein synthesis to reduce the amount of misfolded proteins and thus lowering the need for molecular chaperones as indicated the RpoB58 RNA-Seq analysis suggesting a 'stringent' state (Cho & Misra, 2021).

One of the most prominent features of the stringent response is the downregulation of genes involved in protein synthesis (Cashel et al., 1996). RNA-Seq analysis carried out with RpoB58 revealed that expression of several ribosomal and tRNA genes, as well as genes involved in tRNA modification and the translation elongation step is significantly down-regulated (Cho & Misra, 2021). Downregulation of protein synthesis in RpoB58 was further corroborated by using the *rrnB* P1::*lacZ* reporter gene construct (Fig. 11). Moreover, expression data from *rrnB* P1::*lacZ* confirmed that growth in glucose minimal medium reduces protein synthesis and this further correlated with the observation that minimal medium also eliminates the temperature-dependent

growth defects associated with the absence of DnaJ (Fig. 11, 12). Thus, both minimal medium and the *rpoB58* mutation similarly reduce protein synthesis. Together, these results support the hypothesis that reduced protein synthesis may contribute to reduced protein misfolding, thus lessening the need for molecular chaperones. In the future, this hypothesis can be further tested by either mildly inhibiting protein synthesis using translation-specific antibiotics (e.g. chloramphenicol) or using mutations that are known to lower protein synthesis. Additionally, as the stringent response can be induced by a variety of stresses aside from nutrient starvation, it is possible that the stringent RpoB58 RNAP may prove advantageous against other stresses such as acid or oxidative stress.

During both short-term extreme heat shock and ethanol shock, the RpoB58 mutant showed higher viability than RpoB-WT. At the extreme growth-limiting temperature of 55°C, RpoB58 demonstrated 100-fold more viability after 20 minutes of exposure. Similarly, RpoB58 demonstrated 100-fold greater viability when exposed to 25% ethanol for 30 minutes. Exposure to ethanol is known to trigger the heat shock response among other stress responses triggered during osmotic or acid stress (Neidhardt et al., 1984; Brisette et al., 1990). Two common denominators between heat shock and ethanol shock are protein misfolding and membrane damage. Tolerance against these two short-term shocks indicated that RpoB58 is preadapted to such stresses. While lower protein synthesis may explain partial protection against protein misfolding as described above, it is unclear whether the *rpoB58* mutation also changes membrane lipid composition to make the envelope more tolerant to heat and ethanol shock. Future work on lipid analysis may reveal this possibility.

CHAPTER 4

MATERIALS AND METHODS

4.1 Strains and Strain Construction

All strains were either of *Escherichia coli* K-12 MC4100 or MG1655 origin. Additional strains were obtained from the *Escherichia coli* Genetic Stock Center at Yale University. The two most commonly referred to strains within this study are RpoB-WT, which is the strain with the wild-type *rpoB* allele, and RpoB58, the isogenic strain instead containing the missense *rpoB*G449V allele.

Standard genetic manipulations were performed as described in Silhavy et al., 1984. When needing to delete or move a gene into another background, successful P1 transduction was identified using kanamycin or tetracycline with a concentration of 25 μg/mL.

LacZ fusions (*rrnB* P1) were provided by RL Gourse. The complete strain list and the genotypes used in this study can be found in Table S1.

4.2 Media

Unless otherwise specified, lysogeny broth (LB) and lysogeny broth agar (LBA) plates were used for culturing. LB was made from Difco LB EZMix. To make LBA, 1.5% agar (Becton, Dickinson) was added to the LB mix.

Minimal media was composed of 0.2% glucose with additional M63 salts, thiamine, and MgSO4.

Cultures and plates were typically incubated overnight for 18-21 hours at 37°C unless stated otherwise.

To induce expression of *dnaKJ* in the *dnaKJ* depleted constructs, 100 μM of IPTG was added to the LBA.

4.3 Growth Curves

Cultures were diluted into LB to an OD_{600} of 0.075 from overnight cultures grown at 37°C. Time points were taken every 30 minutes at OD_{600} until cultures reached stationary phase approximately five hours later. The obtained growth curves are from an average of at least three biological replicates.

For RpoB-WT and RpoB58 growth curves at higher temperatures, growth rates were determined by the exponential trendline from the linear portion of the semi-log growth curve. Other methods, such as fitting an equation that would best fit a typical sigmoidal curve, were not possible at the tested higher temperatures (46°C, 47°C) as the growth curves became linearized. To determine viability, the stationary phase cultures were serially diluted and plated on LBA to be incubated overnight at 37°C before counting. To compare liquid versus solid growth, five microliters of stationary phase cultures were streaked and incubated at the respective higher temperature and another five at 37°C.

4.4 Imaging Cells

Stationary phase cultures (after approximately five hours of growth at the respective temperature) were pelleted and washed using 20 mM phosphate buffer solution (PBS). After pelleting and washing twice, cells were fixated in 2% glutaraldehyde.

Cells were first examined using differential interference contrast (DIC) under 100x magnification for consistency between cultures before one RpoB-WT and one

RpoB58 sample at each temperature was sent to the ASU School of Life Science Electron Microscopy Department. The Department then performed scanning electron microscopy (SEM) imaging. Cell dimensions (length and width) were analyzed using ImageJ software.

4.5 Heat Shock and Ethanol Shock

Heat shock and ethanol shock survival curves were plotted using data from the average of at least three biological replicates.

From an overnight culture, cells were diluted before being grown to an exponential OD_{600} of 0.4-0.5 before being exposed to 55°C. Samples were then serially diluted after 0, 10, 20, and 30 minutes before plating on LBA.

A similar method was followed for the ethanol shock. Overnight cultures were first diluted and grown to an exponential OD_{600} of 0.4-0.5. The cultures were then exposed to 25% (v/v) ethanol. Samples taken after 0, 10, and 30 minutes of exposure prior to serial plating onto LBA.

4.6 Beta-galactosidase Assays

Beta-galactosidase assays were used to monitor the transcription of ribosomal RNA promoter *rrnB* P1. Assays were performed on three independent overnight cultures in duplicate as described by Miller (1992).

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APPENDIX A

SUPPLEMENTARY FIGURES

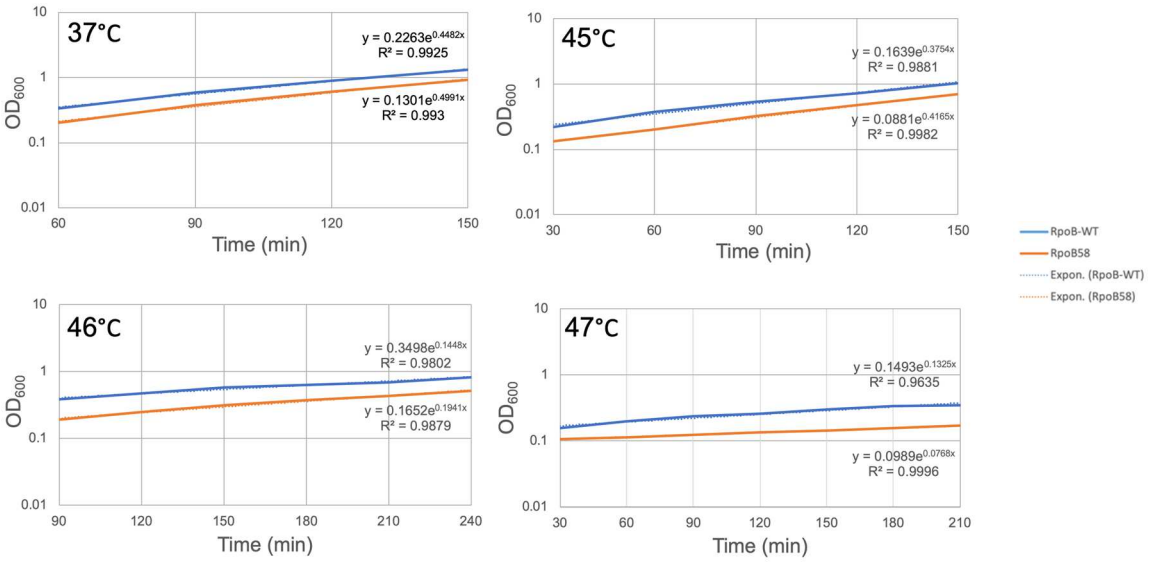

Figure S1. Growth rates of RpoB-WT and RpoB58 using the linear portion of the semi-log growth curves at 37°C, 45-47°C. Exponential trendlines have also been included.

Table S1. List of strains referenced in study

Strain Name	**Relevant genotype**	**Source / Reference**
RAM1292	MC4100Δ*ara*714	Werner & Misra, 2005
RAM3027, referred to as RpoB-WT	RAM1292 btuB:Tn10 (Tc^r)	Laboratory collection
RAM3028, referred to as RpoB58	RAM1292 btuB:Tn10*rpoB*58[*rpoB*G449V] (Tc^r)	Laboratory collection
RpoB-WTΔ*dnaJ*	RAM3027Δ*dnaJ* (Kan^r)	This study
RpoB58Δ*dnaJ*	RAM3028Δ*dnaJ* (Kan^r)	This study
RpoB-WTΔ*cbpA*Δ*dnaJ*	RAM3027Δ*cbpA*Δ*dnaJ* (Kan^r)	This study
RpoB58Δ*cbpA*Δ*dnaJ*	RAM3028Δ*cbpA*Δ*dnaJ* (Kan^r)	This study
P_{IPTG}*dnaKJ*	P_{IPTG}*dnaKJ* (Kan^r) [chromosomal *dnaKJ* under an IPTG promoter]	Deuerling et al., 1999
RpoB-WTP_{IPTG}*dnaKJ*	RAM3027P_{IPTG}*dnaKJ* (Kan^r)	This study
RpoB58P_{IPTG}*dnaKJ*	RAM3028P_{IPTG}*dnaKJ* (Kan^r)	This study
RLG4996	VH1000[MG1655 *pyrE+ lacZ- lacI-*] *rrnB* P1::*lacZ*	Paul et al., 2004
RpoB-WT *rrnB* P1::*lacZ*	RLG4996*rpoB*-WT	This study
RpoB58 *rrnB* P1::*lacZ*	RLG4996*rpoB58*btuB:Tn10	This study

Table S2. Statistics of cell dimensions for RpoB-WT and RpoB58 at 37 and 46°C

A) Average cell dimensions and variation for RpoB-WT and RpoB58 at 37 and 46°C

		Length (nm)	Width (nm)
RpoB-WT at 37°C	Average	1055	584
	Standard Dev.	196.1	36.1
RpoB58 at 37°C	Average	1102	541
	Standard Dev.	184.8	29.6
RpoB-WT at 46°C	Average	3435	920
	Standard Dev.	924.7	86.5
RpoB58 at 46°C	Average	2650	756
	Standard Dev.	631.8	75.1

B) Comparison of cell dimensions for RpoB-WT and RpoB58 at 37 and 46°C

Average Ratio	Length	Width
(RpoB-WT at 46°C)/(RpoB-WT at 37°C)	3.26	1.58
(RpoB58 at 46°C)/(RpoB58 at 37°C)	2.40	1.40
(RpoB-WT at 37°C)/(RpoB58 at 37°C)	1.03	0.93
(RpoB-WT at 46°C)/(RpoB58 at 46°C)	1.30	1.22

Cell dimensions were obtained using ImageJ software on SEM images.